UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

Exploring new targets for developing cannabinoid-based therapies in Parkinson's disease

Explorando nuevas dianas para el desarrollo de terapias basadas en cannabinoides en la enfermedad de Parkinson

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Santiago Rodríguez Carreiro

DIRIGIDA POR

José Javier Fernández Ruiz
Elisa Navarro González de Mesa

Madrid

© Santiago Rodríguez Carreiro, 2025

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA

TESIS DOCTORAL

Exploring new targets for developing cannabinoid-based therapies in Parkinson's disease

Explorando nuevas dianas para el desarrollo de terapias basadas en cannabinoides en la enfermedad de Parkinson

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Santiago Rodríguez Carreiro

DIRECTORES

José Javier Fernández Ruiz

Elisa Navarro González de Mesa

Madrid, 2025

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA

Programa de doctorado de Bioquímica, Biología Molecular y Biomedicina

TESIS DOCTORAL

Exploring new targets for developing cannabinoid-based therapies in Parkinson's disease

Explorando nuevas dianas para el desarrollo de terapias basadas en cannabinoides en la enfermedad de Parkinson

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Santiago Rodríguez Carreiro

DIRECTORES

José Javier Fernández Ruiz

Elisa Navarro González de Mesa

Madrid, 2025

TABLE OF CONTENTS

SUMMARY/*RESUMEN*

EXPLORING NEW TARGETS FOR DEVELOPING CANNABINOID-BASED THERAPIES IN PARKINSON'S DISEASE

Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterised by hypokinetic motor symptoms and various non-motor manifestations. Its main neurohistopathological hallmarks include (i) the loss of dopaminergic neurons in the substantia nigra (SN) pars compacta, leading to striatal dopamine depletion, and (ii) the accumulation of misfolded α-synuclein aggregates, known as Lewy bodies. While the exact cause of this selective neurodegeneration remains uncertain, major risk factors include ageing, genetic predisposition, and environmental influences. Contributing mechanisms such as impaired proteostasis, neuroinflammation, mitochondrial dysfunction, oxidative stress, and excitotoxicity interact and worsen neuronal damage, disrupting basal ganglia circuits and motor control. In the absence of a cure, PD motor symptoms are commonly managed with dopamine replacement therapies, particularly levodopa. However, long-term use leads to complications that reduce therapeutic efficacy. Hence, there is an urgent need for disease-modifying strategies, a goal hindered by late diagnosis and complex pathogenesis.

Among emerging therapeutic avenues, modulation of the endocannabinoid system (ECS) has gained attention. The ECS comprises cannabinoid receptors, endocannabinoids, and related enzymes, playing a central role in neurobiological processes. Alterations in the ECS have been described in PD, and several cannabinoids have shown neuroprotective properties, including anti-inflammatory, antioxidant, autophagy-promoting, and/or anti-excitotoxic actions, mainly in preclinical models. Despite this, few cannabinoid-based therapies have reached clinical trials.

This doctoral thesis aims to contribute to cannabinoid-based therapeutic development for PD by exploring novel targets within the ECS or related pathways. We conducted a comprehensive preclinical evaluation of their neuroprotective and anti-inflammatory effects using relevant *in vitro* and *in vivo* PD models. The work is structured into three chapters:

- **Chapter 1**: Evaluation of cannabinoid type-1 receptor (CB1R) activation and the role of its interactor BiP in an *in vivo* proteinopathy model.
- **Chapter 2**: Study of G protein-coupled receptor 55 (GPR55) as a novel pharmacological target using two murine models of proteinopathy and mitochondrial dysfunction.
- **Chapter 3**: Assessment of the anti-inflammatory and neuroprotective potential of the (+)-*trans* enantiomeric series of cannabidiol (CBD) and four analogues.

In Chapter 1, we explored CB1R activation in the context of protein homeostasis disruption, using a mouse model with local overexpression of α-synuclein with the mutation A53T (α-syn^{A53T}) in the SN via adeno-associated viral vectors (AAV). We evaluated the effect of the selective CB1R agonist ACEA and the partial deletion of BiP, an intracellular CB1R interactor. ACEA provided modest neuroprotection, preserving some dopaminergic neurons, but did not prevent motor deficits. ACEA also increased LAMP-1 and GFAP immunoreactivity, suggesting enhanced degradative flux and astrocytic activity. However, α-synuclein levels also rose, indicating possibly

ineffective lysosomal clearance. Furthermore, the α-syn^{A53T} lesion altered unfolded protein response gene expression in the striatum, including BiP, while ACEA had minimal effect. Partial BiP deletion did not significantly affect neurodegeneration or glial activation in the SN, although it did modify microglial Iba1 immunoreactivity. These results suggest that CB1R activation offers limited neuroprotection in this model, highlighting the need for alternative targets.

In the search for more effective cannabinoid-related targets, Chapter 2 focuses on GPR55, an orphan G protein-coupled receptor often considered an atypical cannabinoid receptor. It binds various cannabinoids, is highly expressed in the SN and striatum, and plays roles in motor control and inflammation. Although no robust changes in GPR55 expression were detected in *post-mortem* human samples or in murine models of PD, based on AAV9-α-syn^{A53T} or 6-hydroxydopamine (6-OHDA) lesions in the SN or striatum, respectively, functional studies using GPR55 knockout mice subjected to these two models revealed motor impairments, particularly in the rotarod test, with more pronounced deficits observed in females, despite unaltered performance in other motor assessments. In the α-syn^{A53T} model, GPR55 deletion improved performance in the cylinder rearing test and was associated with reduced dopaminergic loss and sex-specific changes in astroglial immunoreactivity and microglial morphology, particularly in females. Increased IL-1β and IFN-γ expression was also observed in the female GPR55-deficient mice. In the 6-OHDA model, milder improvements were noted, including better performance in the pole test, slight neuroprotection, but no glial or cytokine alterations. These data support a role for GPR55 in modulating inflammation and neurodegeneration, suggesting its inhibition as a therapeutic strategy in PD.

Lastly, Chapter 3 shifts the focus toward cannabinoid compounds with novel pharmacological profiles. CBD, a non-psychoactive compound found in *Cannabis sativa*, lacks direct CB1R/CB2R activity. However, modifying its stereochemistry has been shown to alter its pharmacological properties. We therefore studied the (+)-*trans* enantiomers of CBD and CBDV, along with three analogues (CBDA-Me, CBDA-Gly, and CBDA-Hyp), for their effects on neuroinflammation. *In vitro*, the (+)-enantiomers showed greater affinity for CB1R and CB2R, particularly the latter. (+)-CBD, (+)-CBDV, and (+)-CBDA-Me acted as CB2R agonists, reducing proinflammatory gene expression in microglial-like cells and protecting neurons in co-culture, in a CB2R-dependent manner. *In silico* analyses revealed favourable pharmacokinetics for (+)-CBD and (+)-CBDV. These two were selected for *in vivo* testing in the LPS-induced inflammation model. *In vivo*, neither compound significantly reduced GFAP or CD68 immunoreactivity in the SN. However, (+)-CBD preserved dopaminergic neurons and improved motor performance, indicating its potential as a neuroprotective and anti-inflammatory agent. These promising results merit further exploration of the therapeutic utility of stereochemically modified cannabinoids in PD.

In conclusion, this thesis reinforces the therapeutic promise of the ECS in PD. While CB1R activation provided limited benefit, targeting GPR55 or using stereochemically optimised cannabinoids such as (+)-CBD may offer novel, disease-modifying strategies. Continued research is warranted to translate these findings into effective treatments for PD.

EXPLORANDO NUEVAS DIANAS PARA DESARROLLAR TERAPIAS BASADAS EN CANNABINOIDES EN LA ENFERMEDAD DE PARKINSON

La enfermedad de Parkinson (EP) es el segundo trastorno neurodegenerativo más frecuente, caracterizado por síntomas motores hipocinéticos y diversas manifestaciones no motoras. Sus principales signos neurohistopatológicos incluyen: (i) la pérdida de neuronas dopaminérgicas en la sustancia negra (SN) *pars compacta*, lo que conlleva una disminución de dopamina en el cuerpo estriado, y (ii) la acumulación de agregados de α-sinucleína mal plegada, conocidos como cuerpos de Lewy. Aunque se desconoce la causa exacta de esta degeneración neuronal selectiva, los principales factores de riesgo incluyen el envejecimiento, la predisposición genética y ciertos factores ambientales. Además, múltiples mecanismos patológicos, como la disfunción de la proteostasis, la neuroinflamación, la alteración mitocondrial, el estrés oxidativo y la excitotoxicidad, interactúan y agravan el daño neuronal, afectando el funcionamiento de los ganglios basales y el control motor. En ausencia de una cura, los síntomas motores se tratan habitualmente con terapias sustitutivas de dopamina, especialmente con levodopa. Sin embargo, su uso prolongado conlleva complicaciones que reducen su eficacia terapéutica. Por ello, existe una necesidad urgente de estrategias modificadoras de la enfermedad, un objetivo obstaculizado por el diagnóstico tardío y la compleja patogénesis.

Entre las vías terapéuticas emergentes, la modulación del sistema endocannabinoide (SEC) ha cobrado relevancia. El SEC está compuesto por receptores cannabinoides, endocannabinoides y enzimas relacionadas, y desempeña un papel central en diversos procesos neurobiológicos. Se han descrito alteraciones en el SEC en la EP, y diversos cannabinoides han mostrado efectos neuroprotectores, incluyendo acciones antiinflamatorias, antioxidantes, promotoras de la autofagia y antiexcitotóxicas, principalmente en modelos preclínicos. No obstante, pocas terapias basadas en cannabinoides han llegado a ensayos clínicos.

Esta tesis doctoral tiene como objetivo contribuir al desarrollo de terapias basadas en cannabinoides para la EP mediante la exploración de nuevas dianas dentro del SEC o en rutas relacionadas. Para ello, se llevó a cabo una evaluación preclínica exhaustiva de su potencial neuroprotector y antiinflamatorio en modelos pertinentes de EP, tanto *in vitro* como *in vivo*. El trabajo se estructura en tres capítulos:

- **Capítulo 1**: Evaluación de la activación del receptor cannabinoide tipo 1 (rCB1) y del papel de su interactor BiP en un modelo de proteinopatía *in vivo*.
- **Capítulo 2**: Estudio del receptor acoplado a proteínas G 55 (GPR55) como nueva diana farmacológica usando dos modelos murinos de proteinopatía y disfunción mitocondrial.
- **Capítulo 3**: Análisis del potencial antiinflamatorio y neuroprotector de la serie enantiomérica (+)-*trans* del cannabidiol (CBD) y cuatro análogos.

En el Capítulo 1, se exploró la activación del rCB1 en un contexto de alteración de la homeostasis proteica, utilizando un modelo murino con sobreexpresión local de α-sinucleína con la mutación A53T (α-syn^{A53T}) en la SN mediante vectores virales adeno-asociados (AAV). Se

evaluaron los efectos del agonista selectivo del rCB1 ACEA y de la eliminación parcial de BiP, un interactor intracelular de rCB1. ACEA proporcionó una neuroprotección modesta, preservando parcialmente las neuronas dopaminérgicas, pero no evitó los déficits motores. Además, aumentó la inmunorreactividad de LAMP-1 y GFAP, lo que sugiere un incremento del flujo degradativo y de la actividad astrocitaria. Sin embargo, también aumentaron los niveles de α-sinucleína, lo que podría indicar una degradación lisosomal ineficaz. La lesión inducida por α-syn^{A53T} alteró la expresión de genes relacionados con la respuesta a proteínas mal plegadas en el estriado, incluyendo BiP, y ACEA tuvo un efecto modulador mínimo. La eliminación parcial de BiP no afectó de forma significativa la neurodegeneración ni la activación glial en la SN, aunque sí alteró la inmunorreactividad microglial de Iba1. Estos resultados indican que la activación de rCB1 ofrece una neuroprotección limitada en este modelo, lo que resalta la necesidad de explorar otras dianas terapéuticas.

En busca de nuevas dianas más eficaces relacionadas con los cannabinoides, el Capítulo 2 se centra en GPR55, un receptor huérfano acoplado a proteínas G, a menudo considerado como un receptor cannabinoide atípico. Este receptor, que se une a diversos cannabinoides, se expresa en alta proporción en la SN y el cuerpo estriado, y participa en la coordinación motora y la inflamación. Aunque no se detectaron cambios robustos en la expresión de GPR55 en muestras humanas *post-mortem* ni en modelos murinos de la EP, basados en la lesión con AAV9-α-syn^{A53T} o en la lesión con 6-hidroxidopamina (6-OHDA) en la SN o el cuerpo estriado, respectivamente, los estudios funcionales realizados en ratones *knockout* para GPR55 sometidos a estos dos modelos revelaron alteraciones motoras, especialmente en la prueba de rotarod, con déficits más acusados en hembras, a pesar de no observarse cambios en otras evaluaciones motoras. En el modelo de α-syn^{A53T}, la eliminación de GPR55 mejoró el rendimiento en la prueba del cilindro, y se asoció con una menor pérdida dopaminérgica y cambios en la inmunorreactividad astrocitaria y morfología microglial dependientes del sexo, más evidentes en hembras. También se observó un aumento de la expresión de IL-1β e IFN-γ en hembras *knockout*. En el modelo 6-OHDA se observaron mejoras más suaves, como un mejor rendimiento en la prueba del poste, leve neuroprotección y sin cambios gliales ni en citocinas. Estos datos apoyan un papel de GPR55 en la modulación de la inflamación y la neurodegeneración, sugiriendo que su inhibición podría ser una estrategia terapéutica en la EP.

Finalmente, el Capítulo 3 cambia el enfoque hacia compuestos cannabinoides con perfiles farmacológicos novedosos. El CBD, compuesto no psicoactivo presente en *Cannabis sativa*, carece de actividad directa sobre rCB1/rCB2. Sin embargo, se ha observado que modificar su estereoquímica puede alterar sus propiedades farmacológicas. Por ello, se estudiaron los enantiómeros (+)-*trans* de CBD y CBDV, junto con tres análogos (CBDA-Me, CBDA-Gly y CBDA-Hyp), por su efecto sobre la neuroinflamación. *In vitro*, los enantiómeros (+) mostraron mayor afinidad por rCB1 y especialmente por rCB2. (+)-CBD, (+)-CBDV y (+)-CBDA-Me actuaron como agonistas de rCB2, reduciendo la expresión de genes proinflamatorios en una línea celular microglial y protegiendo a las neuronas en co-cultivo, de forma dependiente de rCB2. Los análisis

in silico revelaron perfiles farmacocinéticos favorables para (+)-CBD y (+)-CBDV. Estos dos compuestos se seleccionaron para evaluación *in vivo* en el modelo inflamatorio inducido por LPS. Aunque ninguno redujo significativamente la inmunorreactividad de GFAP o CD68 en la SN, (+)-CBD protegió a las neuronas dopaminérgicas y mejoró el rendimiento motor en el modelo de LPS, lo que indica su potencial como agente neuroprotector y antiinflamatorio. Estos resultados prometedores justifican una investigación más profunda sobre el uso terapéutico de cannabinoides estereoquímicamente modificados en la EP.

En conclusión, esta tesis refuerza el potencial terapéutico del SEC en la EP. Aunque la activación de rCB1 ofreció beneficios limitados, la inhibición de GPR55 y el uso de cannabinoides optimizados como (+)-CBD podrían constituir estrategias modificadoras de la enfermedad. Se necesita más investigación para trasladar estos hallazgos a tratamientos efectivos contra esta patología tan compleja.

LIST OF ABBREVIATIONS

6-OHDA	6-Hydroxydopamine	**AAV**	Adeno-associated virus
AC	Adenylate cyclase	**ACEA**	Arachidonyl-2'-chloroethylamide
AD	Alzheimer's disease	**ADMET**	Absorption, distribution, metabolism, excretion and toxicity
AEA	N-arachidonoylethanolamine	**Akt**	Protein kinase B
BBB	Blood-brain barrier	**BSA**	Bovine serum albumin
C3	Complement component 3	**cAMP**	Cyclic adenosine monophosphate
CB1R	Cannabinoid type-1 receptor	**CB2R**	Cannabinoid type-2 receptor
CBD	Cannabidiol	**CBDA**	Cannabidiolic acid
CBDA-Gly	Cannabidiol-glycol ester	**CBDA-Hyp**	Cannabidiol-hydroxypentyl ester
CBDA-Me	Cannabidiol-methyl ester	**CBDV**	Cannabidivarin
CBG	Cannabigerol	**CBN**	Cannabinol
cDNA	Complementary DNA	**CNS**	Central nervous system
COX-2	Cyclooxygenase 2	**CREB**	cAMP response element-binding
CRT	Cylinder Rearing Test	**CSF**	Cerebrospinal fluid
Ct	Threshold cycle	**DA**	Dopamine
DAG	Diacylglycerol	**DBS**	Deep brain stimulation
DMSO	Dimethylsulfoxide	**EBST**	Elevated-body Swing Test
eCB	Endocannabinoid	**ECS**	Endocannabinoid system
eQTL	Expression quantitative loci	**ER**	Endoplasmic reticulum
ETC	Electron transport chain	**FAAH**	Fatty acid amide hydrolase
GC	Genome copy	**GCase**	Glucocerebrosidase
GFAP	Glial fibrillary acidic protein	**GPCR**	G protein-coupled receptor
GPe	Globus pallidus externus	**GPi**	Globus pallidus internus
GPR55	G protein-coupled receptor 55	**GWAS**	Genome-wide association studies
i.p.	Intraperitoneal	**IL**	Interleukin
INF-γ	Interferon γ	**iNOS**	Inducible nitric oxide synthase
KO	Knock-out	**KPBS**	Potassium phosphate-buffered saline
LAMP-1	Lysosomal-associated membrane protein 1	**LB**	Lewy body
L-DOPA	Levodopa	**LPI**	Lysophosphatidylinositol
LPS	Lipopolysaccharide	**LRRK2**	Leucine-rich repeat kinase 2
MAGL	Monoacylglycerol lipase	**MHC**	Major histocompatibility complex
MPP^+	1-methyl-4-phenylpyridinium	**MPTP**	Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSN	Medium spiny neuron	**mtDNA**	Mitochondrial DNA
MTT	Methylthiazolyldiphenyl-tetrazolium bromide	**NAPE**	N-arachidonoylphosphatidylethanolamine
NHP	Non-human primate	**NLRP3**	Nucleotide-binding domain, leucine-rich repeat, and pyrin domain-containing protein 3
NMDA	N-methyl-D-aspartate	**PBS**	Phosphate-buffered saline
PD	Parkinson's disease	**PFF**	Preformed fibril

LIST OF ABBREVIATIONS

PI3K	Phosphoinositide 3-kinase	**PKA**	cAMP-dependent protein kinase
PLC	Phospholipase C	**PPARγ**	Peroxisome proliferator-activated receptor γ
PPMI	Parkinson Progression Marker Initiative	**ROS**	Reactive oxygen specie
RT-qPCR	Reverse transcription polymerase chain reaction	**SEM**	Standard error of the mean
SNpc	Substantia nigra pars compacta	**SNpr**	Substantia nigra pars reticulata
STN	Subthalamic nucleus	**TBS**	Tris-buffered saline
TH	– Tyrosine hydroxylase	**TNF-α**	Tumour necrosis factor α
TRPV1	Transient receptor potential cation channel subfamily V member 1	**UPR**	Unfolded protein response
WT	Wild-type	**α-Syn**	α-Synuclein
Δ^9-THC	Δ^9-Tetrahydrocannabinol	**Δ^9-THCV**	Δ^9-Tetrahydrocannabivarin

INTRODUCTION

1. PARKINSON'S DISEASE

In 1817, a 62-year-old British surgeon, Dr. James Parkinson, authored a case series on six individuals who were suffering from a distressing medical condition. This monograph, titled 'An Essay on the Shaking Palsy,' is widely regarded as the first-ever description in medical literature of what we now recognize as Parkinson's disease (PD). Despite its lack of detail and the omission of many symptoms associated with the disease nowadays, Dr. Parkinson skilfully integrated various symptoms previously thought to be distinct conditions into a single disorder. Furthermore, he made a remarkable distinction in describing tremors, differentiating those '*produced by attempts at voluntary motion versus those which occur whilst the body is at rest*' (Parkinson, 1817, reedited as Parkinson, 2002). It was only towards the end of the 19th century that our understanding of PD significantly advanced. This progress was marked by the recognition of rigidity and bradykinesia as essential clinical features and a better understanding of the involvement of the *substantia nigra pars compacta* (SNpc) in the disease's pathogenesis (Trétiakoff, 1919). Since those early observations, notable strides have been made in comprehending the disease, along with the development of various therapeutic approaches. In this section, a brief examination will be conducted regarding the existing knowledge of this *paralysis agitans*.

1.1. Epidemiology

Surpassed only by Alzheimer's disease (AD), PD stands out as one of the most common neurodegenerative disorders worldwide. The estimated incidence of PD is approximately 14 new cases per 100,000 people each year in the general population. Nevertheless, this incidence increases significantly with age, reaching around 160 new cases per 100,000 individuals yearly in those aged over 65 (Nicoletti et al., 2023). Advances in healthcare and increased life expectancy have led to a doubling of PD prevalence over the past 25 years (World Health Organization, 2023). This upward trend is expected to continue in the coming decades, from 6.2 million cases in 2015 to 12.9 million by 2040 (Dorsey & Bloem, 2018). Mortality rates are not elevated during the first decade after disease onset and diagnosis. However, over time, mortality in individuals with PD eventually doubles in comparison to the non-affected population, resulting in an average life expectancy of 70 years (Poewe et al., 2017).

In addition to aging, epidemiological data reveal sex-based variations. As such, most populations report a higher incidence and prevalence in men than women, with a pooled male-to-female ratio of 3:2 (Hirsch et al., 2016). These disparities in sex prevalence may be attributed to a combination of biological (effects of sex hormones), genetic (sex-associated genes) or societal (gender-specific differences in exposure to environmental elements and healthcare) factors. Similar influences may also underlie the epidemiological specificities observed in diverse ethnicities and geographically defined populations (Van Den Eeden et al., 2003).

1.2. Aetiology and determinants

As with other neurodegenerative disorders, PD can be classified based on its aetiology into two categories: familial forms, which account for approximately 5-10% of all cases; and sporadic forms, which encompass most diagnosed cases.

1.2.1. Familial Parkinson's disease

Heritable forms of PD involve a clear genetic origin, resulting in an earlier onset of the disease and often more severe symptoms. As these familial cases imply a well-known genetic basis, they represent a key approach to understand the molecular mechanisms behind the pathogenesis of PD. For instance, the identification of *SNCA* as the first gene underlying autosomal dominant PD helped to elucidate α-synuclein (α-syn) inclusions as a main neuropathological feature of this disorder (Polymeropoulos et al., 1997).

Most of the familial cases are monogenic, displaying rare mutations in well-established Mendelian genes, which have received the acronym of PARK (**Table 1**). These causative genes display both autosomal dominant (*SNCA*, *LRRK2, VPS35*) and autosomal recessive (*PRKN*, *PINK1*, *DJ1, ATP13A2*) inheritance patterns. However, disease penetrance can vary and may be affected by factors such as age, specific pathogenic variant, patients' ethnicity, and a complex interplay between causal and risk variants (Jia et al., 2022). The functions of the proteins encoded by these genes, as well as the pathological consequences of their mutations in PD, will be discussed in greater detail in subsequent sections.

1.2.2. Sporadic Parkinson's disease

Besides monogenic forms of the disease, PD is typically an idiopathic condition, with a later onset and a more progressive course compared to familial forms. These predominant sporadic cases are characterised by an unknown origin and a multifactorial aetiology, suggesting that they result from the complex interplay between environmental and genetic factors, which mutually influence each other. Genetic susceptibility, albeit significant, has been estimated to explain only one-quarter of the risk of developing PD (De Miranda et al., 2022). Consequently, non-genetic contributors, collectively referred to as the exposome, account for a significant portion of the risk. Furthermore, given that idiopathic PD develops over several decades, a wide range of exogenous factors can influence disease progression, including exposure to toxins, infections, pollutants, and lifestyle.

1.2.2.1. Aging

Epidemiological studies indicate that aging is the most significant factor influencing the occurrence of PD. Aging is a complex process that involves a plethora of changes, many of which may contribute to the pathogenesis of the disease. One extensively studied hallmark of aging is mitochondrial dysfunction, which arises from accumulated defects and failures in mitochondrial quality control. When combined with other factors, mitochondrial dysfunction can significantly

Table 1. PARK genes involved in familial PD. *PARK3 PARK10, PARK12, PARK16, and PARK22 are not included in this table, as they correspond to identified risk variants whose affected gene has not been identified. AD: autosomal dominant, AR: autosomal recessive.*

PARK Locus	Map position	Gene	Inheritance	Description
PARK1/PARK4	4q21	*SNCA*	AD	α-syn
PARK2	6q25.2-q27	*Parkin*	AR	parkin RBR E3 ubiquitin protein ligase
PARK5	4p14	*UCH-L1*	AD	ubiquitin C-terminal hydrolase L1
PARK6	1p35-p36	*PINK1*	AR	PTEN-induced putative kinase 1
PARK7	1p36	*DJ-1*	AR	Parkinsonism-associated deglycase
PARK8	12q12	*LRRK2*	AD	LRRK2
PARK9	1p36	*ATP13A2*	AR	Cation-transporting ATPase 13A2
PARK11	2q36-q37	*GIGYF2*	AD	GRB10 interacting GYF protein 2
PARK13	2p13	*HTRA2*	AD	HtrA serine peptidase 2
PARK14	22q13.1	*PLA2G6*	AR	Calcium-independent phospholipase A2 enzyme
PARK15	22q12-q13	*FBXO7*	AR	F-box protein 7
PARK17	16q11.2	*VPS35*	AD	Vacuolar protein sorting-associated protein 35
PARK18	3q27.1	*EIF4G1*	AD	Eukaryotic translation initiation factor 4 gamma 1
PARK19	1p31.3	*DNAJC6*	AR	HSP40 Auxilin
PARK20	21q22.11	*SYNJ1*	AR	Synaptojanin 1
PARK21	3q22.1	*DNAJC13*	AD	Receptor-mediated endocytosis 8
PARK23	15q22.2	*VPS13C*	AR	Vacuolar protein sorting-associated protein 13C

contribute to the development of age-related diseases (Guo et al., 2023). Additional age-related changes associated with neurodegeneration include elevated oxidative stress, disrupted proteostasis, altered inflammatory responses, diminished sensitivity to neurotrophic factors, iron accumulation, and age-related genetic alterations (Hindle, 2010). However, in addition to these non-specific detrimental aging-associated processes, a distinct mechanism of age-related vulnerability involving neuromelanin has been specifically described for PD. Neuromelanin is an intracellular pigment exclusive to humans that accumulates in dopaminergic neurons of the SNpc throughout life as a byproduct of dopamine (DA) metabolism. During PD, neuromelanin decreases as these neurons die, serving as a marker of disease progression. However, recent studies suggest that neuromelanin accumulation in dopaminergic neurons of the SNpc is sufficient to trigger neuronal dysfunction and death, emphasizing the relevance of this lifelong accumulating pigment in the pathogenesis of PD (Carballo-Carbajal et al., 2019).

1.2.2.2. Genetic susceptibility

Despite the occurrence of clear causative genes, PD genetics reveal a greater complexity than was initially apparent. Genetic contributions to PD exist on a spectrum, ranging from highly penetrant rare variants linked to familial cases, as mentioned earlier (**Table 1**), to common variants with smaller individual effects but with the potential to interact with other genetic or environmental

factors (**Figure 1**). These common variants are usually found in sporadic PD cases and have been mostly identified using genome-wide association studies (GWASs). To date, ninety GWAS variants associated with PD have been confirmed across 78 loci (Nalls et al., 2019). However, these findings are limited to cohorts of European ancestry, with relatively little known about the genetics of PD in other ethnic populations and needing to diversify these studies.

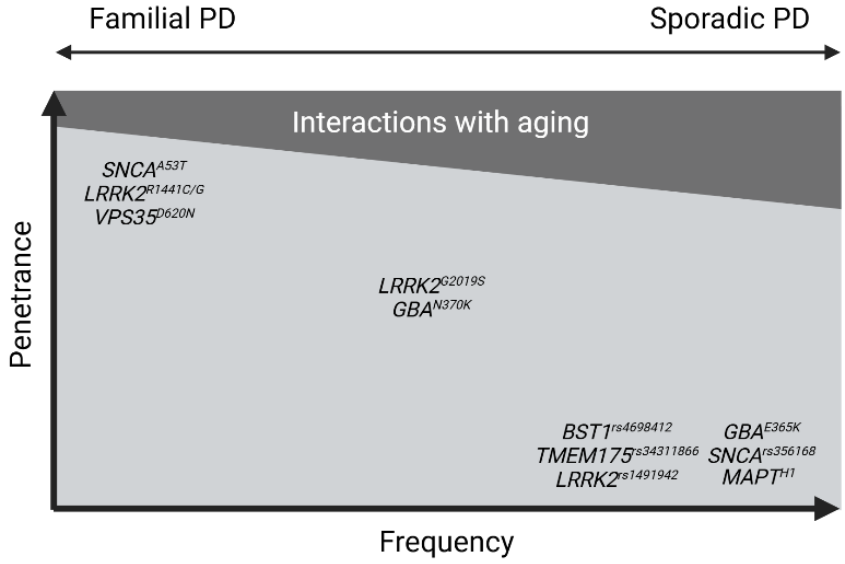


Figure 1. A simplified scheme of genetic architecture of PD. *PD risk alleles show diversities in frequency and penetrance, which can also be modified by age. Highly penetrant rare mutations would be responsible for familial conditions, while low-impact common variants would contribute to idiopathic cases. (Adapted from Ye et al., 2023).*

Several of the identified loci are located near genes involved in mitochondrial and lysosomal function. The two main PD risk genes, *LRRK2* and *GBA1*, harbor mutations associated with approximately 10% of sporadic cases. These mutations are thought to exacerbate α-syn toxicity and amplify the neuroinflammatory response (Kaiser et al., 2023). *LRRK2* is responsible for encoding leucine-rich repeat kinase 2 (LRRK2), a complex protein with kinase and GTPase activity, involved in cytoskeleton dynamics, vesicular traffic, autophagy, immune response and mitochondrial function (Esteves et al., 2014). *LRRK2* mutations display diverse frequencies, penetrance, and effect sizes, with reported increases in kinase activity or reductions in GTPase, although findings have not always been consistent across studies (Ito & Utsunomiya-Tate, 2023). Notably, in idiopathic PD patients, the LRRK2 kinase activity has been found to be aberrantly increased in vulnerable nigrostriatal dopaminergic neurons (Di Maio et al., 2018). *GBA1* encodes glucocerebrosidase (GCase), a lysosomal enzyme responsible for sphingolipids metabolism, whose dysfunction has been related to Gaucher's disease, and more recently to the risk of developing PD (Do et al., 2019). Specifically, *GBA* mutations result in a nearly 4-fold increase in PD risk, loss of lysosomal enzyme activity, and an elevated risk of developing dementia (Simon et al., 2020). Of note, idiopathic PD patients exhibit GCase deficiency, even without *GBA* mutations (Gegg et al., 2012).

While common genetic variability is estimated to contribute to approximately 25% of the heritability of PD, the GWAS loci currently identified explain only a small fraction of this heritability (Blauwendraat et al., 2020). This suggests the presence of many undiscovered risk

variants. Additionally, acquired mitochondrial DNA (mtDNA) mutations (Müller-Nedebock et al., 2019) and epigenetic factors (Song et al., 2023) are also believed to play a role in the pathogenesis of PD.

1.2.2.3. Environmental factors

In addition to aging and genetic susceptibility, the past four decades have seen the identification of an expanding list of modifiable environmental factors associated with PD risk. Meta-analyses have demonstrated that various aspects of rural living, such as the use of well water, farming, and exposure to pesticides or herbicides like rotenone and paraquat, both inhibitors of the mitochondrial complex I, are linked to increased risk (Priyadarshi et al., 2001). Other well-documented environmental risk factors include exposure to metals, toxins such as methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and drugs like methamphetamine or antipsychotics. Additionally, certain medical conditions such as head trauma, melanoma, peripheral inflammation, and even gut microbiota dysbiosis, have been associated with increased PD risk (Simon et al., 2020; Krasowska et al., 2021; Wang et al., 2025). Conversely, several epidemiological studies have pointed to a significantly reduced risk of PD among tobacco smokers, coffee drinkers, and individuals treated with anti-inflammatory agents (De Lau & Breteler, 2006; Simon et al., 2020). Engaging in physical activity and following diets rich in fruits, vegetables, and whole grains have also been identified as lifestyle factors associated with a reduced risk of PD. Nevertheless, despite the breadth of the exposome, it is important to note that, in most cases, a complex interplay between environmental factors and genetic susceptibility is crucial to understanding and elucidating the aetiology of PD (Polito et al., 2016).

1.3. Clinical features

PD ranks as the first disorder affecting basal ganglia, a brain structure essential for motor control. Therefore, PD is primarily recognised as a movement disorder, defined by parkinsonism, a clinical syndrome characterised by bradykinesia, rigidity, rest tremor, and postural instability. Idiopathic PD is the most common form of parkinsonism, but there are other conditions with overlapping symptoms, hindering its accurate diagnosis (**Box 1**). Essential tremor, secondary parkinsonism (vascular, drug-induced) or atypical parkinsonism (dementia with Lewy Bodies, multiple system atrophy) are examples of other pathologies that may include parkinsonism (Keener & Bordelon, 2016; Poewe et al., 2017). Differential diagnosis between PD and other clinical forms of parkinsonism typically relies on the coexistence of specific features and the responsiveness of motor symptoms to DA replacement therapies (Keener & Bordelon, 2016).

1.3.1. Motor symptoms

The primary clinical manifestations of PD are predominantly characterised by motor dysfunction. Indeed, the clinical diagnosis is typically established upon the emergence of asymmetric rigidity and bradykinesia, with or without tremor at rest (Bloem et al., 2021). Bradykinesia refers to the slowing of movement and a decrement in amplitude or speed of movement, often observed as

BOX 1. CLINICAL DIAGNOSTIC CRITERIA FOR PARKINSON'S DISEASE

Several scientific societies and academies have developed their own sets of criteria for PD diagnosis. Here, those established by the Movement Disorder Society will be presented.

Step 1: Identification of parkinsonism

- Presence of bradykinesia
- Coexistence with rest tremor and/or rigidity

Step 2: Verification of PD with two levels of diagnostic certainty

Diagnosis of clinically established PD requires:

- Absence of absolute exclusion criteria. These criteria include clinical or imaging evidence supporting alternative diagnoses of parkinsonism, such as atypical parkinsonism, drug-induced parkinsonism, or essential tremor.
- At least two supportive criteria. These criteria include responsiveness of DA replacement therapy, the presence of classic rest tremor or the occurrence of either olfactory loss or cardiac sympathetic denervation.
- No red flags. This refers to features that are atypical but not entirely exclusionary for Parkinson's disease, such as the rapid progression of gait impairment necessitating wheelchair use or the onset of severe autonomic failure within five years of disease onset.

Diagnosis of clinically probable PD requires:

- Absence of absolute exclusion criteria (mentioned above).
- Presence of red flags (mentioned above) counterbalanced by supportive criteria.
    - If one red flag is present, there must also be at least one supportive criterion.
    - If two red flags, at least two supportive criteria are needed.
    - No more than two red flags are allowed for this category.

A full listing of absolute exclusion criteria, red flags and supportive criteria is presented in Postuma et al., 2015.

impaired facial dynamics. This manifests as a less expressive face or 'masked' face, along with monotone and softer speech intonation, where words may blend together (Hayes, 2019). In terms of tremor, its frequency ranges between 4 and 6 Hz and initially presents unilaterally, most prominently in the distal upper extremity, sometimes involving a single finger (Keener & Bordelon, 2016; Hayes, 2019).

Clinical observations of PD reveal significant heterogeneity in motor features and cohort studies have led to an empirical categorization into two major subtypes: tremor-dominant PD, characterised by a relative absence of other motor symptoms, and non-tremor-dominant PD, which is marked by postural instability and gait disorder (Keener & Bordelon, 2016; Hayes, 2019). Although both subtypes are primarily based on symptomatologic classification, they also differ in progression and prognosis. For instance, tremor-dominant PD tends to exhibit a slower progression and less functional disability compared to non-tremor-dominant PD (Kalia & Lang, 2015). Moreover, it is hypothesised that the different subtypes of PD have distinct aetiologies and pathophysiological mechanisms.

As PD advances, there is a progressive exacerbation of motor disabilities, initially responsive to symptomatic therapeutic interventions. In the later stages of PD, treatment-resistant symptoms are prominent, encompassing axial motor symptoms such as postural instability, freezing of gait, falls, dysphagia (with choking), and speech dysfunction (**Figure 2**). These final features are normally the strongest predictors of institutionalization and death (Kalia & Lang, 2015).

1.3.2. Non-motor symptoms

In addition to the cardinal motor features, most PD patients also exhibit non-motor symptoms. These symptoms affect a wide range of bodily functions, including disturbances in the sleep-wake cycle, cognitive decline, mood and affective disorders, and autonomic dysfunction (urinary incontinence, symptomatic postural hypotension), as well as fatigue and pain (Kalia & Lang, 2015). Indeed, many of these non-motor symptoms can manifest before the appearance of classic motor features and may even precede the diagnosis of the disease (**Figure 2**). The most common symptoms during this prodromal or pre-motor phase of the disease, which can extend for years or even decades, include hyposmia or impaired olfaction, constipation, depression, anxiety, excessive daytime sleepiness and rapid eye movement sleep behaviour disorder (Poewe et al., 2017). Notably, both mood disorders and constipation have been linked to a two-fold increased risk of developing PD in the future (Noyce et al., 2012), whereas the sleep disturbances and the olfactory impairments are increasingly recognised as potential early biomarkers for PD (Orso et al., 2024).

As the disease progresses, non-motor symptoms become increasingly prevalent, with dementia being particularly widespread (Poewe et al., 2017). These non-motor features are strongly associated with diminished health-related quality of life, significantly contributing to overall disability progression and serving as key determinants of nursing home placement and mortality (Kalia & Lang, 2015). Nevertheless, these symptoms also offer a valuable opportunity for earlier PD diagnosis, thereby providing a broader temporal window for therapeutic interventions.

1.3.3. Long-term therapy complications

In advanced stages of PD, the worsening of both motor and non-motor symptoms is often accompanied by the emergence of complications arising from a prolonged DA replacement therapy. As previously noted, this dopaminergic treatment allows for the management of the motor symptoms of the disease for several years. However, it has been extensively reported how chronic administration of these drugs frequently leads to severe adverse effects, ranging from motor issues, such as motor fluctuations and dyskinetic movements, to cognitive disturbances, with psychosis and visual hallucinations being particularly prominent (**Figure 2**) (Poewe et al., 2017).

These therapy-induced complications, combined with non-motor symptoms and motor disabilities that respond poorly to treatment, contribute to the cumulative burden of disability suffered by PD patients (Kalia & Lang, 2015).

1.4. Pathophysiology

Two major neuropathological hallmarks necessarily must occur when facing PD: the loss of neurons in specific regions of the SN and the widespread accumulation of intracellular α-syn (**Figure 3**). Neuronal degeneration and cell death are observed specifically in dopaminergic neurons within the SNpc, resulting in denervation and depletion of DA in the striatum. This

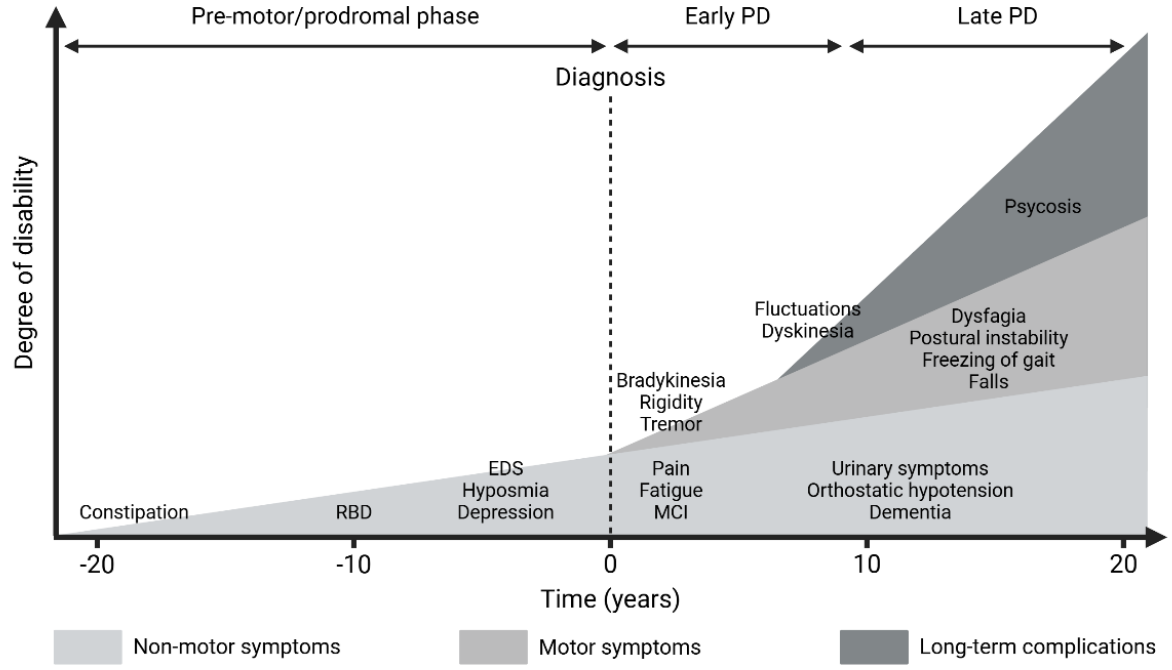


***Figure 2. Time progression of clinical symptoms in PD**. Diagnosis of PD typically occurs when motor symptoms begin, but before that moment, a prodromal stage of 20 years or more could have developed, with several non-motor features. With disease progression, both motor and non-motor manifestations progress while new ones appear, causing significant disability in advanced PD. Long-term complications of dopaminergic therapy further contribute to disability. (Adapted from Kalia & Lang, 2015). Created with BioRender.com.*

specific neuronal loss triggers the onset of clinical motor symptoms, which become apparent when approximately 50% of nigrostriatal neurons have been lost (Fearnley & Lees, 1991) and striatal dopaminergic terminals are significantly depleted (Brooks & Pavese, 2011). The delay in the emergence of motor symptoms is attributed to compensatory mechanisms within the dopaminergic pathways that sustain circuitry function until DA levels are very low (Yee et al., 2000). In the early stages of the disease, the loss of dopaminergic neurons is predominantly confined to the ventrolateral SNpc, which contains neurons projecting to the dorsal putamen of the striatum, with a relative sparing of other midbrain dopaminergic neurons (Damier et al., 1999). However, as the disease progresses to advanced stages, dysfunction extends beyond the dopaminergic system, involving other neurotransmitter pathways and peripheral mechanisms, which may account for some of the non-motor symptoms (Sveinbjornsdottir, 2016).

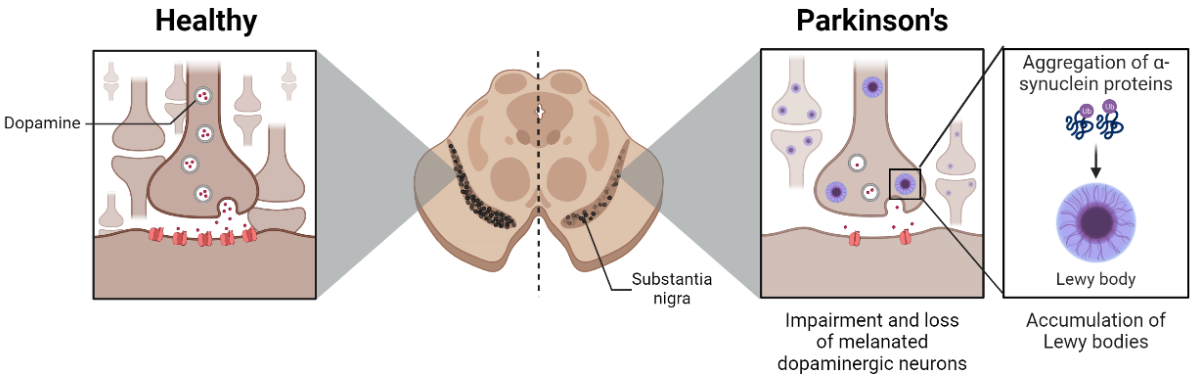


***Figure 3. Main neuropathological hallmarks in PD**. The current pathological criteria for PD require the identification of two key histopathological features: functional and structural loss of dopaminergic neurons within the SNpc, and Lewy pathology, with insoluble inclusions of α-syn within the neurons. Adapted from template of BioRender.com.*

The presence of intracellular inclusions containing aggregates of α-syn is another criterion for a definitive (*post-mortem*) neuropathological diagnosis of PD (Kalia & Lang, 2015). These

proteinaceous inclusions, known as Lewy bodies (LBs), are thought to result from a severe proteinopathy, referred to as Lewy pathology. However, the pathophysiology of PD is multifaceted, involving cytotoxic mechanisms beyond protein dysregulation, such as mitochondrial dysfunction, oxidative stress, neuroinflammation, excitotoxicity, and impaired Ca^{2+} homeostasis (Poewe et al., 2017). Notably, these pathological processes should not be considered isolated events; instead, they should be viewed as an interconnected damaging network, where each event interacts and amplifies the others, creating a synergistic effect that drives neurodegeneration in a self-perpetuating cycle (**Figure 4**).

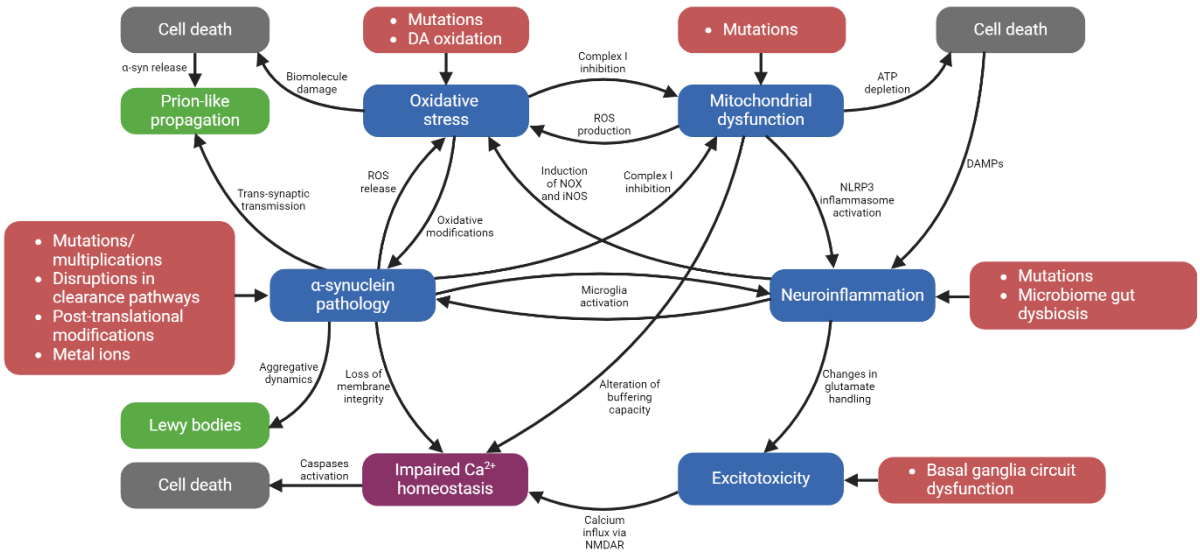


Figure 4. Molecular mechanisms implicated in the PD pathophysiology*. Five major cytotoxic events contribute to the neurodegeneration, including protein dysregulation, mitochondrial dysfunction, neuroinflammation, oxidative stress, and excitotoxicity. All these mechanisms can exhibit external causalities such as genetic alterations, even though they also interact with each other, generating a complex network of interactions that creates a vicious cycle. Thus, cell death is a consequence of the synergy of multiple processes. Created with BioRender.com.*

1.4.1. Basal ganglia circuit dysfunction

As previously mentioned, the SNpc represents the distinctive region undergoing degeneration in PD. This area is integrated into the basal ganglia, a subcortical structure responsible for regulating actions and goal-directed behaviour. Anatomically, it is organised by the flow of cortical inputs to subcortical outputs, which then return to the cerebral cortex, suggesting a 'filter-like' function for coordinating movement (Alexander et al., 1990). Different models have been devised to explain how the neural circuitry within the basal ganglia regulates movement, with the classical model being the most accepted one (McGregor & Nelson, 2019). According to this model of the basal ganglia-thalamocortical loop, cortical activity is transmitted to subcortical regions by three streams that act in concert to shape action dynamics: the direct, indirect and hyperdirect pathways. The direct and indirect pathways provide cortical inputs to the basal ganglia via the striatum. Initially, information flow involves corticostriatal glutamatergic projections from the motor-related cortex to GABAergic striatal medium spiny neurons (MSNs). DA released from the SNpc regulates the activity of these striatal MSNs, which can be categorised into two subtypes. The first subtype, which releases the neuropeptides Substance P and dynorphin, expresses activating DA D1 receptors, coupled to G_S proteins, and directly projects to GABAergic neurons in globus

pallidus internus (GPi)/SN *pars reticulata* (SNpr). This organization represents the direct pathway. The second subtype, which releases the neuropeptide enkephalin, expresses inhibiting DA D2 receptors, coupled to G_i proteins, and projects to GABAergic neurons in globus pallidus externus (GPe), eventually reaching GPi/SNpr via the subthalamic nucleus (STN) as a glutamatergic relay. This configuration constitutes the indirect pathway. Finally, GPi and SNpr serve as the main output nuclei of the basal ganglia, projecting to the ventrolateral thalamus, and ultimately back to motor cortex. The last basal ganglia circuit to be described, the hyperdirect pathway, consists of glutamatergic connections from the frontal cortex to the STN, without involving striatal areas and providing rapid inhibition for action suppression (Nambu et al., 2002).

In a physiological context (**Figure 5A**), DA levels in the striatum are normal. Thus, striatal dopaminergic tone properly regulates the GABAergic output activity of the striatum, activating the D1-mediated direct pathway, and inhibiting the D2-mediated indirect pathway. The outcome of these summative effects is the reduction of the GPi/SNpr GABAergic output and the subsequent disinhibition of the thalamocortical feedback projections. In the parkinsonian condition (**Figure 5B**), the loss of dopaminergic neurons in the SNpc results in a nigrostriatal DA deficiency. This situation causes hypoactivity of the direct pathway and hyperactivity of the indirect pathway, with a net effect of a strong increase in the firing rate of GABAergic basal ganglia output nuclei, which over-inhibit thalamocortical projections, leading to inhibition of motor cortex, suppression of movements and onset of parkinsonian symptoms (McGregor & Nelson, 2019).

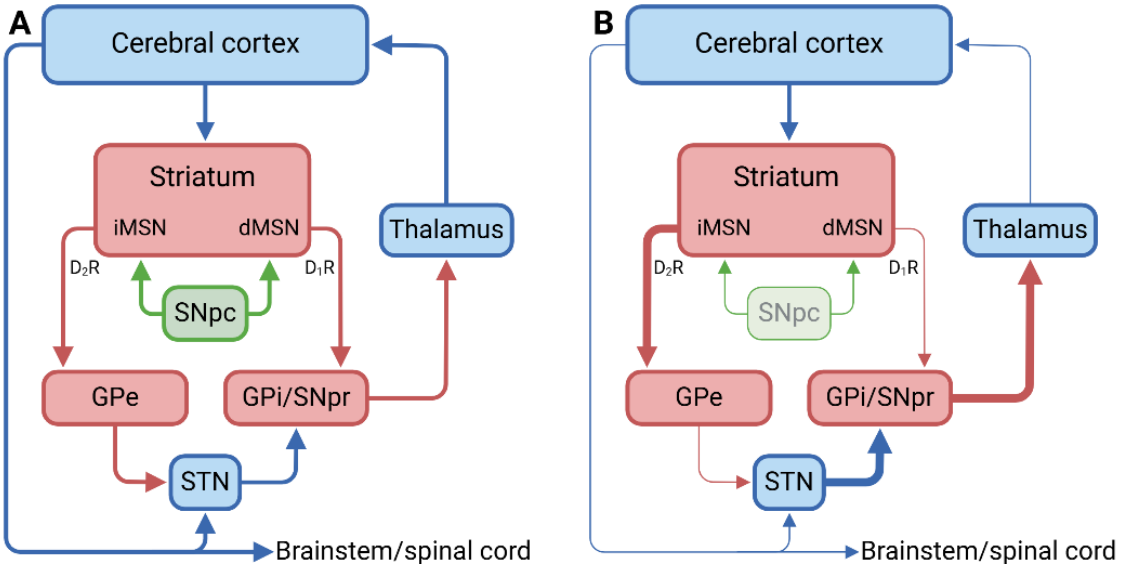


***Figure 5. Classical model of the basal ganglia circuit**. (A) Under healthy conditions, dopaminergic neurons from the SNpc to the striatum activate direct pathway MSNs (dMSNs) via dopamine D1 receptors (D_1R) and inhibit indirect pathway MSNs (iMSNs) via dopamine D2 receptors (D_2R). These striatal GABAergic projections decrease GPi output either directly or indirectly, by means of STN inhibition mediated by the GPe. (B) In the parkinsonian condition, loss of SNpc dopaminergic neurons leads to hypoactivity of the direct pathway and hyperactivity of the indirect pathway, causing excessive GPi output and resulting in excessive inhibition of the thalamus and cortex. Green arrows: dopaminergic projections; red arrows: GABAergic projections; blue arrows: glutamatergic projections; thin arrows: reduced effect; thick arrows: increased effect. (Adapted from McGregor & Nelson, 2019). Created with BioRender.com.*

1.4.2. Protein dysregulation and α-synuclein aggregation

As with most neurodegenerative disorders, PD displays a significant proteinopathy-like pathology, characterised by intraneuronal protein aggregates found in all patients. Specifically, PD is

considered a synucleinopathy, given that protein inclusions are largely composed of α-syn (Spillantini et al., 1997). This small protein (14.5 kDa), consisting of 140 amino acids, is divided into three distinct regions: a lysine-rich amphipathic N-terminal lipid-binding domain, enabling interaction with membranes and vesicles; a hydrophobic non-amyloid component, with a high tendency to aggregate; and a proline-rich acidic C-terminal binding domain, which contains a highly flexible intrinsically disordered region, allowing interaction with metals, small molecules and proteins (Vidović & Rikalovic, 2022). In solution, monomeric α-syn exists as an intrinsically disordered or unfolded soluble protein, while interaction with lipid membranes drives the transition of the N-terminus and non-amyloid component into stabilised helical structures. This helical conformation may form non-pathogenic homotetramers (Nuber et al., 2018). Regarding the physiological function of α-syn, it has not been fully elucidated, but it appears to regulate synaptic plasticity and function as well as vesicle trafficking, supported by its pre-synaptic location. This protein also seems to participate in mitochondrial function and may act as a potential chaperone (Poewe et al., 2017).

In PD, α-syn misfolds and aggregates into insoluble amyloid fibrils rich in β-sheets. During this pathogenetic process, soluble monomers form intermediate, transient oligomeric structures, which then progressively assemble into protofibrils, ultimately resulting in large ordered insoluble α-syn strains (Vidović & Rikalovic, 2022). It remains debatable which α-syn assemblies are most relevant to neurotoxicity, although oligomers of lower molecular mass seem to be more unstable and toxic (Chen et al., 2022). Several factors have been identified as triggers for the misfolding, accumulation, and aggregation of α-syn, including *SNCA* mutations (A30P, E46K, A53T), gene locus multiplications, post-translational modifications (phosphorylation, ubiquitination, acetylation, glycation), interaction with metal ions (Fe, Cu, Mn), and defects in molecular clearance pathways (Vidović & Rikalovic, 2022). α-Syn degradation is mainly driven by the ubiquitin–proteasome system, with the involvement of E3-ubiquitin ligases such as parkin; and autophagy-lysosomal pathways, particularly both chaperone-mediated autophagy and macroautophagy (Xilouri et al., 2013). Several lines of evidence suggest that impairment of clearance pathways might enhance α-syn accumulation. In patients and experimental models, a reduction in lysosomal enzyme activity (GCase or cathepsin D), a decrease in markers of chaperone-mediated autophagy (HSC70 or LAMP2A), and an accumulation of autophagosomes have been observed (Hou et al., 2020). Furthermore, a vicious cycle is established, as neurotoxic forms of α-syn can also disrupt proteostasis (Winslow et al., 2010). The accumulation of misfolded proteins in the endoplasmic reticulum (ER) provokes stress within this organelle, triggering a protective response known as unfolded protein response (UPR). This repair process aims to correct the misfolded proteins and restore proteostasis by temporarily reducing the synthesis of non-essential proteins, activating chaperones such as BiP (**Box 2**) to assist in protein folding, and increasing the expression of factors that promote protein maturation and quality control (Melo et al., 2022). Clinical reports indicate a strong correlation between ER stress and the pathogenesis of PD. For instance, in the SNpc of PD patients, markers of ER stress and UPR, including BiP, were

found to be elevated and co-localised with α-syn (Wang et al., 2023a), revealing another mechanism involved in LB pathology.

BOX 2. CHAPERONE BiP IN ENDOPLASMIC RETICULUM STRESS

The molecular chaperone binding immunoglobulin protein (BiP), also known as glucose-regulated protein 78 kDa (GRP78) or heat shock protein family A member 5 (HSPA5), is primarily localised in the ER. It serves as a chaperone by facilitating the transport of proteins into the ER lumen and assisting in the removal of misfolded proteins by directing them to the cytoplasm. Additionally, as mentioned above, BiP plays a pivotal role in the UPR. In the absence of ER stress, BiP inhibits the activation of three key UPR factors: inositol-requiring enzyme 1 (IRE1), protein kinase R-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). Under conditions of ER stress, BiP dissociates from these three factors, thereby allowing them to become activated and trigger adaptive responses, such as the upregulation of genes encoding UPR-related proteins, including chaperones that facilitate proper protein folding (**Figure 6**) (Shen et al., 2002; Kopp et al., 2019).

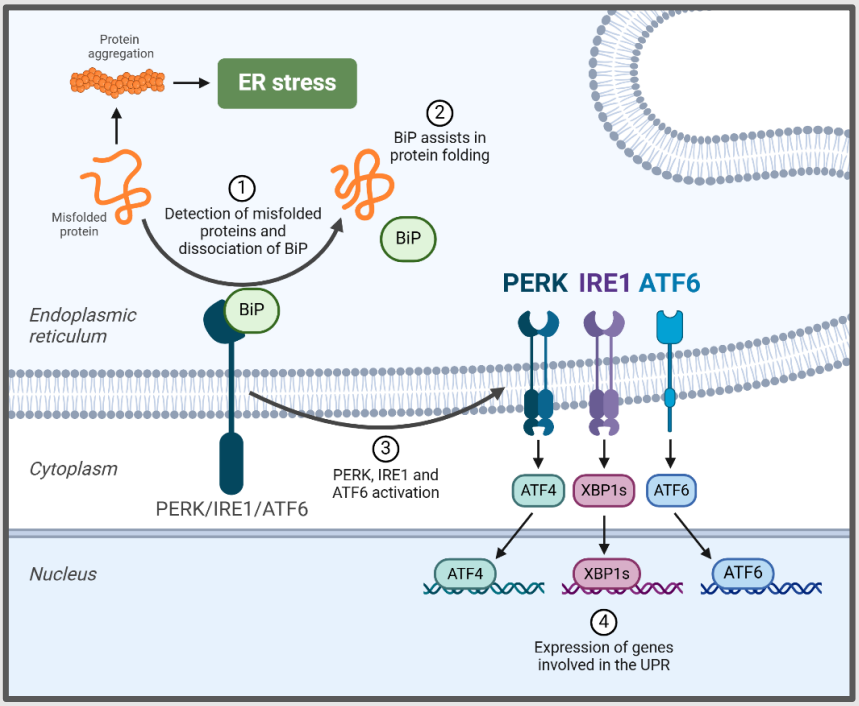


***Figure 6**. Diagram of the mechanism of action of BiP in the UPR. Adapted from template of BioRender.com.*

Although BiP expression decreases during healthy aging, it appears to either increase or decrease in neurodegenerative conditions, depending on the stage of the disease and the associated pathological processes. In this specific context, modulation of BiP levels has been shown to offer therapeutic benefits in various neurodegenerative diseases. For instance, BiP induction in PD models or models of retinal degeneration resulted in reduced apoptosis and increased neuronal survival (Gorbatyuk & Gorbatyuk, 2013). In contrast, a reduction in BiP levels improved memory deficits in a Huntington's disease model (Espina et al., 2023).

In addition to accumulation and aggregation, α-syn appears to exhibit transneuronal propagation in a prion-like manner. Thus, aggregated α-syn fibrils can act as 'seeds' that escape from their original neuron to be captured by neighbouring neurons and, once inside, induce normal α-syn to misfold and aggregate in the new host cell (Chung et al., 2019). In this way, the pathological process initiated by a few cells containing misfolded α-syn may progressively evolve into a widespread synucleinopathy, affecting multiple brain regions as the disease advances and reflecting clinical observations (Braak et al., 2003). The transmission can occur synaptically or take place intercellularly through heparan sulfate proteoglycans, tunnelling nanotubes or exosomes (Chen et al., 2022). Moreover, this prion-like hypothesis supports the current notion

that the α-syn aggregation and propagation are initiated peripherally, particularly in the enteric nervous system and the olfactory bulb (Hawkes et al., 2007). Exposure to toxicants, such as the inhalation of pesticides, herbicides, and the role of the gut microbiota could potentially trigger α-syn pathology in these sites, before it spreads, eventually reaching the SN and leading to motor disturbances (Wang et al., 2025). These early events are consistent with prodromal signs such as anosmia or constipation.

When α-syn misfolds and aggregates, it acquires cytotoxic properties, but the precise mechanism by which oligomers and aggregates promote neuronal death remains unclear. Numerous studies have provided insight into this process, revealing the involvement of various cellular mechanisms. These include impaired protein degradation and proper folding, with interference in ubiquitin–proteasome system (McKinnon et al., 2020); loss of membrane integrity, leading to increased permeability and elevated basal intracellular Ca^{2+} levels (Angelova et al., 2016); mitochondrial dysfunction, affecting mitochondrial dynamics and inhibiting complex I activity (Rocha et al., 2018); oxidative stress, promoting the release of reactive oxygen species (ROS) (Deas et al., 2016); neuroinflammation, activating microglia to release inflammatory factors and exert cytotoxic effects (Hoenen et al., 2016); and disruption of vesicle trafficking and axonal transport (Volpicelli-Daley et al., 2014).

1.4.3. Mitochondrial dysfunction

Mitochondria are highly dynamic and multifunctional organelles, involved in a wide range of functions, including energy production, calcium homeostasis, metabolic and redox signalling, and apoptosis. Proper mitochondrial function is especially crucial for neuronal activity and survival. Neurons have a high bioenergetic demand to maintain ionic homeostasis and synaptic function, which is largely met through mitochondrial oxidative phosphorylation for ATP production. For that reason, mitochondrial dysfunction can impact neurons more severely than other cell types. More specifically, nigral dopaminergic neurons display some features that make them particularly vulnerable to energy deficiency: (i) they are continuously active, with constant calcium oscillations and calcium extrusions that incur high energy costs (Pissadaki & Bolam, 2013); and (ii) they have long unmyelinated axons and extensive axonal arborization with a massive number of synaptic sites, all of which require substantial energy to be mantained (Bolam & Pissadaki, 2012). Therefore, mitochondrial impairment may play a key role in the selective vulnerability of the nigrostriatal pathway during PD.

Indeed, the disruption of mitochondrial function and integrity has long been implicated as a key mechanism in the pathogenesis of PD. Numerous studies have explored the relationship between mitochondria dysfunction and PD, identifying environmental, genetic, and biological factors that contribute to this association. MPTP was the first environmental toxicant discovered to induce parkinsonian symptoms by inhibiting the mitochondrial complex I of the electron transport chain (ETC) (Langston et al., 1983). After that, several other toxic compounds that inhibit the complex I (rotenone, paraquat, 6-hydroxydopamine) were identified as risk factors for

PD development and as agents capable of inducing PD-like symptoms in preclinical models (Lapointe et al., 2004; Ossowska et al., 2005; Simola et al., 2007). These findings are further supported by reports of decreased complex I activity in SN tissue samples from idiopathic PD patients (Janetzky et al., 1994). In the case of genetic forms of PD, several causative genes, including *Parkin* (PARK2), *PINK1* (PARK6), *DJ-1* (PARK7) and *LRRK2* (PARK8), are directly involved in mitochondrial dysfunction. Mutations in these genes appear to promote neurodegeneration by disrupting complex I activity and impairing key mitochondrial processes, such as mitophagy, mitochondrial dynamics (fission and fusion), and biogenesis (Henrich et al., 2023). In addition to nuclear gene mutations, defects in mtDNA have also been implicated. These defects are found to accumulate with age and are particularly prominent in SN dopaminergic neurons of PD patients (Bender et al., 2006). Such deleterious mutations have been shown to impair mitochondrial protein synthesis, ultimately leading to structural and functional mitochondrial abnormalities (Moradi Vastegani et al., 2023).

Furthermore, a substantial body of evidence supports the notion that α-syn pathology is a major contributor to mitochondrial dysfunction, thereby reinforcing the central role of mitochondria in PD pathogenesis. Multiple mechanisms through which α-syn impairs mitochondrial activity have been described (Henrich et al., 2023). For example, pathological α-syn has been shown to reduce complex I activity in a dose-dependent manner (Liu et al., 2009). In addition, α-syn oligomers are thought to interact with proteins on the mitochondrial outer membrane, disrupting mitochondrial protein import, impairing ETC function, promoting the accumulation of ROS, and leading to a loss of mitochondrial membrane potential (Di Maio et al., 2016). Moreover, α-syn has also been implicated in the dysregulation of calcium exchange between the mitochondria and the ER, thereby compromising mitochondrial respiration (Erustes et al., 2021).

Several mechanisms explain how mitochondrial dysfunction contributes to PD pathogenesis and dopaminergic neurodegeneration. Impaired mitochondrial respiration leads to ATP depletion and excessive production of ROS, resulting in metabolic and oxidative stress that culminates in neuronal death (Tretter et al., 2004). Mitochondrial membrane damage, caused by ROS or pathological α-syn, disrupts calcium uptake and buffering, promoting cytosolic calcium overload and excitotoxicity (Carbone et al., 2017). Moreover, disturbances in mitochondrial quality control mechanisms, such as mitophagy, fusion, fission, and biogenesis, are associated with increased neuronal vulnerability and degeneration (Moradi Vastegani et al., 2023). Mitochondria also contribute to neuroinflammation via activation of the NLRP3 inflammasome (Litwiniuk et al., 2021), and play a pivotal role in apoptosis by releasing cytochrome c into the cytoplasm (Wolf et al., 2022), further amplifying neurodegenerative cascades.

1.4.4. Neuroinflammation

Neuroinflammation happens to be a key feature shared by most neuropathologies, including PD. Physiologically, this immune response has the primary objective of protecting central nervous

system (CNS) parenchyma from harmful stimuli and promoting tissue repair and homeostasis. However, this controlled phenomenon can evolve into an excessive and deleterious response if it becomes chronic, finally exacerbating cytotoxic events and neuronal death. In the context of PD, brain inflammation is a well-established contributor to progressive neurodegeneration, finding increased levels of proinflammatory factors and activated immune cells in the blood, cerebrospinal fluid (CSF) and brain tissue from PD patients (Wang et al., 2022a). Furthermore, several studies have found a significant reduction in PD incidence among regular nonsteroidal anti-inflammatory drug users (Wahner et al., 2007; Gagne & Power, 2010), which reinforces the role of neuroinflammation in PD development. These findings are consistent with the observation that dopaminergic neurons in the SN may be particularly susceptible to neuroinflammation, partly due to the region-specific characteristics of inflammatory glial cells (microglia and astrocytes) in the basal ganglia (De Biase et al., 2017). Additionally, catecholaminergic neurons in PD patients have been shown to exhibit increased expression of major histocompatibility complex class I (MHC-I) proteins, which exposes them to cytotoxic T cell-mediated attack (Cebrián et al., 2014).

At present, a growing body of evidence has shown the involvement of both innate and adaptive immune system in PD. Microglia represent the first line of the immune response in the CNS, and an increased activation of microglia has been observed in PD patients (Terada et al., 2016). In the context of PD, the activation of immune cells can be induced because of neurodegeneration, by molecules released by dead or damaged neurons (ATP, mtDNA, misfolded and aggregated α-syn). Nevertheless, microglial alterations can also occur prior to neuronal death/damage. Indeed, a number of genetic risk variants identified by GWAS (Nalls et al. 2019) affect immune-related genes, such as *LRRK2*, *Parkin*, *PINK1* and *HLA* (Araújo et al., 2022; Wang et al., 2022a). Moreover, through expression quantitative trait loci (eQTL) studies, it has been demonstrated that PD risk variants affect gene expression specifically in immune cells, including microglia and monocytes, but not in other cell types (Navarro et al., 2021; Lopes et al., 2022). In neurodegenerative diseases, microglia acquire a phagocytic and pro-inflammatory phenotype, producing cytokines like tumour necrosis factor α (TNFα), interleukin (IL) 1β, IL-6, interferon γ (IFN-γ), among others (Zhang et al., 2023). These pro-inflammatory factors generate local inflammation that affects neighbouring cells, favouring a neurotoxic environment. For instance, these inflammatory factors can transform astrocytes into a toxic phenotype. Astrocytes are glial cells that perform a variety of functions, including providing structural and trophic support to neurons, maintaining blood-brain barrier (BBB) integrity, and regulating synaptic activity, while also exhibiting inflammatory capabilities (Escartin et al., 2021). These cells become reactive primarily due to a pro-inflammatory environment generated by responding microglia, a phenomenon that ultimately exacerbates neurotoxic inflammation (Liddelow et al., 2017). However, other microglia-independent processes have been reported to trigger harmful responses in astrocytes, such as interaction with α-syn (Chavarría et al., 2018). Distinct α-syn aggregates have been identified in astrocytes of PD patients (Altay et al., 2022), owing to these glial cells' ability to take up this neuronal-origin protein (Loria et al., 2017). Once α-syn is ingested, astrocytes, like microglia, can act as a double-

edged sword in the pathology. On one hand, they can degrade α-syn via proteasomal and lysosomal pathways, even more efficiently than neurons (Loria et al., 2017), contributing to aggregate clearance. On the other hand, excessive uptake may overwhelm lysosomal capacity, leading to the depletion of protein degradation systems and the formation of intracellular deposits in astrocytes (Lindström et al., 2017). Indeed, α-syn has been shown to inhibit autophagic flux (Di Domenico et al., 2019) and promote ER stress in astrocytes (Lee et al., 2019). The eventual accumulation of damage associated with protein overload drives a detrimental, disease-accelerating phenotype in both microglia and astrocytes, disrupting glial homeostasis and facilitating α-syn spreading to recruit additional help for clearance (Lindström et al., 2017). The ability of astrocytes to connect with neurons, microglia, and other astrocytes via tunnelling nanotubes positions them as major spreading agents in PD (Abounit et al., 2016).

The loss of integrity of BBB allows the infiltration of peripheral activated monocytes and T cells into the SN (Harms et al., 2018). Microglia and monocytes can induce adaptive immune responses by expressing MHC-II molecules, which activate $CD4^+$ T cells through antigen presentation. Indeed, it has been reported that T cells from PD patients can recognize α-syn peptides (Sulzer et al., 2017). After being activated, T cells proliferate and differentiate into Th1 and Th17 cells. The former release pro-inflammatory factors, exacerbating the activation of microglia, astrocytes and monocytes, and amplifying the central innate immune response. Th17 cells, together with cytotoxic $CD8^+$ T cells, can directly harm neurons. Indeed, the activation of $CD8^+$ T cells is triggered by MHC-I present on the neuronal surface (Wang et al., 2022a).

There is a close link between α-syn pathology, mitochondrial dysfunction and neuroinflammation. A point of convergence among these three processes may be the inflammasome formation. Specifically, the NLRP3 inflammasome can be activated after mitochondrial dysfunction via ROS production, mtDNA release or mitochondrial membrane depolarization, but also by α-syn through the Fyn-1β molecular pathway (Panicker et al., 2019). However, α-syn can also enhance inflammation through other signalling pathways (Wang et al., 2022a). Interestingly, inflammation can also promote α-syn misfolding, enhancing α-syn pathology and generating a self-aggravating cycle (Poewe et al., 2017). Considering this, peripheral tissue inflammation has been suggested to trigger a sufficient level of α-syn misfolding and aggregation, from which the α-syn pathology can expand. Indeed, gut microbiome dysbiosis, chronic inflammation of the intestinal mucosa and accumulation of α-syn in the enteric nervous system have been observed in PD patients (Lin et al., 2019). As the intestinal microbiota plays a critical role in microglia maturation, differentiation and function (Erny et al., 2015), alterations in the microbiome are expected to induce and modulate the neuroinflammatory response, establishing a gut-brain communication, with the intestinal tract as a potential initial site of inflammation (**Box 3**).

BOX 3. MICROBIOTA-GUT-BRAIN AXIS IN PARKINSON'S DISEASE

The gut is often referred to as the 'second brain' due to its significant influence on brain health and neurological functions. This relationship, known as the gut–brain axis, highlights the bidirectional communication between the gastrointestinal tract and the CNS. The gut microbiota plays a critical role in this interplay, as evidenced by studies on germ-free animals, which show alterations in behavior and essential processes such as myelination and microglial maturation (Cryan et al., 2019). In the context of PD, numerous studies have provided compelling evidence of disruptions in microbiota composition (dysbiosis) in PD patients compared to healthy controls (Hill-Burns et al., 2017; Barichella et al., 2019; Mehanna et al., 2023). Specific gut microbiota signatures have been identified and correlated with distinct clinical phenotypes (Scheperjans et al., 2015). Remarkably, fecal microbiota transplantation from PD patients into mice induces motor deficits, and neuroinflammation can be alleviated by antibiotic treatment (Sampson et al., 2016).

The mechanisms linking gut microbiota to PD are not yet fully understood, but they appear to involve multiple overlapping pathways (**Figure 7**). PD-associated dysbiosis is generally characterised by a reduction in beneficial bacteria such as *Prevotellaceae* and an increase in pro-inflammatory microbes like *Akkermansia* and *Desulfovibrio*. These alterations may lead to excessive production of lipopolysaccharide, elevated levels of pro-inflammatory cytokines (TNFα, IL-1β), and dysregulated microbial metabolites (short-chain fatty acids (SCFAs), bile acids, and amino acids). This inflammatory environment can extend to the systemic circulation, as dysbiosis also compromises the integrity of the intestinal barrier, ultimately reaching the brain and promoting neuroinflammation (Wang et al., 2025). Furthermore, the dysregulation of pro-inflammatory cytokines and microbial metabolites may trigger the misfolding and aggregation of native α-synuclein in the enteric nervous system, initiating Lewy pathology peripherally. Over time, this pathology can propagate to the brain via the vagus nerve, aligning with Braak's hypothesis (Wang et al., 2025).

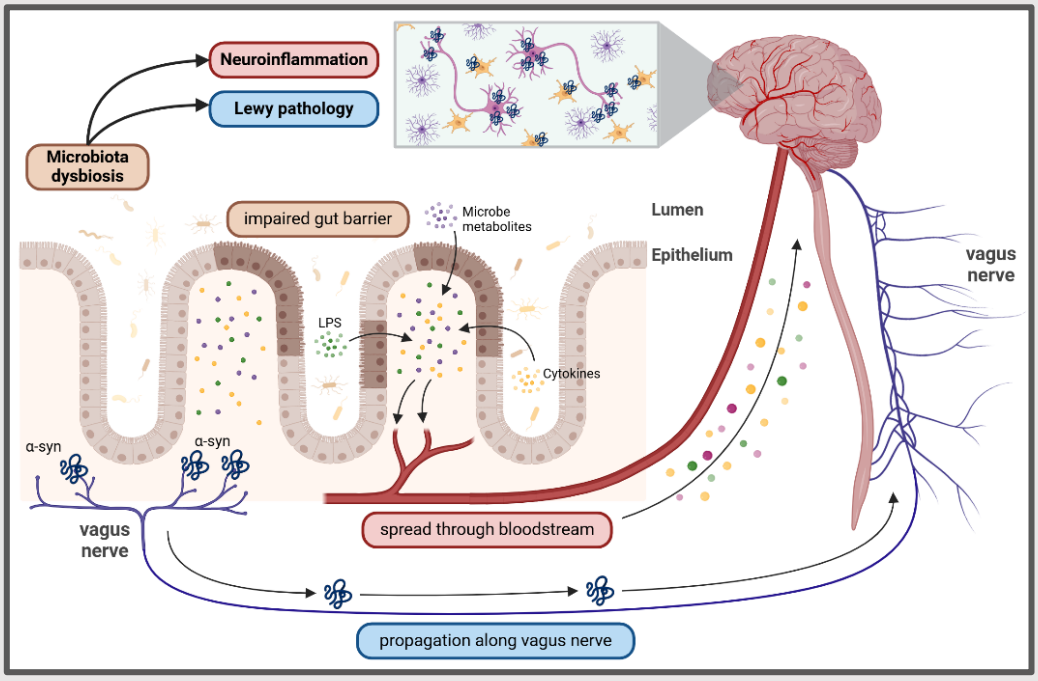


***Figure 7**. Overview of the underlying mechanisms of the microbiota–gut–brain axis in PD. (Adapted from Klann et al., 2022). Created with BioRender.com.*

1.4.5. Oxidative stress

ROS and reactive nitrogen species are physiologically produced within the cell as they are signalling molecules (D'Autréaux & Toledano, 2007). However, when accumulating, these chemically reactive molecules trigger tissue toxicity, harming DNA, proteins and lipids. This imbalanced situation is known as oxidative stress and can be the result of an exacerbated production of ROS and/or a reduced antioxidant capacity. Indeed, there is extensive evidence about the oxidative damage in PD. *Post-mortem* brain samples from PD patients have shown higher

levels of oxidised biomolecules and lower levels of glutathione, as well as an accumulation of iron in the SN that contributes to ROS production by Fenton's reaction (Dias et al., 2013). Moreover, mutations in *DJ-1* (PARK7), encoding a putative antioxidant, lead to familial forms of PD characterised by increased cellular oxidative stress (Skou et al., 2024).

Considering that mitochondria are the main source of ROS production, disturbances in mitochondrial function, especially in the ETC, as those found in patients and neurotoxin-based PD models, lead to an increase in ROS production, which in turn inhibits complex I (Moradi Vastegani et al., 2023), creating a vicious cycle of energetic deficiency and oxidative damage. Nevertheless, mitochondrial dysfunction is not the only source of oxidative stress. Thus, through the chronic activation of microglia, neuroinflammation can be responsible for a conspicuous production of ROS, contributing to amplifying the oxidative environment. More precisely, reactive microglia is associated with the activation of the NAPDH oxidase and the inducible nitric oxide synthase (iNOS), both being up-regulated in *post-mortem* samples and toxin-induced animal models (Dias et al., 2013; Chakrabarti & Bisaglia, 2023).

Besides the mitochondrial and glial origin of ROS, in dopaminergic neurons, cytosolic DA can trigger auto-oxidation, producing ROS and reactive DA quinones. The spontaneous oxidation of DA is favoured by its accumulation in the cytosol, which can be promoted by impairments in its catabolism, transport into synaptic vesicles or reuptake from the synaptic cleft (Chakrabarti & Bisaglia, 2023). Both DA-derived ROS and DA quinones have been implicated in oxidative damage within the cell, reacting with mitochondria and targeting different proteins, including α-syn (Dias et al., 2013). Interestingly, non-enzymatic oxidative modifications, such as methionine oxidation, nitration, oxidative tyrosine dimerization or oxidative DA adduction, affect the aggregative dynamics of α-syn. In fact, one suggested mechanism responsible for dimerization and oligomerization of α-syn is the dityrosine crosslinking promoted by nitration and oxidation of the protein (Vidović & Rikalovic, 2022).

1.4.6. Excitotoxicity

Excitotoxicity or glutamate-mediated toxicity is a common phenomenon behind neurodegeneration in multiple pathological disorders. The excessive activation of glutamatergic receptors by glutamate triggers intracellular processes related to calcium overload and bioenergetic changes, which ultimately increase oxidative burden and apoptotic cascades (Ambrosi et al., 2014). The over-stimulation of glutamate receptors is a direct consequence of a glutamate accumulation at the synapse. This impaired glutamate homeostasis has been described in PD, observing increased glutamate neurotransmission in brain tissue and elevated glutamate levels in plasma (Iovino et al., 2020). The basal ganglia circuit includes glutamatergic inputs, mainly coming from the cortex and the STN. The loss of dopaminergic neurons in the SNpc has an impact on this striatal circuitry, leading to the disinhibition of the STN and an increase in its glutamate release, an alteration that has been related to the parkinsonian tremor (McGregor & Nelson, 2019). Since this glutamatergic nucleus has a direct connection with the SNpc (Kreitzer,

2009), the hyperactivity of the STN can further promote dopaminergic neuronal loss via excitotoxicity. Nevertheless, besides the dysregulation of the glutamatergic neurotransmission, excitotoxicity can also result from an impaired glial function. Indeed, most extracellular glutamate is buffered by astrocytes, facilitating its reuptake via excitatory amino acid transporters and engaging its conversion into glutamine (Verkhratsky & Nedergaard, 2018). However, it has been observed how astrogliosis and the transformation into neurotoxic astrocytes can negatively impact their ability to reuptake glutamate (Howland et al., 2002). Moreover, when microglia become reactive, they express glutamate transporters and also play a role in glutamate signalling (Chrétien et al., 2004). Therefore, neuroinflammatory events may exacerbate glutamate-induced neurotoxicity in PD by promoting changes in astrocytes and microglia that affect their glutamate handling.

1.5. Current therapies

At present, no cure is available for PD. This disorder is commonly diagnosed once motor symptoms have appeared and massive neuronal death has occurred, rendering it impossible to initiate early treatments aimed at halting or slowing down neurodegeneration. This underscores the critical need for diagnostic strategies capable of identifying PD in its prodromal phase, when therapeutic interventions may still have the potential to alter the disease trajectory. To this end, the discovery and validation of robust and specific biomarkers, spanning molecular, imaging, and immunological domains, are essential not only for early and accurate diagnosis but also for monitoring disease progression and stratifying patients for clinical trials aimed at developing disease-modifying therapies. However, available treatments for this neurodegenerative disorder remain focused on symptomatic relief, mainly through dopaminergic replacement therapy, but alternative therapeutic strategies have increasingly been explored in recent years.

1.5.1. Symptomatic dopaminergic therapies

Dopaminergic therapies represent the first-line treatment strategy, with DA replacement by levodopa (L-DOPA) administration as the gold standard for PD (Poewe et al., 2017). L-DOPA is a DA precursor that crosses the BBB, unlike DA itself, and is converted to DA within the remaining dopaminergic neurons of the SNpc, restoring DA tone in the basal ganglia. In this way, motor symptoms are improved, and disability is reduced, yet the neurodegenerative process persists, without exhibiting disease-modifying effects. Notably, not all patients with PD respond equally to L-DOPA treatment, with a subset, particularly those exhibiting a more rapid motor progression, showing poor responsiveness (Malek et al., 2019). Moreover, side effects include hallucinations, sleepiness or confusion, likely due to alterations in other neurotransmission systems, such as serotonergic or noradrenergic (Balestrino & Schapira, 2020). Furthermore, the discontinuous drug delivery, because of the short half-life of L-DOPA and the variability in its gastrointestinal absorption, promotes a non-physiological pulsatile striatal DA receptor stimulation and the evolution of motor complications, such as motor response oscillation and dyskinesias (Bloem et al., 2021). To avoid these undesirable effects, strategies to increase the bioavailability and half-

life of L-DOPA have been developed, including continuous delivery systems (either intestinally via percutaneous endoscopic gastro-jejunostomy tubes or subcutaneously via mini-pumps) (Freitas et al., 2016) or the use of adjuvants that inhibit degradation enzymes (Fox et al., 2011). These inhibitors act peripherally on the aromatic amino acid decarboxylase (benserazide, carbidopa) and the catechol-O-methyltransferase (tolcapone, entacapone, opicapone), as well as centrally on the glial monoamine oxidase type B (selegiline, rasagiline, safinamide) (Balestrino & Schapira, 2020). DA receptor agonists (ropinirole, pramipexole, rotigotine, apomorphine) have also become an important medical therapy for motor symptoms, as they are associated with a lower risk of dyskinesia (their longer half-life and the use of continuous infusion lead to more constant levels and less discontinuous phasic striatal stimulation) (Poewe et al., 2017) (**Figure 8**). However, they also display lower effectiveness than L-DOPA, so that they are usually considered as monotherapy in earlier phases of the disease or in combination with L-DOPA in patients with motor fluctuations (Jankovic & Poewe, 2012). Moreover, DA agonists have other relevant drawbacks, especially considering their potential to induce impulse control disorders, derived from an excessive stimulation of dopaminergic receptors in the reward circuits (Staubo et al., 2024). This often manifests as compulsive behaviours, addictive-like patterns, or even psychiatric comorbidities (Pettorruso et al., 2016).

1.5.2. Complementary pharmacological interventions

Complementary to dopaminergic therapy, other pharmacological systems need to be targeted to address additional symptoms, such as L-DOPA-induced complications, L-DOPA-resistant motor features, and non-motor manifestations. Amantadine, an N-methyl-D-aspartate (NMDA) receptor antagonist, is currently the most effective and available pharmacological treatment for L-DOPA-induced dyskinesia (**Figure 8**), even though adenosine, serotonin, or adrenergic receptors may be considered (Balestrino & Schapira, 2020). Cholinergic and noradrenergic targets are also used to address tremor, gait dysfunctions, and falls (Poewe et al., 2017). Finally, many non-motor symptoms are unaffected or even aggravated by DA replacement therapy, requiring consideration of targets such as cholinesterase inhibitors for cognitive disturbances and dementia, serotonin receptor ligand clozapine for psychotic features, and adrenergic, noradrenergic, or muscarinic agents, among others, for autonomic failures (Seppi et al., 2011).

1.5.3. Complementary non-pharmacological interventions

Other strategies different from pharmacotherapy are being employed for PD treatment, including neurosurgery and physical activity. Deep brain stimulation (DBS) involves high-frequency electrical stimulation of specific brain areas by the implantation of an electrode. This technique can mimic the effects of a lesion without damaging brain tissue, making it reversible and adjustable as the disease progresses (Church, 2021). DBS is typically used in patients experiencing long-term complications from L-DOPA therapy, aiming to alleviate fluctuations and dyskinesia, and allowing for a reduction in medication. It is not prescribed for patients insensitive to dopaminergic treatment (Malek, 2019). The brain targets chosen for DBS are the STN and, less frequently, GPi.

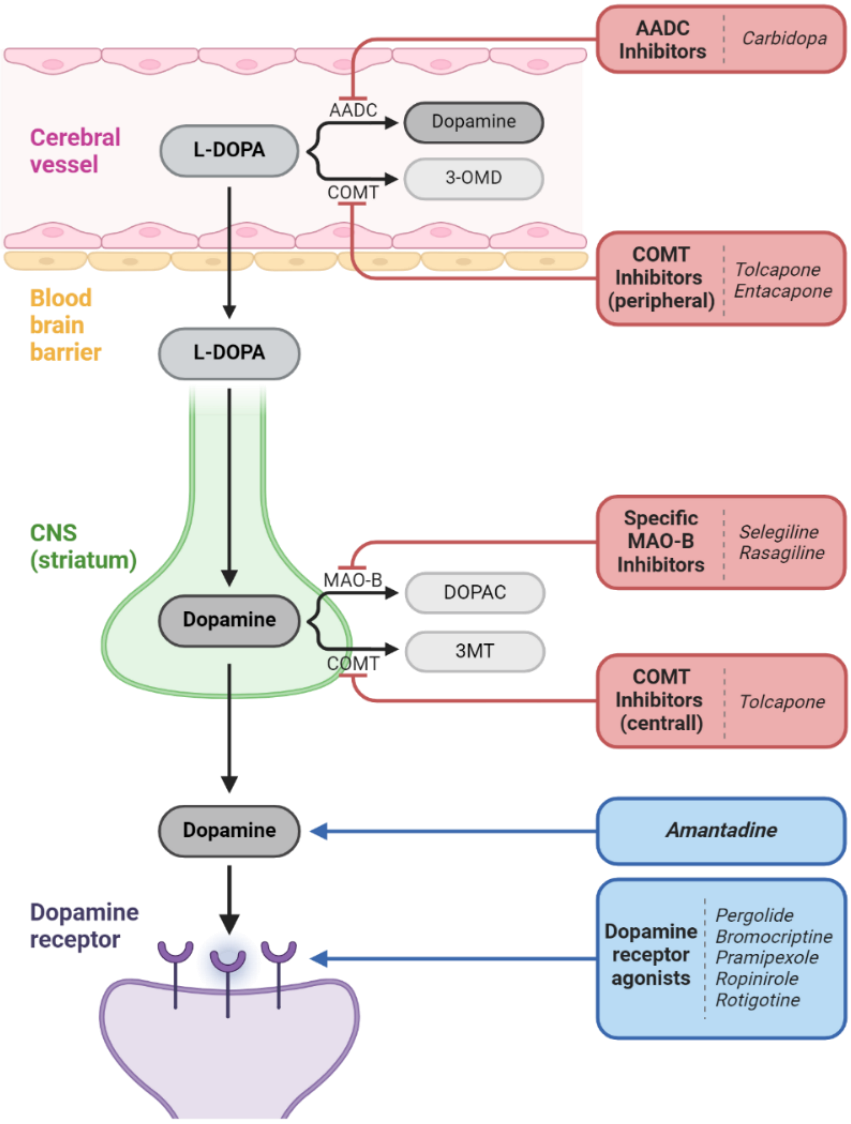


***Figure 8. Dopaminergic therapy for PD**. Multiple drugs are used to manage PD by increasing DA signalling. This pharmacological strategy uses L-DOPA as the gold standard. This compound acts as a DA precursor in dopaminergic neurons, temporarily supplementing the deficiency of this neurotransmitter within the basal ganglia. However, L-DOPA is typically administered with adjuvants that inhibit degradation enzymes peripherally or centrally. DA agonists can also be prescribed, as well as other drugs that improve the action of L-DOPA. AADC: aromatic L-amino acid decarboxylase; COMT: catechol-O-methyltransferase; MAO-B: monoamine oxidase type B. Adapted from template of BioRender.com.*

Thalamic DBS is rarely used but is effective against tremor (Bloem et al., 2021). Adverse events are associated with the surgical intervention itself (intracranial bleeding, infection, device displacement), which can be mitigated by using focused ultrasound, a less invasive technique. This alternative is currently under investigation (Martínez-Fernández et al., 2023). More conventional surgical interventions, such as pallidotomy and, to a lesser extent, thalamotomy, both of which involve the permanent destruction of a small portion of the GPi or thalamus, respectively, may be considered in cases where DBS is not a viable option or has proven ineffective (Lozano et al., 2018).

Exercise-based treatment complements the current therapy for PD and includes physiotherapy and rehabilitation. Physiotherapy strategies seek the management and compensation of some disabilities that affect gait, mobility, balance, speech, or swallowing, intending to bypass the defective basal ganglia function and improving the quality of life of patients (Bloem et al., 2021).

On the other hand, multiple studies provide evidence for the benefits of regular and aerobic exercise as symptomatic treatment (Tsukita et al., 2022). High-intensity activity seems to be more effective than lower-intensity options (Schenkman et al., 2018; Chrysagis et al., 2024). These findings align with the neuroplastic and potential neuroprotective effects of exercise (Ahlskog, 2011). Moreover, epidemiological evidence supports a lower risk of PD associated with physical activity (Xu et al., 2010). Additionally, a considerable number of clinical trials are currently underway to develop new therapies for PD (**Box 4**).

BOX 4. CLINICAL TRIALS IN PARKINSON'S DISEASE

Despite available therapeutic approaches, there is still a need to enhance current symptomatic treatments and, more urgently, to develop effective disease-modifying treatments that stop, slow, or even reverse the progression of the disease. The collective effort made in this field is evident in the 136 clinical trials targeting PD registered in 2023 (**Figure 9**). When considering clinical trials aimed at long-term disease-modifying therapies (n = 60; 44%), a wide array of strategies has been explored. These include cell therapy, microbiome- or gastrointestinal-related agents, α-syn or LRRK2-targeting strategies, anti-inflammatory compounds, antioxidants, kinase inhibitors, neurotrophic factors, glucagon-like peptide-1 receptor agonists, GBA-inducing strategies, energy metabolism-related approaches and gene therapy, among others (McFarthing et al., 2024). Nevertheless, despite efforts, the high rate of drug failures highlights the need for more diverse studies to improve effectiveness and approval rates. Early biomarkers and prodromal signs could enable early diagnosis and intervention, boosting treatment effectiveness. Additionally, limitations in preclinical models hinder predicting human outcomes, driving the development and refinement of new models.

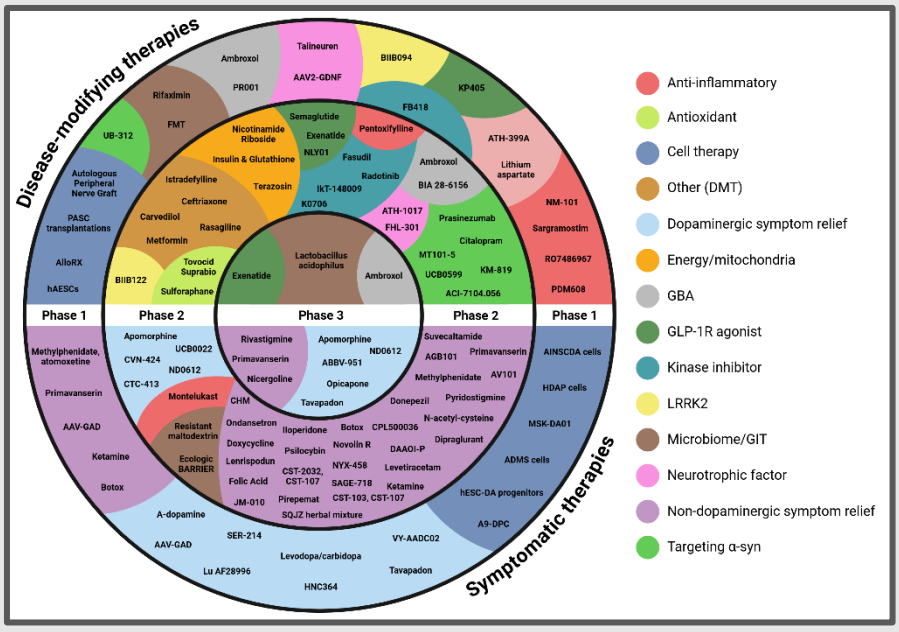


***Figure 9. PD drug therapies in clinical trials**. In 2023, 136 clinical studies were being carried out, exploring therapies that counteract the symptomatology (lower half; n = 76, 56%) or that modify disease progression (upper half; n = 60, 44%). This myriad of pharmacological agents is spread across Phase 1 (n = 41, 30%), Phase 2 (n = 79, 58%) and Phase 3 (n = 16, 12%), and includes novel therapies (n = 51, 38%), repurposed drugs (n = 52, 38%), reformulations (n = 26, 19%) and new claims (n = 7, 5%). (Adapted from McFarthing et al., 2024). Created with BioRender.com.*

1.6. Experimental Parkinson's disease

Clinical trials are necessary to increase opportunities for developing effective strategies against neurodegeneration. However, extensive preclinical studies are crucial before clinical trials can begin. These studies provide the foundation for the benefits of therapeutic approaches, but also

for identifying new therapeutic targets. Different experimental tools have been designed to preclinically study PD, ranging from cell cultures to patients samples. However, animal models are by far the most used method to replicate the PD pathology. The primary limitation of PD models is their incapacity to fully represent the whole physiopathology of the disease, as the pathogenic mechanism in humans remains unknown. Although a completely valid model is elusive, existing models are continually improved thanks to advances in scientific knowledge and techniques.

1.6.1. Animal models

PD naturally occurs almost exclusively in humans. Therefore, disease modelling is required to emulate a PD-like pathology in non-human subjects. Rodents are the most popular animal models used across research groups due to their ease of maintenance and reasonable similarities to humans. Consequently, rats (De la Rosa et al., 2022) and mice (Leem et al., 2022) are widely used for PD modelling. On the other hand, non-human primates (NHPs), despite their close phylogenetic relationship with humans, are much less used, mainly for ethical concerns and logistical challenges, but they offer valuable insights for understanding PD pathology. Commonly used NHPs in PD research include macaques (Davin et al., 2022) and marmosets (Oh et al., 2022). Finally, small non-mammalian animals, such as *C.* (*Caenorhabditis*) *elegans* (Choong et al., 2023), zebrafish (Matsui et al., 2021) and *Drosophila melanogaster* (J. He et al., 2021), can be used in modelling the basics of PD, mostly involving genetic manipulations.

Regardless of the animal species, the induction of PD experimental models can be achieved through different approaches, including genetic manipulation, pharmacological intervention, or the combination of both. Notably, given that each model type offers specific advantages and reflects distinct aspects of the disease, integrating multiple complementary models is crucial for achieving a more holistic understanding of PD pathophysiology.

1.6.1.1. Genetic models

As mentioned earlier, rare mutations in causal genes can give rise to familial forms of PD. The identification of these genetic variations has enabled the creation of genetic models of the disease through gene manipulation. This mutation model system has been used to identify and comprehend mechanistic insights into molecular pathways underlying heritable PD, and by extension, some of the mechanisms shared with sporadic forms. The primary limitation of this type of PD models is the poor dopaminergic neurodegeneration achieved. Gene manipulation typically involves the overexpression or replacement of mutant variants in autosomal dominant genes (*SNCA* and *LRRK2*), and the suppression or deficiency of autosomal recessive genes (*Parkin*, *PINK1*, and *DJ-1*). The application of techniques like CRISPR also allows for advances towards more precise and efficient gene editing in PD models, as currently established in the case of human induced pluripotent stem cells (Calatayud et al., 2017).

1.6.1.1.1. *SNCA* (PARK1/4)

As the major component of LB (Spillantini et al., 1997), many models have been produced by altering α-syn expression. Specifically, overexpression models have been used to promote the protein inclusions observed in PD, using both wild-type (WT) (Chesselet et al., 2012) and mutant (A53T, A30P, E46K) (Giasson et al., 2002; Gomez-Isla et al., 2003; Greenbaum et al., 2005) variants, expressed under different neural promoters. Although they do not reproduce the decrease in dopaminergic neurons, these models display alterations in striatal DA, motor dysfunction, neuroinflammation, and α-syn aggregates, more widely reported in mutant than in WT overexpression models. Notably, the expression of α-syn under the tyrosine hydroxylase (TH) promoter was used to limit its expression within dopaminergic neurons, but no strong phenotypes were observed (Matsuoka et al., 2001).

1.6.1.1.2. *LRRK2* (PARK8)

LRRK2 is a multidomain protein with kinase and GTPase activity, which has gained relevance in PD due to its mechanistic role in the pathogenesis of the disease (Usmani et al., 2021). Mutations in the *LRRK2* gene imply alterations in the kinase activity and interaction capabilities of the protein (Cookson, 2015). Therefore, most models focus on expressing mutated versions of the protein (G2019S, R1441C/G), using traditional knock-in (Yue et al., 2015) or bacterial artificial chromosome transgenic models (Li et al., 2009). These models lead to a reduction in DA release and impaired motor function, but none have reported any dopaminergic cell loss or α-syn pathology (Chang et al., 2022).

1.6.1.1.3. *Parkin* (PARK2), *PINK1* (PARK6), *DJ-1* (PARK7)

Parkin (a ubiquitin ligase), PINK1 (a kinase), and DJ-1 (an antioxidant) are believed to collectively play a role in regulating mitochondrial quality within the cell (Trempe & Fon, 2013). The recessive inheritance pattern has favoured knock-out (KO) models to mimic an expected loss-of-function. However, individual (Goldberg et al., 2003; Chandran et al., 2008; Gispert et al., 2009) or combined (Kitada et al., 2009) deletion of these genes in rodents does not result in neuronal loss in the SNpc, reduced striatal DA levels, LB-like structures, nor significant behavioural disturbances. Nevertheless, these models are suggested to represent an early stage of the disease, proving valuable for the study of compensatory mechanisms, pre-motor symptoms, and early alterations (Lechner et al., 2024).

1.6.1.2. Toxin-based models

In addition to gene manipulation, PD can also be mimicked through pharmacological intervention, typically involving the administration of neurotoxins or preformed fibrils (PFF) of α-syn within specific areas of the CNS (striatum, medial forebrain bundle, SNpc), or even systemically. Furthermore, intracerebral lesions can be performed bilaterally or unilaterally, depending on the experiment's requirements and the chosen model. Generally, neurotoxin-based models aim to induce rapid and increased nigrostriatal dopaminergic loss, although they lack a clear

pathophysiological or etiological correlation, with most of them not displaying α-syn pathology. Despite this drawback, these models are commonly employed for analysing therapeutic targets and screening pharmacological treatments.

1.6.1.2.1. 6-Hydroxydopamine

The addition of an extra hydroxyl group in DA is sufficient for the molecule to selectively display toxicity in dopaminergic neurons. 6-Hydroxydopamine (6-OHDA) is taken up by the DA transporter, and once inside the cell, inhibits complexes I and IV of the mitochondrial ETC. It also accumulates in the cytoplasm, where it rapidly oxidizes and promotes ROS production (Simola et al., 2007), leading to cell death via mitochondrial dysfunction and oxidative stress. Due to its inability to cross the BBB, this neurotoxin must be directly delivered to target regions, basically the striatum, SNpc, and medial forebrain bundle, resulting in different patterns of neuronal degeneration. While the injection into the SNpc or medial forebrain bundle produces a prominent lesion and rapid cellular death (Yuan et al., 2005; Athari et al., 2023), striatal inoculation allows for retrograde neurodegeneration, a phenomenon observed in PD, with primary damage occurring in axon terminals and subsequent cell body loss (Lee et al., 1996). Striatal administration also modulates the lesion level in a dose-dependent manner (Przedborski et al., 1995). The 6-OHDA lesion reproduces the motor impairments of the disease but fails to replicate LB formation (Alvarez-Fischer et al., 2008).

Several considerations should be noted. 6-OHDA can internalize into catecholaminergic neurons, including dopaminergic and noradrenergic populations (Luthman et al., 1989). To ensure the lesion selectivity only in dopaminergic neurons, a norepinephrine transporter inhibitor (such as desipramine) must be co-administered with the neurotoxin. Additionally, unilateral lesioning is recommended, as bilateral injection into the striata results in severe absence of thirst and hunger, which ultimately leads to death due to the animal's inability to care for itself (Khan et al., 2023).

1.6.1.2.2. Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

The relationship between MPTP and PD was discovered accidentally after individuals rapidly developed severe parkinsonian symptoms from exposure to heroin adulterated with MPTP (Langston et al., 1983). MPTP is a lipophilic molecule that easily crosses the BBB, allowing for systemic administration. This molecule is oxidised to 1-methyl-4-phenylpyridinium (MPP^+) by the astrocytic MAO-B. MPP^+ shares structural similarity with DA and can be captured by dopaminergic neurons via the DA transporter. This neurotoxin primarily induces neuronal death through mitochondrial impairment and energy depletion, inhibiting the mitochondrial complex I. Additionally, there is an oxidative stress component, as MPTP displaces DA from vesicles via vesicular monoamine transporter and enhances DA auto-oxidation (Schildknecht et al., 2017). The pathological features of this model depend on the treatment regime. The common acute model (high dose and short duration) produces non-progressive and aggressive neurodegeneration but

results in minor motor complications (Vingill et al., 2018). In contrast, chronic exposure to MPTP (low dose and extended duration) generates a PD model closely resembling the original pathology, with more progressive neuronal loss and motor dysfunctions (Muñoz-Manchado et al., 2016). According to Lewy pathology, the presence of α-syn inclusions is still controversial in this model.

When considering this chemical-induced model, other factors should be considered. While C57Bl/6 mice exhibit effective vulnerability to MPTP, sensitivity varies among different strains of mice, whereas rats are insensitive to it. NHPs are the most sensitive animals to MPTP and can display behavioural and neuroanatomical properties like those of humans (Khan et al., 2023). In addition to the chosen animal species, it has been observed that age increases the susceptibility of the animal to the toxin (Jarvis & Wagner, 1985).

1.6.1.2.3. Rotenone

Similar to MPTP, the insecticide rotenone is a lipophilic compound, capable of crossing the BBB and provoking cellular death by inhibiting complex I in the mitochondria. However, other effects have also been described, such as the blockage of proteasome activity, resulting in proteolytic stress as well (Ibarra-Gutiérrez et al., 2023). In contrast to the previous neurotoxins, rotenone lacks selectivity for the dopaminergic population, causing neurodegeneration beyond this neurotransmitter system (Höglinger et al., 2003) and leading to systemic toxicity (Lapointe et al., 2004). Nevertheless, chronic administration at low doses has been reported to induce selective cell degeneration in the nigrostriatal region. In addition to this loss of dopaminergic neurons, other PD-related features have been observed, such as motor deficits, catecholamine depletion and α-syn inclusions resembling LBs in the surviving neurons (Khan et al., 2023).

1.6.1.2.4. Paraquat

Structurally similar to MPP^+, the herbicide paraquat has demonstrated neurotoxic actions, resulting in the loss of dopaminergic neurons and exhibiting several behavioural features observed in PD (Nixon et al., 2018). This molecule can cross the BBB through a neutral amino acid carrier and exerts its toxic effects by altering the redox cycling of glutathione and thioredoxin, impairing the ability of the cell to combat oxidative stress (Niso-Santano et al., 2010). The nigral dopaminergic loss induced by paraquat occurs without any striatal DA depletion, suggesting distinct neurochemical effects (Khan et al., 2023). However, chronic administration appears to lead to persistent neurodegeneration and DA depletion (Ossowska et al., 2005).

1.6.1.2.5. Lipopolysaccharide

In exploring the role of neuroinflammation in PD pathogenesis, numerous studies have utilised lipopolysaccharide (LPS), a gram-negative bacterial component, to simulate inflammatory events associated with the disease. LPS serves as a potent activator of microglial cells, activating the toll-like receptor 4 signalling pathway on these cells. This activation triggers the production and release of cytokines (i.e., TNFα, IL-6, IL-1β) and free radicals (NO, O^{2-}) within the brain. Reactive

microglia, in turn, stimulate the activation of astrocytes, further contributing to the inflammatory response by producing additional cytokines and free radicals. The excessive release of these molecules may pose toxicity to neurons, compromising mitochondrial function and inducing oxidative stress (Deng et al., 2020).

LPS has been administered through stereotaxic surgery in various brain regions, such as intrapallidal (Huo et al., 2018) and intracerebroventricular (Zhou et al., 2019), although the inoculation into the SNpc or striatum remains the most common to mimic PD. This procedure induces degeneration of nigral dopaminergic neurons and their projections to the striatum, accompanied by a reduction in striatal DA levels and subsequent motor dysfunction (Hunter et al., 2017; Bao et al., 2018). Additionally, α-syn pathology has been observed (Choi et al., 2009), though further investigation is required to substantiate these findings. Alternative administration routes, including systemic (intraperitoneal, *i.p.*) (Liu et al., 2008) or intranasal (Li et al., 2015) inoculation, have been employed to examine potential environmental aetiology and the progression of PD. These approaches have successfully induced nigrostriatal neurodegeneration, motor disturbances, and α-syn pathology, contributing to a comprehensive understanding of PD pathogenesis (Liu et al., 2008).

1.6.1.2.6. α-Synuclein preformed fibrils

Most neurotoxin-induced models of PD fail to recapitulate α-syn pathology and lack the prion-like propagation observed clinically. Thus, over the last decade, new approaches have been developed to reproduce the aggregation and transmission of α-syn *in vivo*. These animal models are based on the inoculation of PFFs of α-syn, which are aggregates formed by misfolded proteins. They can be obtained from brain homogenates of synucleinopathy patients (Recasens et al., 2014) or symptomatic animals (Luk et al., 2012a), or as pre-aggregated recombinant α-syn (Luk et al., 2012b). The injection of these fibrillar α-syn into the animal brain induces dopaminergic neuronal loss, neuroinflammatory events, motor impairment and Lewy-like pathology, including the formation of α-syn-positive inclusions and the progressive spread of proteinopathy. Interestingly, alternative routes of administration have been explored, proving the propagation of α-syn from the periphery (Sacino et al., 2014) or gastrointestinal tract (Holmqvist et al., 2014), as speculated in clinical forms.

1.6.1.3. Viral vector-mediated models

While gene manipulation can be targeted to specific neuronal populations using various promoters, the injection of viral vectors encoding different proteins of interest into selected brain areas represents a widely employed alternative to classical genetic models. Lentivirus (Lo Bianco et al., 2002) and herpes simplex virus (Lee et al., 2010) have been utilised for transfection, but the most common choice is adeno-associated viruses (AAVs). Regarding the carried genes, mutated *LRRK2* (Dusonchet et al., 2011) or RNA interference of *Pink1* (Haque et al., 2012) have been employed. However, the most widely used and characterised gene is *SNCA,* encoding both WT and mutated (A53T) α-syn, leading to its local overexpression. In general, overexpression of α-syn in

the SNpc results in progressive nigrostriatal degeneration within weeks to months, depending on the host species (Dovonou et al., 2023). Factors such as virus titre or serotype, as well as the age, sex, and strain of the animal, can also impact the outcomes obtained. Most studies have been conducted in rodents, where unilateral inoculation into the SN with various AAV serotypes carrying the *SNCA* gene induced motor impairments, loss of 40–60% of nigral dopaminergic neurons, and depletion of striatal dopaminergic terminals (Liu et al., 2008; Koprich et al., 2010; Decressac et al., 2012; Ip et al., 2017; Castro-Sánchez et al., 2018). These changes were accompanied by mitochondrial dysfunction, impaired autophagy, LB-like inclusions and inflammatory responses (Dovonou et al., 2023). Notably, the post-injection timeframe varies across studies, ranging from a few weeks to a couple of months. Intranigral injections of AAV-α-syn can also be performed in NHPs, though less frequently. These injections lead to dopaminergic neuron loss and progressive motor deficits, albeit with greater variability compared to their rodent counterparts (Kirik et al., 2003; Eslamboli et al., 2007; Yang et al., 2015). Lastly, a more refined system encoded in viral vectors has been recently designed, successfully inducing α-syn aggregation via optogenetic stimulation and enabling real-time monitoring of LB-like structure formation with precise spatiotemporal control (Bérard et al., 2022).

Another cargo besides α-syn has been tested, yielding promising results: the human tyrosinase, responsible for the synthesis of neuromelanin. AAV-mediated overexpression of this enzyme in the SNpc of rats led to age-dependent intracellular accumulation of neuromelanin within nigral dopaminergic neurons, followed by the formation of LB-like α-syn inclusions within neuromelanin-positive cells, as well as nigrostriatal neurodegeneration, hypokinetic symptoms, and other pathological events such as mitochondrial and autophagic dysfunction and neuroinflammation (Carballo-Carbajal et al., 2019). Therefore, this *in vivo* model supports a key role of this human-exclusive pigment in the PD pathogenesis.

1.6.1.4. Double hit models

Despite the availability of diverse animal models for PD, none has fully replicated the pathophysiology of the disease. Consequently, there is a growing trend towards combining pharmacological interventions with genetic manipulations. Examples of these types of interventions could be *PINK1* heterozygous KO mice treated with rotenone, which show an accelerated progression of synaptic plasticity alterations (Martella et al., 2016); or mutant α-syn mouse models combined with iron overload (Billings et al., 2019) or neuroinflammation via LPS injection (Gao et al., 2011), used to explore the interplay between these two factors and to develop more comprehensive models for evaluating pharmacological treatments (Billings et al., 2019). Moreover, the double-hit paradigm can also imply two simultaneous genetic alterations: for example, the human α-syn overexpression alongside downregulation of endogenous GCase (Polissidis et al., 2022). These double-hit approaches are supported by several reasons. First, they are useful to investigate how genetic manipulation may heighten susceptibility to neurotoxins. Second, they allow the generation of animal models that better resemble the multifactorial pathogenesis associated with PD (Khan et al., 2023).

1.6.2. Human samples

While animal models have been fundamental in understanding the underlying mechanisms of PD and testing therapeutic interventions, their ability to fully replicate the complexity of human pathology remains limited. Consequently, the use of human samples has become an essential tool for validating pre-clinical findings and bridging experimental research into the clinical reality of the disease. Thus, prior to clinical trials, PD patients can contribute to research by providing samples from their own tissues. *Post-mortem* human samples, particularly brain tissue, are of particular value, as they enable the study of pathological changes and the identification of abnormalities in various regions of the CNS (Dobricic et al., 2022; Tu et al., 2022). Interestingly, the application of *omics* techniques to these *post-mortem* tissues (Smajić et al., 2021) is generating a wealth of information, offering a more comprehensive understanding of PD pathophysiology and aiding in the identification of potential disease-specific biomarkers.

In addition to *post-mortem* samples, various other types of biological samples can be collected from living PD patients, primarily to investigate potential biomarkers that offer diagnostic and prognostic insights. These biological sources include blood (Sulzer et al., 2017; Navarro, Udine, de Paiva Lopes, et al., 2021; Riboldi et al., 2022), serum (Jiang et al., 2020), CSF (Nilsson et al., 2023), urine (Kumari et al., 2020), and even saliva (Fernández-Espejo et al., 2021). Tissues like blood or skin can indeed be used to generate human induced pluripotent stem cells (Sulzer et al., 2017; Sun et al., 2021; Grigor'eva et al., 2022), creating an *in vitro* model for further disease research (**Box 5**). Other assessments different from collecting biological samples are imaging tests, such as magnetic resonance imaging or positron emission tomography scans, which have been showing their potential for early diagnosis of PD and monitoring disease progression (Furukawa et al., 2022). Notably, in 2010, the Michael J. Fox Foundation initiated the Parkinson Progression Marker Initiative (PPMI), an international, multicenter study aimed at identifying biomarkers of PD progression to enhance understanding of its pathophysiology and improve the success of disease-modifying therapies. The cohort consists of over 1,500 individuals, including PD patients and healthy controls, from whom clinical, imaging, and biospecimen data are collected using standardised protocols. All data are made publicly accessible via the PPMI database (Parkinson Progression Marker Initiative, 2011).

Human samples present several challenges, including their limited availability and the complexity of obtaining them, which often involves extensive bureaucratic procedures. Additionally, variability in extraction methods and timepoints, both between and within biobanks, can introduce inconsistencies in the outcomes. Nonetheless, human samples remain an invaluable resource, as they offer direct and unparalleled insights into the actual human pathology, making them crucial for advancing the understanding of neurodegenerative diseases like PD.

BOX 5. *IN VITRO* APPROACHES FOR PARKINSON'S DISEASE RESEARCH

In vitro models are based on cells, tissues, or organs that are cultured outside of a living organism. These models provide a simplified and controlled environment for investigating the cellular and molecular mechanisms underlying disease pathogenesis. They also serve as an initial platform for pharmacological screening and therapeutic intervention development. In PD research, *in vitro* models primarily focus on dopaminergic neurons and glial cells, which may originate from various sources. Additionally, organotypic cultures and organoids have been utilised to study PD, offering more complex and physiologically relevant systems (Smits et al., 2019; Elfarrash et al., 2021). This box summarizes the main *in vitro* approaches used in PD research.

Cell lines. Immortalised cell lines are modified, either intentionally or spontaneously, to proliferate indefinitely under appropriate culture conditions. These cell lines can be derived from human or animal sources and are often used for PD research via neurotoxin exposure, viral gene expression or genetic transfection. Some examples of cell lines used in the context of PD research are dopaminergic SH-SY5Y cell line (Burgaz et al., 2021a), microglial BV-2 cell line (Li et al., 2023), astrocytic U251 cell line (Zheng et al., 2023), or oligodendroglial MO3.13 cell line (Yang et al., 2024).

Primary cultures. Primary cells are derived directly from embryonic or neonatal rodent brain tissue, offering greater physiological and morphological relevance than immortalised cell lines. These cultures encompass dopaminergic neurons (Slanzi et al., 2020), microglia (Ho et al., 2017), astrocytes (Giehrl-Schwab et al., 2022) and oligodendrocytes (Kisos et al., 2012). PD-like alterations can be induced by exposing these cell cultures to neurotoxins, viral gene expression, transfection, or by using cells derived from transgenic animals carrying PD-associated genetic mutations. Additionally, mixed cultures, such as primary neurons and microglia (Liu & Hong, 2003), enable more comprehensive systems.

Human-induced pluripotent stem cells (hiPSCs). The reprogramming of somatic cells into pluripotent stem cells has revolutionised PD research. hiPSCs enable the generation of patient-specific dopaminergic neurons (Grigor'eva et al., 2022) and glial cells (Azevedo et al., 2022; Ohtonen et al., 2023; Yarkova et al., 2023). These cells replicate the genetic variability and physiological characteristics of sporadic or genetic PD patients more accurately than other *in vitro* systems. In addition to reprogrammed neuronal and glial models, **human-derived samples**, often involving innate immune cells such as peripheral monocytes or lymphocytes, can also be used to investigate systemic aspects of the disease (Sulzer et al., 2017; Muñoz-Delgado et al., 2021; Navarro et al., 2021). These approaches are particularly valuable for exploring inflammatory processes, thereby offering further insight into the immunopathological mechanisms underlying PD.

2. CANNABINOIDS AND ENDOCANNABINOID SYSTEM

The plant *Cannabis sativa* has been widely used for textile, medicinal, and recreational purposes since ancient times. The first documented reference to the medical applications of cannabis appeared in China about five thousand years ago, where its cultivation was generally associated with obtaining fibre and oil from the seeds. However, it was in India where a considerable degree of knowledge was reached on the therapeutic properties of cannabis, particularly in the context of alterations related to the nervous system (cramps, infantile convulsions, migraine, hysteria, neuralgia, sciatica and tetanus). Thus, cannabis use rapidly spread throughout Asia and Middle East but did not reach Europe until centuries later. During the 19th century, the English colonial presence in India and Napoleon's expeditions to Egypt brought the medical and recreational applications of cannabis to Europe, and subsequently to the United States. Nevertheless, the clinical use of cannabis began to decline in the early 20th century with the emergence of new compounds with greater therapeutic efficacy, such as opioids (Ramos Atance & Fernández Ruiz, 2000). The research interest in cannabinoids reemerged between 1940 and 1950, with the identification and characterization of cannabidiol (CBD) (Adams et al., 1940a) and cannabinol (CBN) (Adams et al., 1940b). After two decades, the main psychoactive component of cannabis,

Δ^9-tetrahydrocannabinol (Δ^9-THC), was isolated (Gaoni & Mechoulam, 1964). These early discoveries boosted research in the field of cannabinoids over the following decades (**Figure 10**), particularly into their biological effects, ultimately culminating in the identification of an entire endogenous system involved in these actions: the endocannabinoid system (ECS). It represents a complex intercellular communication system that spans the entire organism, playing a pivotal role in different physiological and pathological processes. Despite its wide distribution, the ECS appears to hold a special position within the CNS, where it acts as a master regulator in modulating neuronal activity and network function (Lu & Mackie, 2021). Among the major components of the ECS are the cannabinoid receptors, their endogenous ligands known as endocannabinoids (eCBs), and the enzymes responsible for the biosynthesis and inactivation of eCBs.

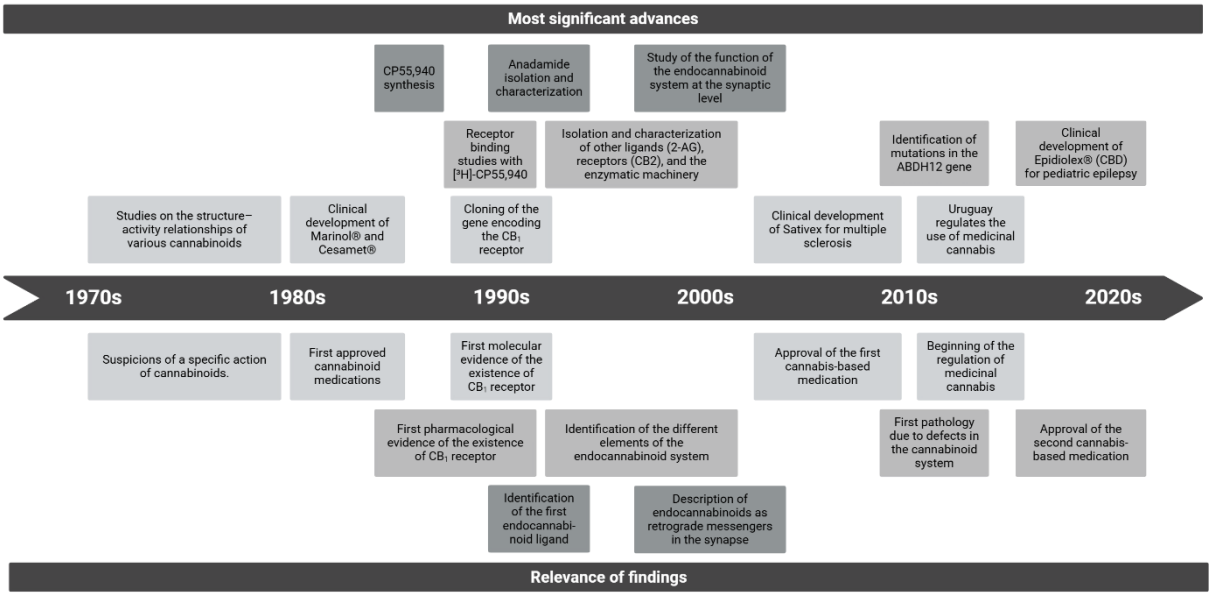


Figure 10. Chronology of the most important milestones in the research on the mechanisms of action of phytocannabinoids and in the discovery of the endocannabinoid system*. Created with BioRender.com.*

2.1. Cannabinoid receptors

The identification of stereochemical requirements for the psychotropic activity of cannabinoids, along with the structure–activity relationships observed across various compounds, led to the hypothesis that cannabinoids exert their effects through specific receptors (Mechoulam et al., 1980). To date, after prolonged research, two receptors have been formally recognised as cannabinoid receptors: cannabinoid type-1 receptor (CB1R) and cannabinoid type-2 receptor (CB2R). Both are G protein-coupled receptors (GPCRs), that is, integral membrane proteins with seven transmembrane domains, bound in their intracellular C-terminal to specific G proteins that trigger a wide variety of signalling pathways (Mackie, 2005).

2.1.1. Cannabinoid type-1 receptor

CB1R was first detected via pharmacological binding in 1988 (Devane et al., 1988), whereas its cloning took a few more years (Matsuda et al., 1990; Gérard et al., 1991). Ultimately, not long ago, its crystal structure was elucidated (Hua et al., 2016), completing the molecular characterization

of this cannabinoid receptor. CB1R is composed of a single polypeptidic chain of 472 amino acids in humans, encoded by the *CNR1* gene mapped to 6q15 in humans (GeneID 1268). Its sequence is well-conserved among vertebrates, with 97-99% of identity between humans and rodents (Howlett et al., 2002).

CB1R is widely distributed throughout the body, even though it is especially prevalent within the CNS. Indeed, it is considered one of the most abundant GPCRs in the mammal brain (Herkenham et al., 1991a). The brain areas with the highest levels of CB1R are the hippocampus, basal ganglia, and cerebellum, whereas other regions such as cortex, amygdala, thalamus, hypothalamus, brainstem, and spinal cord display lower expression of this cannabinoid receptor (Mackie, 2005). This anatomical distribution accounts for its involvement in multiple neurobiological functions, including movement control (Sañudo-Peña et al., 2000), nociception (Cravatt & Lichtman, 2004), learning and memory (Marsicano & Lafenêtre, 2009), sleep behaviour (Méndez-Díaz et al., 2021), food intake and appetite regulation (Tarragon & Moreno, 2019), or emotional behaviour (Martin et al., 2002). At the cellular level, CB1R is mostly located to the presynaptic terminals of GABAergic interneurons and, to a lesser extent, in glutamatergic projecting neurons (Tsou et al., 1998), where it mediates the inhibition of neurotransmitter release through retrograde signalling (see **Section 2.3 of INTRODUCTION**). In specific neuronal populations, a diffuse postsynaptic staining has also been reported (Tsou et al., 1998). However, it is not only neurons; CB1R is also expressed in glial cells, including astrocytes, oligodendrocytes, and microglia, as well as neural progenitors, albeit at lower levels than in neuronal cells (Zhang et al., 2014).

As usual for GPCRs, CB1R is primarily localised in the plasma membrane on the cell surface, where it can sense the outer space and trigger signalling cascades inside the cell (**Figure 11**). The canonical signalling pathway begins with the activation of $G_{i/0}$ proteins, whose α subunit inhibits the adenylate cyclase (AC) activity and reduces intracellular cyclic adenosine monophosphate (cAMP). This reduction of cAMP levels suppresses the activity of cAMP-dependent protein kinase (PKA), ultimately affecting the expression of genes regulated by the cAMP response element-binding (CREB) factor (Davis et al., 2003). Likewise, the βγ subunits released upon activation of $G_{i/0}$ proteins trigger the inhibition of calcium influx by blocking voltage-gated calcium channels and the enhancement of potassium efflux by activating inwardly rectifying potassium channels (Guo & Ikeda, 2004). This leads to an overall hyperpolarization of neuronal terminals and the consequent reduction of neurotransmitter release (Castillo et al., 2012). However, other signalling pathways can be induced by the activation of $G_{i/0}$ proteins, including the activation of mitogen-activated protein kinases (Bouaboula et al., 1995) and phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) (Galve-Roperh et al., 2002) pathways, which are key regulators of cell growth, survival and death. Aside from the classical $G_{i/o}$ coupling, in certain contexts the CB1R can also interact with G_s and G_q proteins. In general terms, G_s promotes the activation of AC, increasing cAMP levels (Glass & Felder, 1997); whereas G_q is responsible for activation of phospholipase C (PLC) and Ca^{2+} release from the ER, increasing cytosolic calcium levels (Lauckner et al., 2005).

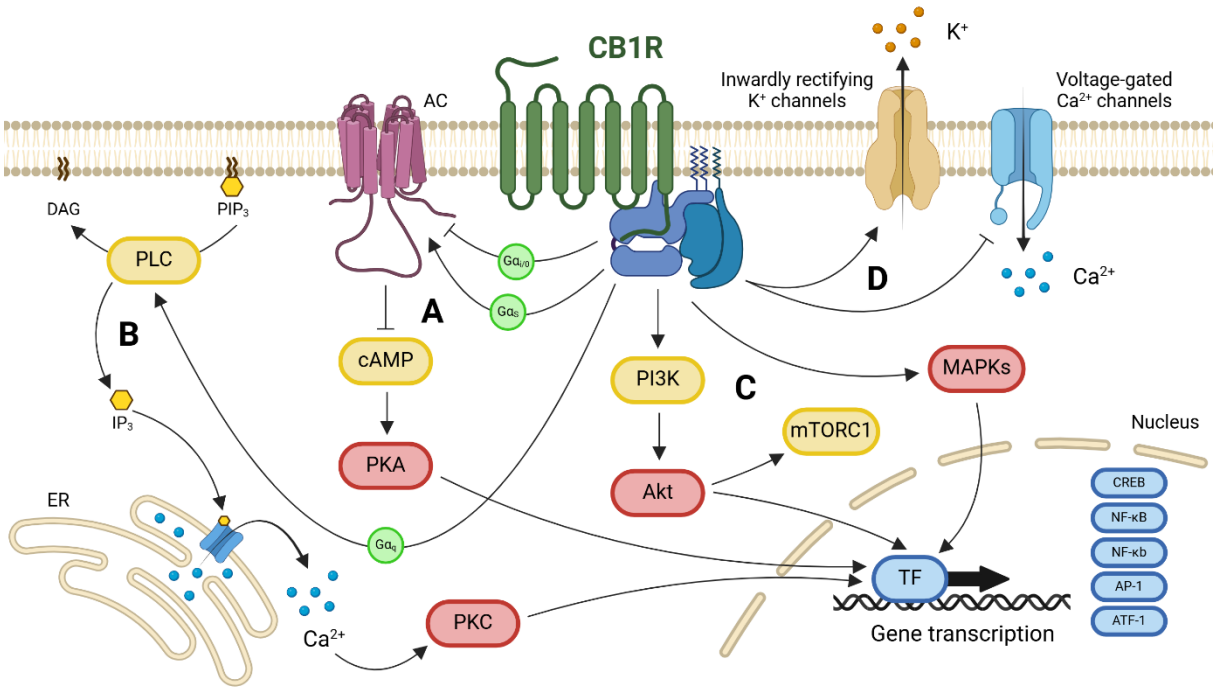


Figure 11. Main signalling pathways triggered by CB1R. *The primary signalling pathways activated by CB1R include (A) the inhibition of voltage-gated calcium channels and activation of inwardly-rectifying potassium channels, leading to a hyperpolarization state; (B) the modulation of AC activity, which impacts cAMP levels and, consequently, PKA activity; (C) the activation of PLC, resulting in the release of calcium stored in the ER via inositol-trisphosphate and the activation of PKC; and (D) the induction of PI3K and MAPK cascades, which further propagate downstream signalling. The modulation of these kinase activities (in red) affects the function of specific transcription factors (in blue), which, in turn, regulate the expression of genes involved in critical cellular processes, such as cell fate (survival vs. death) and inflammation. PIP_3, phosphatidylinositol-trisphosphate; DAG, diacylglycerol; IP3, inositol-trisphosphate; TF, transcription factor. Created with BioRender.com.*

Although CB1R signalling may be multifaceted, several factors contribute to this complexity. The CB1R is expressed in various cell types, each containing a different intracellular composition of signalling molecules, which favour particular pathways in specific cell populations. An example of this cell type-specific functional selectivity is how astrocytic CB1Rs predominantly couple with $G_{q/11}$ proteins, triggering the release of glutamate from astrocytes (Navarrete & Araque, 2008). Additionally, within the same cell there are distinct pools of intracellular CB1Rs separate from those on the plasma membrane, including within mitochondria. Mitochondrial CB1Rs signal differently from their cell surface counterparts, inhibiting a soluble form of AC and reducing PKA activity, which ultimately decreases mitochondrial respiration (Bénard et al., 2012). Beyond contextual factors, GPCRs can adopt multiple conformations based on ligand binding, a phenomenon known as biased signalling. This selective stabilization of a specific receptor conformation by a particular agonist triggers certain downstream pathways, leading to distinct physiological responses (Wouters et al., 2019). Another relevant feature of GPCRs is their ability to form homodimers (Wager-Miller et al., 2002) or heterodimers with other receptors (Callén et al., 2012; Martínez-Pinilla et al., 2014; Viñals et al., 2015). Dimerization alters ligand responses by these receptors, expanding their signalling repertoire. Finally, in addition to associating with other receptors, interactions with regulatory proteins significantly influence CB1R-evoked signalling. Several intracellular proteins have been found to interact with CB1R, including β-arrestins and CRIP1a (Howlett et al., 2010), and more recently BiP/GRP78/HSPA5 (Costas-Insua et al., 2021). This latest interactor has been reported to selectively favour specific G proteins in coupling with CB1R.

2.1.2. Cannabinoid type-2 receptor

A few years after the discovery of CB1R, a second cannabinoid receptor was identified, the so-called CB2R (Munro et al., 1993), whose crystal structure has also recently been published (Li et al., 2019). CB2R consists of a single polypeptidic chain of 360 amino acids encoded by the *CNR2* gene mapping to 1p36.11 in humans (GeneID 1269). It shares 44% sequence identity with CB1R, which rises to 68% within transmembrane regions involved in ligand binding (Montero et al., 2005). When evaluating amino acids sequence homology among species, CB2R displays greater variations in comparison to CB1R, with 81-82% of identity between humans and rodents (Zhang et al., 2015).

Originally, CB2R was described as a peripheral cannabinoid receptor owing to its initial predominant detection in immune cells (Munro et al., 1993; Galiègue et al., 1995). However, the peripheral designation was abandoned after identifying this cannabinoid receptor throughout the CNS, especially in microglia, but also in astrocytes, oligodendrocytes and neural progenitors (Zhang et al., 2014). Regarding neurons, some works have demonstrated CB2R expression in these (Atwood & Mackie, 2010; Stempel et al., 2016), even though there is still some controversy due to the lack of good experimental tools for its cellular detection. In general terms, CB2R exhibits lower expression levels within the CNS in comparison to CB1R or peripheral tissues, at least under physiological conditions. However, in a neuroinflammatory environment, CB2R expression is notably enhanced. This upregulation has been reported specifically in glial cells, both in patients (Benito et al., 2003; 2007; Palazuelos et al., 2009; Rodríguez-Cueto et al., 2014; Gómez-Gálvez et al., 2016; Espejo-Porras et al., 2018) and animal models (Sagredo et al., 2009; Espejo-Porras et al., 2015; Gómez-Gálvez et al., 2016; López et al., 2018) of a plethora of neurodegenerative disorders. The amplification in CB2R expression under some pathological conditions suggests a role for this cannabinoid receptor as a protective mechanism (Fernández-Ruiz et al., 2007; Pacher & Mechoulam, 2011).

The signalling pathways induced upon activation of the CB2R are practically the same as those described for the CB1R. However, there are some differences between the two receptors. Plasmalemmal CB2R does not signal through G_s and G_q proteins, even though it can activate PLC via the release of βγ subunits (Zoratti et al., 2003). More importantly, this receptor cannot regulate calcium and potassium conductance like CB1R, which prevents it from controlling neurotransmitter release via hyperpolarization. Beyond these differences, CB2R remains a GPCR, so similar phenomena contributing to the variety of signalling pathways in CB1R also occur with this receptor; for example, the formation of dimers (Callén et al., 2012; Singh et al., 2012; Moreno et al., 2014) or the existence of intracellular pools of the receptor that signal differently, such as through coupling to G_q proteins (Brailoiu et al., 2014).

2.1.3. Atypical cannabinoid receptors

Throughout the study of cannabinoids, most effects elicited by these compounds have been explained by their interaction with CB1R and CB2R. However, some studies have also reported

that some cannabinoid-evoked actions persist even when these classical receptors are pharmacologically blocked or genetically deleted (Jan et al., 2002; Qiao et al., 2012; Raffa & Ward, 2012). This suggests the involvement of non-canonical cannabinoid receptors in the pharmacology of cannabinoids. Other GPCRs, ion channels, and nuclear receptors have been identified as additional cannabinoid targets (Pertwee, 2015). To maintain conciseness, the primary representative of each receptor type will be described.

2.1.3.1. G protein-coupled receptor 55

The G protein-coupled receptor 55 (GPR55) was discovered as a novel human GPCR gene in 1999 (Sawzdargo et al., 1999). In this first *in silico* work, GPR55 was cloned and mapped to the locus 2q37 in humans, encoding a protein of 319 amino acids that shares the highest sequence identity with GPR35 (37%), GPR92 (30%), P2Y5 receptor (30%), and GPR23 (29%) (Baker et al., 2006). When compared to rodent orthologs, the degree of identity is over 75% (Ryberg et al., 2007).

Interestingly, GPR55 mRNA transcripts were first detected in human brain, exclusively in striatum (Sawzdargo et al., 1999). However, it is currently known that this receptor is expressed throughout the entire CNS, including striatum, hippocampus, forebrain, cortex, cerebellum, GP and SNpc (Wu et al., 2013; Patricio et al., 2022; Sánchez-Zavaleta et al., 2022), albeit at lower levels than CB1R. At the cellular level, GPR55 expression has been reported in both neurons (Chiba et al., 2011; Celorrio et al., 2017; Marichal-Cancino et al., 2017; Martínez-Pinilla et al., 2020; Sánchez-Zavaleta et al., 2022) and microglia (Pietr et al., 2009; Gasperi et al., 2013; Saliba et al., 2018; Shen et al., 2022). Aside from the CNS, GPR55 is also found in adrenal glands, gastrointestinal tract, lung, liver, uterus, bladder, kidney and bone (Shore & Reggio, 2015). Given its ubiquitous distribution, GPR55 appears to be involved in a variety of physiological and pathological processes. In the context of brain functionality, the use of GPR55-deficient animals has pointed to a function in motor coordination (Wu et al., 2013; Hurst et al., 2017; Marichal-Cancino et al., 2017), pain modulation (Staton et al., 2008), neurogenesis (Cherif et al., 2015; Hill et al., 2018; 2019) and immune/inflammatory regulation (Staton et al., 2008; Hill et al., 2019).

Initially, GPR55 was considered a novel cannabinoid-sensitive receptor, displaying affinity for a variety of cannabinoid compounds, with CBD acting as an antagonist (Ross, 2009). Indeed, based on these findings, GPR55 was eventually proposed as cannabinoid type-3 receptor (Moriconi et al., 2010). However, this possibility remains controversial due to the low sequence identity with CB1R (13.5%) and CB2R (14.4%), the lack of the classical cannabinoid binding pocket within its structure (Oka et al., 2007), and its distinct pharmacological profile compared to the canonical cannabinoid receptors (**Figure 12**). More consistent evidence points to GPR55 as the functional and selective receptor for lysophosphatidylinositol (LPI), which acts as a direct agonist (Oka et al., 2007; Henstridge et al., 2009; Kallendrusch et al., 2013; Kurano et al., 2021).

As a member of the GPCR superfamily, GPR55 is coupled to a specific set of G proteins, triggering different signalling cascades upon ligand binding (**Figure 12**). It is preferentially linked to G_q protein, driving an inositol trisphosphate-mediated calcium mobilization from the ER and

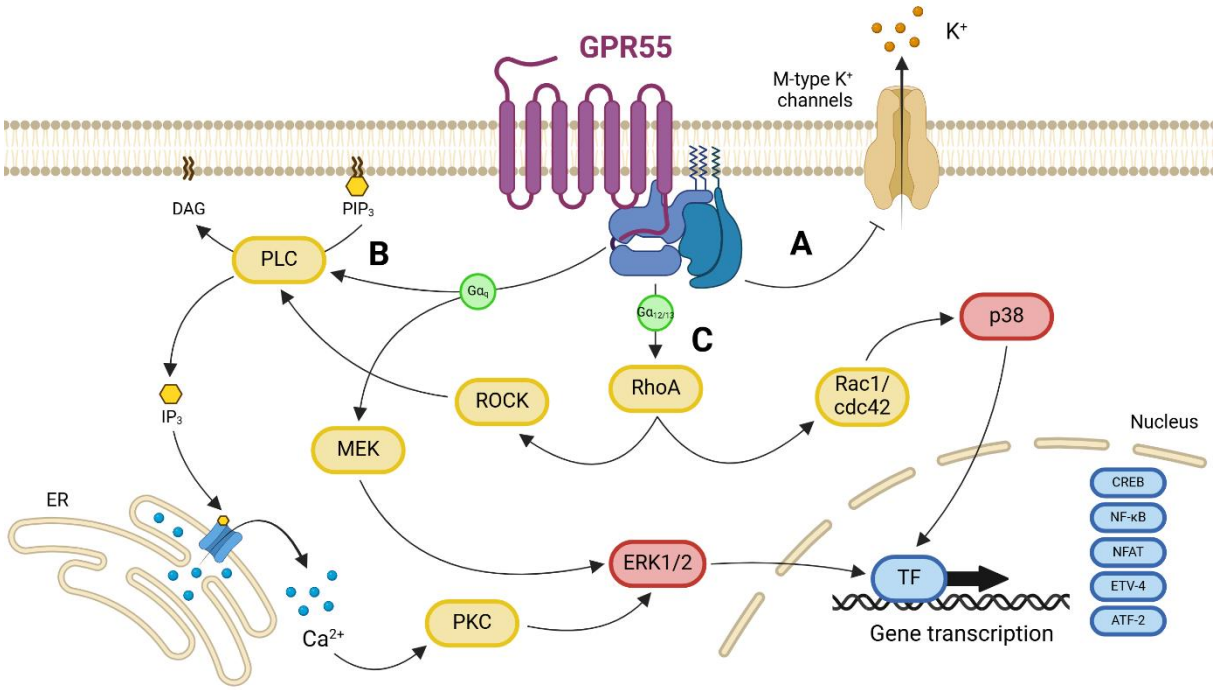


Figure 12. Main signalling pathways triggered by GPR55*. The primary signalling pathways activated by GPR55 include (A) the inhibition of M-type potassium current, resulting in enhanced neuronal excitability; (B) the activation of ERK1/2 via multiple vias, including the induction of PLC, which promotes the release of calcium stored in the ER through inositol trisphosphate, and the activation of MEK; and (C) the activation of the GTPase RhoA, initiating downstream cascades involving ROCK and other GTPases such as Rac1 or cdc42. These latter GTPases activate p38, which together with ERK1/2, regulates the expression of genes involved in critical cellular processes, such as cell fate (survival vs. death) and inflammation. PIP_3, phosphatidylinositol-trishosphate; DAG, diacylglycerol; IP3, inositol-trisphosphate; MEK, MAPK/ERK kinase; ROCK, Rho-associated protein kinase; TF, transcription factor. Created with BioRender.com.*

the phosphorylation of extracellular signal-regulated kinase 1/2 (Oka et al., 2007; Lauckner et al., 2008). These kinases induce downstream various transcription factors, which ultimately regulate cell proliferation and survival, as well as inflammation. However, GPR55 can also signal via $G_{12/13}$ proteins, activating GTPases such as Rac1, RhoA or Cdc42 (Ryberg et al., 2007). These molecules are involved in intracellular calcium mobilization (Henstridge et al., 2009), as well as cytoskeletal remodelling and migration (Lauckner et al., 2008; Balenga et al., 2011). Apart from G protein-mediated signalling, GPR55 has been reported to inhibit potassium currents (Lauckner et al., 2008), which, together with increased intracellular Ca^{2+} concentration, leads to an enhancement of neuronal excitability and neurotransmitter release. After a prolonged activation, GPR55 can interact with β-arrestin and GPCR-associated sorting protein-1, targeting this receptor for internalization and degradation (Kapur et al., 2009; Kargl et al., 2012). It is important to note that heterodimerization, mainly with CB1R (Martínez-Pinilla et al., 2020), as well as biased agonism or cell type-based functional selectivity (Moriconi et al., 2010), may further explain the preferential triggering of specific pathways over others.

In addition, there is a clear and strong association between other orphan GPCRs and eCB-like molecules, particularly in the cases of GPR18 (Kohno et al., 2006) and GPR119 (Overton et al., 2006).

2.1.3.2. Transient receptor potential cation channel subfamily V member 1

The transient receptor potential cation channel subfamily V member 1 (TRPV1), also known as the capsaicin receptor or vanilloid receptor 1, is mainly localised on nociceptive primary afferent

sensory neurons, where its activation is involved in the perception of nociceptive and inflammatory inputs (Caterina et al., 2000). This homotetrameric receptor can be activated by noxious heat, protons, inflammatory mediators, and a variety of chemicals, including capsaicin, the active (hot) component of chili peppers (Caterina et al., 1997). Besides its peripheral location, TRPV1 channels are widely distributed within the CNS, being present in the presynaptic terminals of glutamatergic and GABAergic neurons, where their activation can regulate synaptic transmission (Chahl, 2024). Within these central sites, the eCB anandamide is a full agonist of TRPV1 (Ross, 2003), creating a link between the ECS and this channel, which is then purported to participate in eCB signalling, specifically regarding synaptic transmission and pain regulation (Zou & Kumar, 2018). In fact, TRPV1 channels have been suggested to function as ionotropic cannabinoid receptors (De Petrocellis et al., 2017).

Not only TRPV1, but also other transient receptor potential channels of this subfamily (TRPV2-4) or other subfamilies (ankyrin receptor TRPA1, melastatin receptor TRPM8) have also shown to be regulated by cannabinoids (De Petrocellis et al., 2011; Muller et al., 2018).

2.1.3.3. Peroxisome proliferator-activated receptor γ

The peroxisome proliferator-activated receptor γ (PPARγ) belongs to a family of nuclear receptors that are ubiquitously expressed in the body. They function as transcriptional regulators, with a DNA-binding region that allows them to interact with PPAR response elements present in the genome. Therefore, when an activator binds to PPARs, they form heterodimers with retinoid X receptors, which translocate to the nucleus and trigger transcriptional responses. The target genes appear to be related to lipid metabolism and glucose homeostasis, as well as cell differentiation and inflammation (Mirza et al., 2019). Highly expressed in adipose tissue, PPARγ is also abundant in the CNS, being present in different brain regions, both in neurons and glia, albeit at lower levels in the latter (Warden et al., 2016). Remarkably, this nuclear receptor has been associated with diverse brain processes, such as neuroinflammation (Pizcueta et al., 2023) and neurogenesis (Quintanilla et al., 2014).

PPARγ displays a wide ligandome: endogenous activators include unsaturated fatty acids and eicosanoids, whereas other potent agonists such as thiazolidinediones have been developed as synthetic compounds (Kliewer et al., 1997). These PPARγ-activating compounds have exhibited neuroprotective effects both in the brain and in peripheral systems, regulating anti-inflammatory responses, oxidative stress, neuronal death, neurogenesis, cellular differentiation, and angiogenesis (Villapol, 2018). In addition to these endogenous and synthetic PPARγ ligands, several studies have reported that certain cannabinoids, whether endogenous, plant-derived, or synthetic, can activate PPARγ, as well as PPARα, even though with lower potency than classical PPAR-specific ligands (O'Sullivan, 2016). Activation of PPARγ by cannabinoids has been related to a range of physiological effects, including neuroprotection, anti-inflammatory responses, modulation of reward behaviour, memory, and cognition, analgesia, anti-tumour activity, and regulation of satiety, feeding and metabolism (O'Sullivan, 2016). Notably, a crosstalk between

CB2R and PPARγ signalling has been proposed for the antioxidant and anti-inflammatory effects of beta-caryophyllene (Youssef et al., 2019).

2.2. Cannabinoid ligands

Customarily, the term cannabinoid has been used to refer to a set of more than 100 molecules present in the *Cannabis sativa* plant, which mostly share a terpenophenolic chemical structure. However, this concept has evolved beyond these botanic-origin cannabinoids, so-called phytocannabinoids. Since the discovery of the ECS, two novel categories of compounds, according to their origin, were included in the current heterogenous family of cannabinoids: eCBs, endogenously produced by animals, mainly mammals (including humans); and synthetic cannabinoids, generated by chemical synthesis in the laboratory and designed on purpose to improve any aspect of their activity. Other classifications regarding pharmacological activity and chemical structure can be done.

2.2.1. Endocannabinoids

eCBs are lipid signalling molecules that act as endogenous ligands for the cannabinoid receptors. The major representatives of eCBs are two arachidonic acid derivatives: *N*-arachidonoylethanolamine (AEA), commonly known as anandamide; and 2-arachidonoylglycerol (2-AG). AEA was the first eCB discovered after the identification of CB1R (Devane et al., 1992). It shows relatively low levels in the CNS, where it acts as a partial agonist of CB1R and CB2R, with a weaker potency for the latter (Pertwee et al., 2010). In contrast, 2-AG, identified after the discovery of CB2R (Mechoulam et al., 1995), is more abundant and displays a full agonism for both receptors (Pertwee et al., 2010), thus being considered as the main endogenous cannabinoid agonist. However, the differences found in endogenous concentrations, receptor affinity and potency suggest that both eCBs can exert distinct effects within the body. For instance, in the context of low receptor expression or weak coupling to signalling pathways, AEA may antagonize the effects of other more effective agonists (Lu & Mackie, 2016), while it activates TRPV1 (Ross, 2003), inhibits calcium channels and negatively regulates 2-AG biosynthesis (Maccarrone et al., 2008). Other less studied eCBs include 2-arachidonoylglycerylether (noladin ether), *O*-arachidonoylethanolamine (virodhamine) and *N*-arachidonoyldopamine (Zoerner et al., 2011).

eCBs are produced on demand, synthesised from membrane phospholipids in response to specific stimuli, which normally trigger an elevation of intracellular calcium (Piomelli, 2003). A complex enzymatic machinery participates in the eCB synthesis, which involves multiple interconnected pathways; so, the focus will be on the main routes. AEA synthesis can begin with the transference of an arachidonoyl group to the phospholipid phosphatidylethanolamine, catalysed by an *N*-acyltransferase, giving rise to the intermediate *N*-arachidonoylphosphatidylethanolamine (NAPE); then, NAPE is hydrolysed by the NAPE-specific phospholipase D to finally generate AEA, as well as phosphatidic acid as a by-product (Di Marzo et al., 1994). Regarding 2-AG, the formation of the intermediate diacylglycerol (DAG), thanks to

the hydrolytic activity of PLC on phospholipids, is followed by the lipolytic cleavage of DAG by DAG lipase, which ultimately leads to the production of 2-AG and a free fatty acid (Bisogno et al., 2005).

Once produced, eCBs are immediately released to the extracellular space to exert their actions mainly through CB1R and CB2R. As is the case with classical neurotransmitters, tight regulation of eCB tone is required after receptor activation. The signal termination is initiated with the clearance of eCBs from the extracellular compartment and the subsequent inactivation by cellular enzymes. It remains elusive how eCBs return inside the cell, but several mechanisms have been considered. Passive diffusion across the membrane can be supported by the hydrophobic nature of eCBs and the concentration gradient established by intracellular degradation (Kaczocha et al., 2006). However, pharmacological blockade studies suggest the existence of lipid-carrier proteins that function as eCB membrane transporters (Hillard et al., 1997). Despite this, no specific protein or gene corresponding to a dedicated transporter for AEA or 2-AG has been identified to date. Fatty acid-binding proteins, such as FABP5, have been identified as intracellular carriers of eCBs, which facilitate their solubilization and delivery to sites of degradation or signalling, playing a crucial role in their synaptic transport and retrograde signalling (Oubraim et al., 2025). Upon internalization, eCBs are primarily inactivated via hydrolysis, releasing AA and ethanolamine or glycerol. Two major serine hydrolases have been found to degrade AEA and 2-AG: postsynaptic fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996) and presynaptic monoacylglycerol lipase (MAGL) (Dinh et al., 2002). Enzymatic hydrolysis of 2-AG can be also carried out by postsynaptic α/β-hydrolase domain type 6 and microglial α/β-hydrolase domain type 12 enzymes, albeit to a much lesser extent (Navia-Paldanius et al., 2012). Aside from hydrolysis, eCBs can also undergo oxidation by lipoxygenases, cyclooxygenases and cytochrome P450 oxidases (Guindon & Hohmann, 2008). However, these pathways should not be regarded as degradation routes.

2.2.2. Phytocannabinoids

The first cannabinoid compound isolated and identified was the phytocannabinoid CBN at the end of the 19th century (Ramos Atance & Fernández-Ruiz, 2000). It took a few more decades to discover what are now known as the main cannabinoids derived from the plant *Cannabis sativa*: CBD (Mechoulam & Shvo, 1963) and Δ^9-THC (Gaoni & Mechoulam, 1964). Over the following decades, an expanding array of phytocannabinoids was discovered. These compounds can be classified based on the number of rings in their chemical structure into tricyclics (Δ^9-THC, CBN), bicyclics (CBD), and monocyclics (cannabigerol, CBG). Additionally, the cannabis plant naturally produces varin derivatives characterised by a three-carbon aliphatic side chain, such as Δ^9-tetrahydrocannabivarin (Δ^9-THCV), cannabidivarin (CBDV), and cannabigerovarin, as well as acidic forms, which retain a free carboxyl group, including tetrahydrocannabinolic acid, cannabidiolic acid (CBDA), and cannabigerolic acid. Although some of these minor phytocannabinoids have gained pharmacological interest, such as CBG or Δ^9-THCV, the focus will be on Δ^9-THC and CBD.

Δ^9-THC, in its (–)-*trans*-enantiomeric form (**Box 6**), is the most prevalent phytocannabinoid in *Cannabis sativa* and the primary agent responsible for its psychotropic effects. Pharmacologically, it has been classified as a partial agonist for both CB1R and CB2R, even though it exhibits a certain preference for the former (Pertwee et al., 2010). Δ^9-THC exerts most of its central actions through the stimulation of the CB1R, including the so-called cannabinoid-induced tetrad: hypolocomotion, hypothermia, catalepsy, and antinociception (Martin et al., 1991); as well as the psychotropic effects. In contrast, when interacting with the CB2R, this phytocannabinoid can also display anti-inflammatory actions (Pacher & Mechoulam, 2011). Neuroprotection and neurorestoration have also been linked to Δ^9-THC activity (Fernández-Ruiz et al., 2015). In addition to classical cannabinoid receptors, Δ^9-THC has been reported to engage with other targets, which include orphan receptors (GPR18, GPR55), TRP channels (TRPV2, TRPA1), glycine and serotonin 5-HT_{3A} receptors (Howlett et al., 2002; Pertwee et al., 2010). Remarkably, these off-targets have been mostly substantiated in *in vitro* experiments at high concentrations (micromolar range), so their physiological relevance still needs to be elucidated.

BOX 6. Stereoisomers of phytocannabinoids

Typically, when talking about Δ^9-THC or CBD, it is assumed that reference is being made to the form in which they are naturally found in the plant. However, from a pharmacological perspective, it is important to consider that these phytocannabinoids can acquire other conformations. Both Δ^9-THC and CBD possess two chiral centers (carbons 3 and 4), allowing them to have four potential stereoisomers, and therefore, two pairs of enantiomers: (–)-*trans*-(3R,4R), (+)-*trans*-(3S,4S), *cis*-(3R,4S), and *cis*-(3S,4R)-isomer (**Figure 13**). These stereoisomers are structurally identical but differ in the spatial arrangement around the stereogenic centers, creating mirror images of one another. Moreover, they often show completely different activities despite being the 'same' compound. As regards to Δ^9-THC and CBD, nature seems to prefer (–)-*trans* isomers, as they are the ones found in the plant. Interestingly, the naturally occurring (–)-*trans*-CBD has no affinity for either CB1R or CB2R. Yet, non-natural (+)-*trans*-CBD has shown a stronger affinity for both cannabinoid receptors (Bisogno et al., 2001; Fride et al., 2004; Hanus et al., 2005), completely changing the pharmacology of the molecule when compared with its natural counterpart. Preclinical studies have been performed with (+)-*trans*-CBD and derivatives, confirming various biological activities in different cell lines, which can be mostly addressed to their binding in CB1R/CB2R orthosteric sites. Among the reported effects, those related to the modulation of the immune response stand out, with predominantly anti-inflammatory effects being observed (Kozela et al., 2016; Götz et al., 2019). Thus, it is important to consider the change in stereoisomerism as a source of pharmacological diversity in cannabinoids, with endogenous racemic conversion as a potential mechanism to explore.

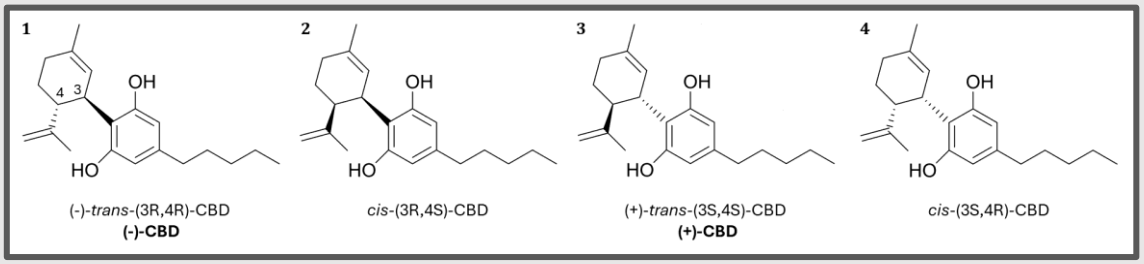


Figure 13. Chemical structures of the four CBD stereoisomers*. The stereocentres are indicated at positions 3 and 4. Compounds 1 and 2 correspond to the trans isomers, while compounds 3 and 4 are the cis isomers (Adapted from Rao et al., 2025).*

Regarding CBD, this is the second most common phytocannabinoid and the major non-psychotropic one. Indeed, CBD lacks affinity for CB1R and CB2R (Pertwee et al., 2010), and consequently, it does not elicit the cannabinoid-induced tetrad nor the psychotropic effects. However, this molecule is considered to have one of the greatest therapeutic potentials,

considering its broad neuroprotective profile and its favourable benefit-to-risk ratio, with no undesired psychotropic side effects. Numerous studies have reported beneficial actions of CBD on inflammation, oxidative stress, excitotoxicity and neurogenesis, among others (Fernández-Ruiz et al., 2015). This pleiotropism is closely associated with a complex pharmacology, as CBD has been found to target GPR18, GPR55, PPARγ, $GABA_A$ and $5\text{-}HT1_A$ receptors, adenosine A_{1A} receptors, and TRPV channels (TRPV1, TRPV2, TRPA1) (Howlett et al., 2002; Pertwee et al., 2010). Moreover, some studies have indicated that CBD may allosterically modulate CB1R (Laprairie et al., 2015) and CB2R (Martínez-Pinilla et al., 2017). In addition to receptor-dependent mechanisms, the chemical structure of CBD provides this molecule with a direct antioxidant profile, as its hydroxyl groups and benzene ring can neutralize free radicals. The antioxidant properties of CBD have even been reported to surpass those of common antioxidant agents, such as ascorbic acid or α-tocopherol (Atalay et al., 2019).

The favorable pharmacological tolerance and safety profile of CBD have led to the development of two pharmaceutical formulations based on this phytocannabinoid: Epidiolex®, a purified oral solution of CBD, prescribed as an anticonvulsant drug (Devinsky et al., 2017); and Sativex®, a 1:1 combination of CBD and Δ^9-THC, administered as a sublingual spray for the treatment of multiple sclerosis-related spasticity (Patti et al., 2016) and oncological pain (Johnson et al., 2013).

2.2.3. Synthetic cannabinoids

Besides natural cannabinoids, a third group has emerged, driven by the need to endow these compounds with new or improved properties. Thus, through either *de novo* synthesis or starting from known molecules, a relevant array of (semi-)synthetic compounds has been generated, which now constitute new pharmacological tools and/or potential drugs.

This massive plethora of synthetic cannabinoid ligands has been classified regarding their pharmacological profile using binding assays (Pertwee et al., 2010). They include non-selective and selective agonists, antagonists, and inverse agonists. In **Table 2**, the most widely used synthetic cannabinoids are listed along with their pharmacological profiles and affinity values at CB1R and CB2R, if applicable. For instance, in this PhD project, among the listed compounds, the non-classical cannabinoid CP55,940 has been used as a radiolabelled ligand in binding assays, as well as the AEA derivative arachidonyl-2'-chloroethylamide (ACEA) to selectively target CB1R in an *in vivo* assay.

Furthermore, in contrast to classical ligands targeting the orthosteric sites of cannabinoid receptors, the development of allosteric modulators and indirect ligands has presented a significant opportunity to expand and diversify pharmacological approaches within the cannabinoid field. A wide range of allosteric modulators has been designed for CB1R since 2005, such as Org27569 (Price et al., 2005), while in the case of CB2R, the development of effective and selective allosteric modulators has lagged further behind (Yuan et al., 2022). Among the compounds that regulate eCB tone are molecules designed to block eCB uptake, such as UCM707 (López-Rodríguez et al., 2003), as well as inhibitors of eCB degradation enzymes, including

Table 2. *Pharmacological profiles and affinity values (K_i) for cannabinoid receptors of main synthetic cannabinoid ligands.*

Ligand	Pharmacological profile	K_i CB1R (nM)	K_i CB2R (nM)	Reference
HU-210	Non-selective CB1R and CB2R	0.1	0.2	Rhee et al., 1997
WIN 55,212-2		4.4	1.2	Hillard et al., 1999
CP 55,940		0.5	2.8	
ACEA	Selective CB1R agonist	1.4	>2000	
Met-AEA		28.3	868	
HU-308	Selective CB2R agonist	>10000	22.7	Hanus et al., 1999
JWH-133		677	3.4	Huffman et al., 1999
JWH-139		2290	14	
SR141716A	Selective CB1R antagonist/inverse agonist	1.8	514	Rinaldi-Carmona et al., 1994
AM-251		7.5	2290	Gatley et al., 1996
SR144528	Selective CB2R antagonist/inverse agonist	>10000	5.6	Rinaldi-Carmona et al., 1998
AM-630		5152	31.2	Pertwee et al., 1995

UBR597, which targets FAAH (Piomelli et al., 2006), and JZL184, a selective MAGL inhibitor (Long et al., 2009). Furthermore, considering atypical cannabinoid receptors, significant efforts have also been made to develop novel compounds that target these cannabinoid-related sites. These include GPR55 agonists (e.g., O-1602) (Johns et al., 2007) and antagonists (e.g., ML193) (Heynen-Genel et al., 2010), as well as PPARγ activators (e.g., VCE-003.2) (García et al., 2018) and inhibitors (e.g., T0070907) (Lee et al., 2002).

2.3. Physiological roles of endocannabinoid system

Given the widespread distribution of the various components of the ECS across a wide range of tissues, organs, and physiological systems, it is unsurprising that this system exerts a modulatory influence on numerous physiological processes. Thus, a regulatory effect has been described at the level of the immune system, where cannabinoids act as anti-inflammatory and immunosuppressive agents (Kasatkina et al., 2021); the cardiovascular system, promoting vasorelaxation and bradycardia (Sierra et al., 2018); the endocrine system, where it modulates neuroendocrine responses (Borowska et al., 2018); the skeletal system, participating in bone remodelling (Raphael & Gabet, 2016); and in other peripheral organs and tissues such as the liver, pancreas, adipose tissue, and skeletal muscle, where eCBs promote gluconeogenesis and fatty acid synthesis (Bellocchio et al., 2008).

However, the first function of the ECS to be described, and the one most pertinent to this doctoral thesis, pertains to its role in the nervous system, where it modulates a wide array of neurobiological processes. In a neuronal context, the primary role of eCBs is to modulate synaptic transmission. Acting as retrograde messengers, eCBs are released by the post-synaptic neuron following depolarization and bind to CB1R located presynaptically, where they suppress neurotransmitter release (Castillo et al., 2012) (**Box 7**). By modulating neuronal activity and synaptic transmission across distinct brain regions, the ECS within the nervous system exhibits pleiotropic properties, influencing multiple neurobiological functions, including motor control,

nociception, memory and learning, endocrine regulation, emesis, thermoregulation, food intake and appetite, sleep, and various emotional and cognitive processes (**Table 3**). Furthermore, the ECS plays a crucial role during brain development, as well as in the survival and integrity of neural cells, promoting apoptosis of tumour neural cells, protecting healthy neurons, and facilitating the repair of damaged cells. These processes hold therapeutic potential in the context of neurological tumours and neurodegenerative diseases.

BOX 7. Endocannabinoid-mediated signalling

eCBs modulate synaptic transmission, acting as synaptic messengers. However, unlike neurotransmitters, they exert retrograde signalling, from postsynaptic to presynaptic terminals (**Figure 14A**). This kind of communication participates in neuronal plasticity processes, such as depolarization-induced suppression of inhibition (Wilson & Nicoll, 2001) and of excitation (Melis et al., 2004), as well as long-term depression at both inhibitory and excitatory synapses (Kreitzer & Regehr, 2001). The eCB-mediated retrograde signalling starts with the depolarization-induced elevation of cytosolic calcium in the postsynaptic compartment, which activates Ca^{2+}-sensitive enzymes for eCB synthesis. Once synthesised, eCBs are released into the synaptic cleft, where they move backwards and bind to presynaptic cannabinoid receptors, mainly CB1R. Upon activation of CB1R, eCBs suppress the release of neurotransmitters via two distinct pathways responsible for short-term and long-term plasticity, respectively. Short-term depression involves βγ subunits of G_i protein, which inhibit Ca^{2+} influx, crucial for neurotransmitter release, and enhance K^+ efflux, promoting a hyperpolarised state. In contrast, long-term depression requires the inhibition of cAMP/PKA pathway via AC inhibition, induced by $G\alpha_i$ (Castillo et al., 2012). In line with what was previously mentioned, 2-AG seems to display a pivotal role in this retrograde signalling.

Paracrine signalling can involve non-neuronal cell types, such as astrocytes (**Figure 14B**). Since astrocytes are part of the tripartite synapse, they are also influenced by eCBs released into the synaptic cleft by neurons. When eCBs bind to astrocytic CB1R, they can regulate astrocyte physiology, triggering gliotransmission and affecting synaptic function (Oliveira da Cruz et al., 2016). Besides their paracrine action, eCBs can also modulate synaptic transmission in an autocrine or non-retrograde manner (**Figure 14C**). These compounds can interact with receptors located postsynaptically, such as TRPV1 or CB1R, resulting in long-lasting forms of synaptic inhibition (Bacci et al., 2004; Grueter et al., 2010).

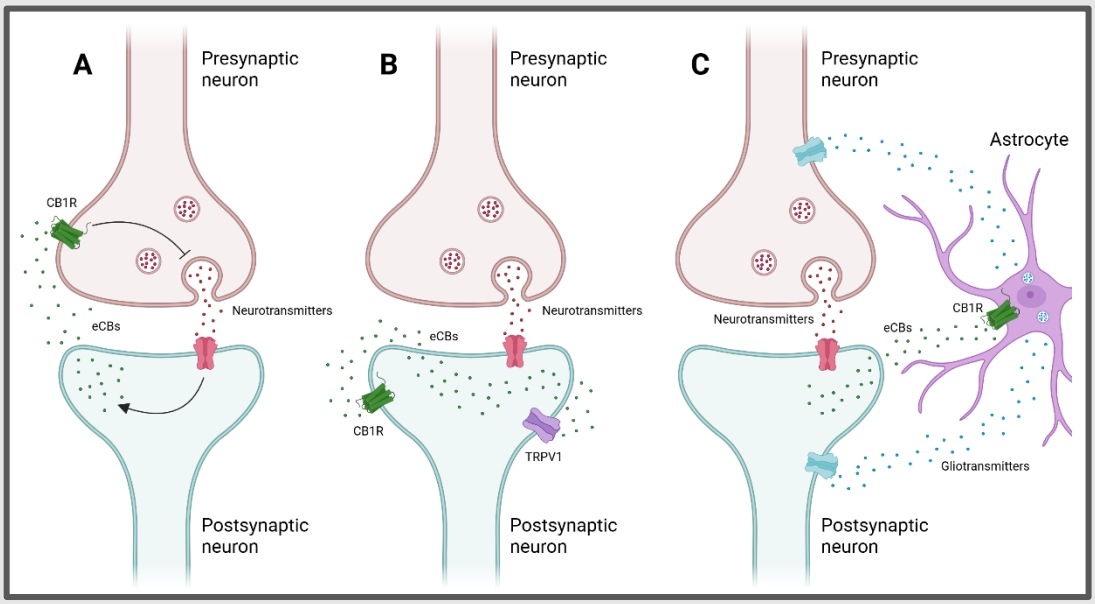


Figure 14. Modulation of synaptic transmission by eCBs *through (A) retrograde signalling, acting on presynaptic CB1R; (B) non-retrograde signalling, acting on postsynaptic receptors, such as CB1R or TRPV1; and (C) neuron-astrocyte signalling, acting on astrocytic CB1R. (Adapted from Castillo et al., 2012). Created with BioRender.com.*

Beyond neurons, the eCB signalling system also plays a crucial physiological role in glial cells, with key components found in astrocytes, oligodendrocytes, and microglia. Specifically, eCBs and cannabinoid receptors modulate the proliferation, differentiation, and survival of glial cells, as well

***Table 3**. Main physiological functions of the ECS in the CNS, along with associated locations and their potential therapeutic applications in various pathologies.*

Process	ECS role	ECS location	Indications	Reference
Motor activity	Modulates and fine-tunes motor activity, by enhancing or suppressing movement, depending on the context and the specific brain regions involved	BG and cerebellum	PD, Huntington's disease, Tourette syndrome, dyskinesias	Fernández-Ruiz et al., 2011
Nociception	Reduces pain perception and inflammation, contributing to the body's natural pain-relief mechanisms	Supraspinal brain and spinal areas, and peripheral sensory nerve terminals	Neuropathic and inflammatory pain	Vučković et al., 2018
Memory and learning	Helps with the selective forgetting of irrelevant memories, facilitating cognitive flexibility and efficient memory processing	Hippocampus	Post-traumatic stress, AD	Marsicano & Lafenêtre, 2009
Neuroendocrine response	Influences hormone secretion, helping to maintain homeostasis in response to environmental and physiological changes	Mediobasal hypothalamus	-	Tasker et al., 2015
Emesis	Acts as a modulator of emesis, helping to control nausea and vomiting	Area postrema	Nausea and vomiting in chemotherapy patients	Parker et al., 2011
Thermoregulation	Helps to balance and regulate body temperature in response to environmental change	Hypothalamic nucleus	-	Rawls & Benamar, 2011
Intake and appetite regulation	Increases food intake by enhancing the sensation of hunger, promoting feed behaviour	Hypothalamic nucleus, limbic structures	Obesity, age-induced anorexia, anorexia in cancer and HIV/AIDS patients	Tarragon & Moreno, 2019
Sleep behaviour	Supports the initiation and maintenance of sleep, enhancing deep sleep and improving overall sleep quality	Anterior lateral hypothalamus	Sleep disorders, insomnia	Méndez-Díaz et al., 2021
Emotional and cognitive processes	Enhances motivation, regulates emotivity by helping balance emotional responses, exerts anxiolytic effects and plays a key role in the reward system, enhancing pleasure and reward responses	Cortex and limbic system	Addiction, psychiatric disorders	Martin et al., 2002

as their specific functions within the CNS. Astrocytes are capable of synthesising their own eCBs (Schüle et al., 2021), and astrocytic CB1R participates in processes such as calcium signalling, gliotransmission, metabolism, and inflammatory responses (Eraso-Pichot et al., 2023). In oligodendrocytes, CB1R and CB2R act synergistically to promote cell survival, proliferation, migration, differentiation, and remyelination (Molina-Holgado et al., 2023). Microglial cells express low levels of cannabinoid receptors when at rest, but upon stimulation, CB2R expression significantly increases, playing a pivotal role in proliferation, migration, and modulation of their inflammatory phenotype (see **Section 2.4.1 of INTRODUCTION**).

2.4. Neuroprotective actions of cannabinoids

The involvement of the ECS in multiple neurobiological processes allows it to exert a plethora of beneficial effects in different pathological conditions. In fact, five cannabinoid-based drugs have already been approved for the treatment of certain symptoms of various pathologies (**Box 8**). However, in the specific context of neurodegenerative diseases, the most relevant function that the ECS can exert is the regulation of cellular fate; that is, controlling the homeostasis, integrity and

survival of neural cells (Garcia-Arencibia et al., 2019). Although this action is not exclusive to neural tissue, since neurons are post-mitotic cells and the replacement of new neurons is limited, if not null, the cytoprotective effects of ECS have gained more interest in the nervous system. Within this system, it has been observed how, in pathological conditions, some elements of the ECS can be modified in response to damage to display neuroprotective mechanisms (Bilsland et al., 2006; Gómez-Gálvez et al., 2016). Advantageously, neuroprotective properties of cannabinoids are pleiotropic, acting synergistically on multiple cytotoxic events that occur simultaneously in neurodegenerative disorders, such as neuroinflammation, oxidative stress, excitotoxicity or protein aggregation (Fernández-Ruiz et al., 2015) (**Figure 15**).

BOX 8. Cannabinoid-based drug development

Based on the solid evidence obtained from preclinical and clinical studies, the ECS has been positioned as a potential target with pharmacological applications in various pathological contexts. To date, five cannabinoid-based drugs have been developed, approved and commercialised (**Table 4**). Some of these are based on phytocannabinoids, whereas the active component in others is a synthetic molecule. Although these cannabinoid-derived drugs are primarily available to address symptoms in different diseases without modifying the progression of the pathology, their validation and marketing help foster consensus on their therapeutic efficacy and safety, while also mitigating the associated social stigma.

***Table 4**. Cannabinoid-derived drugs approved for use in the treatment of various pathologies and symptoms. FDA: Food and Drug Administration; EMA: European Medical Agency,*

Drug	Active molecule	Approved	Mechanism of action	Indication	Reference (Pubchem)
Marinol®	Δ^9-THC or dronabinol (phytocannabinoid from plant extract)	1985 by FDA	Partial agonist at both cannabinoid receptors	Anorexia associated with weight loss in AIDS patients. Nausea and vomiting associated with chemotherapy in patients who have not responded adequately to conventional antiemetic treatments.	CID 16078, 2025
Cesamet®	Nabilone (synthetic)	1981 by EMA		Nausea and vomiting associated with chemotherapy in patients who have not responded adequately to conventional antiemetic treatments.	CID 5284592, 2025
Acomplia®	SR141716A or rimonabant (synthetic)	2006 by EMA, withdrawn in 2008	Inverse agonist at CB1R	Obesity or overweight with associated risk factors, such as type 2 diabetes or dyslipidemia; in combination with diet and exercise.	CID 104850, 2025
Sativex®	Δ^9-THC:CBD (1:1) (phytocannabinoids from plant extract)	2017 by EMA	Partial agonist at both cannabinoid receptors/negative allosteric modulator at CB1R	Spasticity and neuropathic pain in multiple sclerosis.	CID 44148067, 2025
Epidiolex®	CBD (phytocannabinoid from plant extract)	2018 by FDA 2019 by EMA	Not fully understood, but 5-HT_{1A} and CB2R might be involved	Seizures associated with refractory epilepsy in Lennox-Gastaut syndrome or Dravet syndrome; in combination with clobazam, for patients aged 2 years or older.	CID 644019, 2025

2.4.1. Anti-inflammatory effects

The anti-inflammatory action of cannabinoids is one of the most well-documented properties of these molecules. Their activity is based on simultaneously promoting the production of anti-inflammatory mediators, such as IL-4 or IL-10, and inhibiting the release of pro-inflammatory mediators, such as IL-1β or TNF-α (Benito et al., 2008). In the nervous system, the anti-inflammatory action promoted by cannabinoids is primarily due to the activation of CB2R on glial

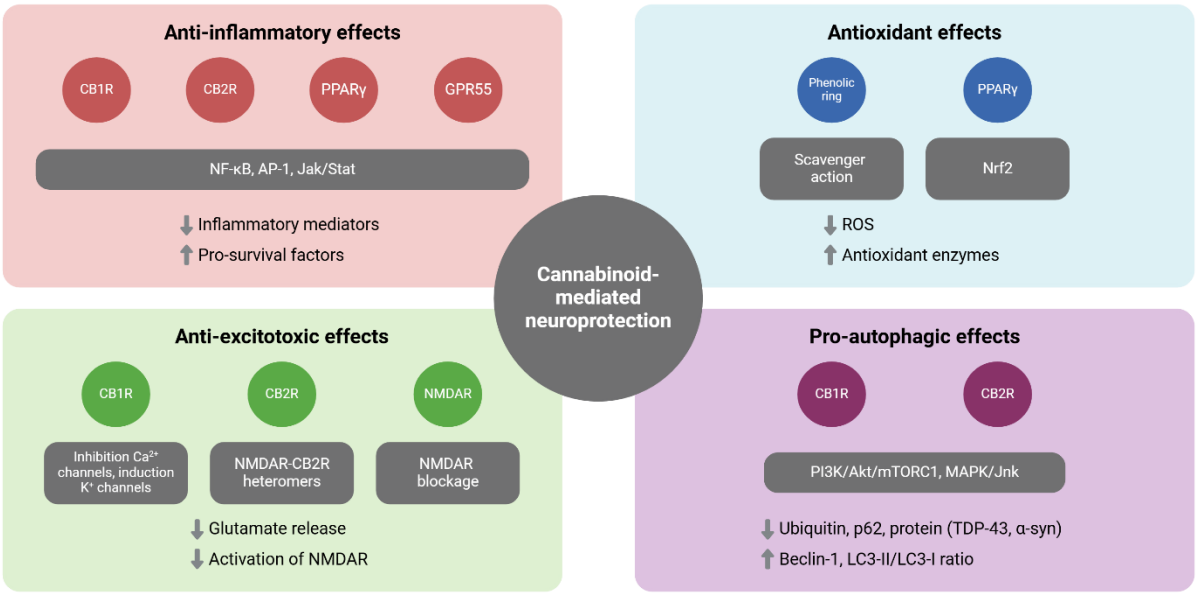


Figure 15. Key mechanisms and outcomes of the neuroprotective effects by cannabinoids*. The main neuroprotective actions of cannabinoids are outlined, highlighting primary targets and receptors (depicted as coloured circles), mechanisms and intracellular pathways (represented in grey boxes), and the observed outcomes. Created with BioRender.com.*

cells (see **Section 2.1.2 of INTRODUCTION**), which undergo up-regulation under pathological conditions ranging from neurodegenerative diseases to more acute neural disorders, such as ischaemia. This overexpression of CB2R can be interpreted as an endogenous protective response, as numerous pharmacological studies have shown that activation of these receptors by different cannabinoids promotes anti-inflammatory effects and reduces the presence of neurotoxic factors such as TNF-α in preclinical models of AD (Tolón et al., 2009; Aso et al., 2013), Huntington's disease (Palazuelos et al., 2009; Sagredo et al., 2009), PD (Gómez-Gálvez et al., 2016; Burgaz et al., 2021b), amyotrophic lateral sclerosis (Espejo-Porras et al., 2018; Rodríguez-Cueto et al., 2021a), and multiple sclerosis (Tiberi et al., 2022); even promoting neuronal survival. Other studies have highlighted that activation of CB2R also preserves the function of the BBB in neuroinflammatory models, both *in vitro* (Bullock et al., 2023) and *in vivo* (Wang et al., 2018). In contrast, genetic deletion of CB2R in murine models of these diseases enhances the inflammatory response, leading to a global worsening of the pathology (Palazuelos et al., 2009; García et al., 2011; Rodríguez-Cueto et al., 2021b). It is important to note that although CB2R is primarily expressed in microglia, its presence in lymphocytes, macrophages, and neutrophils also contributes to the modulation of the inflammatory response (Kasatkina et al., 2021).

In addition to CB2R, there are other cannabinoid-sensitive receptors capable of regulating neuroinflammation. As previously mentioned, the activation of PPAR-γ can regulate immune responses by influencing microglial activation and the differentiation of monocytes and lymphocytes, blocking the NF-κB, AP-1, and Jak-Stat pathways (Carta et al., 2011). Several cannabinoid compounds have demonstrated anti-inflammatory properties as PPAR-γ activators, with the phytocannabinoid CBG (Granja et al., 2012) and its synthetic derivative VCE-003.2 (Burgaz et al., 2019) standing out. GPR55 is also gaining relevance as a potential immunomodulatory target, as it is highly expressed by microglia (Shen et al., 2022). Moreover, microglial depletion attenuates the neuroprotective effects exerted by the activation of this

receptor (Kallendrusch et al., 2013). Stimulation of microglial cells by LPS or IFN-γ appears to mainly lead to an increase in the expression of this receptor, although downregulations have also been observed (Pietr et al., 2009; Gasperi et al., 2013). Several pharmacological studies have shown how targeting GPR55, either with agonists or antagonists, leads to a reduction in pro-inflammatory cytokines and an increase in anti-inflammatory factors, along with morphological changes in microglia consistent with an anti-inflammatory phenotype (Celorrio et al., 2017; Hill et al., 2019; Wang et al., 2022b; Xiang et al., 2022a). Finally, while its role may be considered limited, microglial CB1R also seems to influence the brain's response to inflammation, although in a manner opposite to that of CB2R (De Meij et al., 2021).

2.4.2. Anti-excitotoxic effects

Considering the regulatory role of the ECS in synaptic function, which limits neurotransmitter release in the synaptic cleft through its retrograde signalling, it is plausible to postulate the potential role of cannabinoids in excitotoxicity. Activation of CB1R on presynaptic glutamatergic neurons reduces glutamate release, which in turn decreases the activation of postsynaptic NMDA receptors, ultimately mitigating neuronal excitotoxicity. The anti-excitotoxic action of CB1R agonists has been demonstrated both *in vitro* (Abood et al., 2001) and *in vivo* (Pintor et al., 2006), preventing neuronal damage caused by various excitotoxins. This neuroprotective action is lost in the presence of CB1R antagonists. Similarly, genetic deletion of the CB1R in murine models with excitotoxic damage reinforces the importance of this receptor in the protective effect (Marsicano et al., 2003; Chiarlone et al., 2014).

Within the ECS, other anti-excitotoxic mechanisms exist that are independent of the CB1R. Some cannabinoids, such as AEA or HU-211, can act directly on NMDA receptors, blocking them (Shohami & Mechoulam, 2000). Additionally, the formation of heteromers between NMDA receptors and CB2R in certain neuronal types may help mitigate excitotoxicity by blocking the ionotropic function of the former after activation of the latter (Rivas-Santisteban et al., 2021).

2.4.3. Antioxidant effects

Another neuroprotective property of cannabinoids lies in their ability to act as antioxidant agents. Phytocannabinoids such as CBD and Δ^9-THC, as well as synthetic cannabinoids like CP-55,940, can act as ROS scavengers by having a phenolic ring structure capable of donating electrons to free radicals (Marsicano et al., 2002). However, in addition to this receptor-independent mechanism, some cannabinoids can combat oxidative stress by modulating signalling pathways that regulate antioxidant responses; for instance, the activation of PPARγ and Nrf2 (Gugliandolo et al., 2018). Studies have shown that CBD can induce both transcription factors, which cooperate reciprocally to control the expression of antioxidant enzymes, such as catalase, glutathione S-transferase, and manganese-dependent superoxide dismutase (Atalay et al., 2019). This phytocannabinoid can also reduce oxidative stress by decreasing the expression of enzymes such as cyclooxygenase 2 (COX-2) and iNOS (Castillo et al., 2010; Chen et al., 2016). Regarding cannabinoid receptors, it has

been reported that CB1R and CB2R can differentially modulate ROS production in macrophages, increasing it or decreasing it, respectively, to modulate inflammatory responses (Han et al., 2009).

2.4.4. Pro-autophagic effects

The benefits of cannabinoids in inducing autophagy have been well established in cancer models, where they exert antineoplastic effects by promoting autophagic cell death in tumour cells. This pro-autophagic activity appears to be mediated by the activation of both CB1R and CB2R (Salazar et al., 2009; Velasco et al., 2016; Mao et al., 2019; Ellert-Miklaszewska et al., 2021). However, the role of cannabinoid-induced autophagy in other cellular contexts, such as neurodegeneration, where protein aggregation is a common feature, remains less understood. Only a few studies have investigated the modulation of autophagy by cannabinoids in neurodegenerative disease models. For instance, administration of Sativex®, a 1:1 mixture of Δ^9-THC and CBD, in a complex tauopathy model induced autophagy, as observed with decreased p62 levels, increased LC3-II/LC3-I ratio, and elevated Beclin-1 levels. This resulted in a reduction in tau and amyloid deposition in the hippocampus and cerebral cortex, as well as an improvement in behavioural symptoms (Casarejos et al., 2013). To identify specific targets, subsequent studies employed selective agonists to examine the contribution of each cannabinoid receptor. HU-308, a selective CB2R agonist, increased autophagy markers (LC3-II/LC3-I ratio, Beclin-1 expression) in a murine model of experimental autoimmune encephalomyelitis, whereas opposite effects were observed following genetic deletion of the CB2R, confirming the role of this receptor in modulating autophagy (Shao et al., 2014). Consistent results were observed in a murine PD model where HU-308 normalised lysosomal marker LAMP-1 (*Lysosomal-Associated Membrane Protein 1*) levels (Palomo-Garo et al., 2016). Regarding the CB1R, *in vitro* studies have highlighted its role in inducing autophagy and restoring α-syn levels after overexpression in a neural cell line (Erustes et al., 2024). Moreover, recently published data from our laboratory demonstrate that ACEA, a selective CB1R agonist, induces autophagy, as evidenced by an increased LC3-II/LC3-I ratio, as well as a reduction in aggregation markers such as ubiquitin, p62, and TDP-43, in a mouse model of frontotemporal dementia (Gonzalo-Consuegra et al., 2024). Collectively, these findings underscore the involvement of CB1R and CB2R in cannabinoid-induced autophagy, highlighting the potential of the ECS to target and remove pathological protein aggregates in neurodegenerative disorders.

2.5. Endocannabinoid system and cannabinoids in Parkinson's disease

Across the basal ganglia, the components of the ECS are highly expressed. CB1R is found on striatal GABAergic projection neurons, as well as on subthalamonigral and corticostriatal glutamatergic neurons (Fernández-Ruiz, 2009). Nigrostriatal dopaminergic neurons are likely devoid of CB1R. By contrast, these TH-positive neurons express CB2R and TRPV1 (Fernández-Ruiz, 2009; García et al., 2015; Sierra et al., 2015). Although CB2R is predominantly found in glial cells, particularly in reactive states (Fernández-Ruiz, 2009), specific neuronal subpopulations within the basal ganglia have also been reported to express CB2R, such as pallidothalamic GABAergic neurons (Lanciego et al., 2011). Regarding other cannabinoid-sensitive receptors, their expression profiles

within the basal ganglia remain less well characterised. For example, GPR55 has been detected in MSNs of the striatum and GPe, as well as in the STN and SN (Celorrio et al., 2017; Sánchez-Zavaleta et al., 2022), even though further studies are necessary to validate this.

During the progression of PD, ECS components within the basal ganglia may undergo dynamic changes in their expression and/or localization. These alterations can reflect both pathological processes and compensatory protective mechanisms, evolving alongside disease progression. This section will discuss the observed modifications in ECS elements and the available evidence supporting their potential as therapeutic targets in PD.

2.5.1. Cannabinoid type-1 receptor in Parkinson's disease

The CB1R exhibits dynamic dysregulation in PD. In *post-mortem* patient tissues and experimental models, CB1R upregulation in striatal neurons is consistently observed during intermediate to advanced stages, coinciding with the onset of bradykinesia and widespread neurodegeneration (Lastres-Becker et al., 2001; García-Arencibia et al., 2009a). However, prior to this upregulation, CB1R appears to undergo desensitization or downregulation at early, presymptomatic stages, as neuronal dysfunction begins. This biphasic pattern has been identified in genetic (García-Arencibia et al., 2009b) and neurotoxin-based models (Lastres-Becker et al., 2001; Walsh et al., 2010; Binda et al., 2023). *In vivo* positron emission tomography studies have further confirmed region-specific CB1R alterations in patients (Van Laere et al., 2012; Ajalin et al., 2022). More recently, the downregulation of the CB1R has been linked to gait disturbances, postural instability, and rigidity in PD patients (Ajalin et al., 2024).

2.5.2. Cannabinoid type-2 receptor in Parkinson's disease

The CB2R undergoes distinct alterations in PD, depending on its cellular localization. In glial cells, including astrocytes and microglia at lesion sites, CB2R levels are elevated. This upregulation has been observed in the SN of *post-mortem* PD patients (Gómez-Gálvez et al., 2016; Navarrete et al., 2018) and in animal models exposed to neurotoxins, such as MPTP, rotenone, or LPS (Price et al., 2009; García et al., 2011; Concannon et al., 2016; Gómez-Gálvez et al., 2016; Navarrete et al., 2018). While similar responses have been noted in 6-OHDA-lesioned rats (Concannon et al., 2015), findings in this model remain inconsistent (Gómez-Gálvez et al., 2016). In contrast, neuronal expression of this receptor decreases, reflecting dopaminergic pathway degeneration where receptor presence is detected even in healthy controls (García et al., 2015; Sierra et al., 2015). However, this decline extends to other neuronal populations within the basal ganglia such as pallidothalamic neurons (Lanciego et al., 2011).

2.5.3. Endocannabinoids in Parkinson's disease

Changes in eCBs levels, in parallel WITH their inactivating enzymes FAAH and MAGL, have been robustly reported in PD. Elevated AEA levels have been detected in CSF from patients (Pisani et al., 2010) and in the striatum of rodents with 6-OHDA lesions (Gubellini et al., 2002). Analogously, increases in 2-AG were detected in reserpine-treated rats (Di Marzo et al., 2000) and MPTP-

lesioned marmosets (Van der Stelt et al., 2005). These alterations appear to partially recover after chronic L-DOPA therapy (Maccarrone et al., 2003), and correlate with fluctuations in eCB-inactivating enzymes. FAAH levels are reduced in the striatum of 6-OHDA-lesioned rodents (Gubellini et al., 2002), while MAGL expression decreases in the SN but rises in the putamen of patients (Navarrete et al., 2018). Moreover, loss-of-function mutations in DAG lipase β, involved in synthesising 2-AG, have been linked to early-onset PD in a Chinese cohort (Liu et al., 2022). The overall increase in eCB tone is considered an endogenous protective mechanism, as blocking eCB degradation mitigates disease progression and symptoms (Maccarrone et al., 2003; Fernández-Suárez et al., 2014) (see **Section 2.2.3 of INTRODUCTION**).

2.5.4. Atypical cannabinoid receptors in Parkinson's disease

Other receptors such as PPARγ and GPR55 can also be targeted by cannabinoids. While no alterations have been observed in PPARγ, evidence regarding changes in GPR55 remains limited. In the striatum of 6-OHDA-lesioned mice, GPR55 expression appears unchanged; in contrast, a marked downregulation was reported in LPS-lesioned mice (Burgaz et al., 2021c). Similarly, MPTP-treated mice exhibited reduced striatal GPR55 expression, though no changes were detected in dopaminergic neurons of the SN (Celorrio et al., 2017). In macaques exposed to MPTP, heteromers involving cannabinoid receptors and GPR55 increased in the striatum and nucleus accumbens, returning to baseline levels upon chronic treatment with L-DOPA in dyskinetic animals (Martínez-Pinilla et al., 2020). Human data are more scarce, but a relatively recent study reported reduced GPR55 expression in both direct and indirect pathway MSNs, consistent with findings from murine MPTP and LPS models (Hewer et al., 2023). Nonetheless, further studies are needed to validate and expand upon these observations.

2.5.5. Preclinical evidence of cannabinoid therapeutics in Parkinson's disease

Identifying the alterations in ECS components during PD progression is crucial for developing effective cannabinoid-based pharmacological strategies in preclinical research. Numerous studies have explored ECS modulation as a therapeutic approach for PD, underscoring the complexity of this system and the variability of its responses, which depend heavily on cellular context, disease state, and the specific experimental models used to replicate PD pathology.

Since 2003, multiple studies have demonstrated the neuroprotective effects of phytocannabinoids such as CBD, Δ^9-THC and Δ^9-THCV, highlighting their ability to improve motor function, preserve dopaminergic neurons, and reduce oxidative stress and inflammation (Lastres-Becker et al., 2005; García-Arencibia et al., 2007; García et al., 2011). Extensive research on CBD has emphasised its antioxidant properties, not only as a ROS scavenger but also through Nrf2 signalling (Talebi et al., 2023). Recent findings have confirmed these benefits in 6-OHDA-lesioned rodents (De Mattos et al., 2024) and paraquat-based *in vitro* models (Mendivil-Perez et al., 2023). However, in MPTP models, the efficacy of CBD remains debated. While initial studies showed limited effects (Celorrio et al., 2017), subsequent research reported improvements in motor function and inflammation (Wang et al., 2022c). Beyond its antioxidant role, CBD can also prevent

apoptosis by downregulating Bax and caspase 3, implicating the Akt/mTOR pathway (Gugliandolo et al., 2020), and inhibit GSK-3β, activating WNT/β-catenin signalling (Iuvone et al., 2009; Berwick & Harvey, 2012; Vallée et al., 2021).

In addition to CBD, the phytocannabinoid Δ^9-THCV has also emerged as a potential PD therapy due to its dual activity at CB1R, acting as an antagonist at low doses and an agonist at higher concentrations (>5 mg/kg) (Thomas et al., 2005; Pertwee, 2008; García et al., 2011), while consistently functioning as a CB2R agonist (García et al., 2011). Thus, on the one hand, through CB1R antagonism, Δ^9-THCV alleviates hypokinetic symptoms in 6-OHDA-lesioned mice (García et al., 2011) and reduces L-DOPA-induced dyskinesia (Espadas et al., 2020). On the other hand, its CB2R agonism confers neuroprotection, particularly in inflammatory models, where Δ^9-THCV reversed LPS-induced neuroinflammation and dopaminergic neuron loss (Gómez-Gálvez et al., 2016).

CB1R antagonists have demonstrated efficacy in alleviating hypokinetic symptoms, enhancing the effects of dopaminergic agonists (Cao et al., 2007), but also mitigating dyskinesia following prolonged L-DOPA treatment (Gutiérrez-Valdez et al., 2013). Conversely, CB1R agonists have shown potential in reducing tremors (Sañudo-Peña et al., 1998) and alleviating dyskinetic movements (Morgese et al., 2007; Pérez-Rial et al., 2011), despite concerns about exacerbating bradykinesia and motor deficits (García-Arencibia et al., 2007; Chung et al., 2011). This duality initially raised doubts about the feasibility of targeting CB1R as a neuroprotective strategy in PD (Fernández-Ruiz, 2009). However, studies in CB1R-deficient mice revealed heightened susceptibility to 6-OHDA-induced lesions, suggesting a protective role for CB1R activity in slowing disease progression (Pérez-Rial et al., 2011). Recent *in vitro* findings reinforce this perspective, demonstrating CB1R involvement in regulating autophagy and lysosomal pathways, facilitating the removal of damaged organelles and protein aggregates in neurons exposed to parkinsonian toxins (Gugliandolo et al., 2023). These insights highlight the complex role of CB1R in PD, indicating that therapeutic outcomes may hinge on receptor state, dosage, and disease stage.

Inhibition of FAAH and MAGL, which elevates eCB levels, offers another approach targeting CB1R and to a lesser extent other receptors (CB2R, PPARs). FAAH inhibition has shown neuroprotection in 6-OHDA-lesioned rats (Maccarrone et al., 2003) and MPTP models (Celorrio et al., 2016), while also mitigating L-DOPA-induced dyskinesia through CB1R activation (Solinas et al., 2006). Similarly, MAGL blockade demonstrated protective effects in MPTP-lesioned mice (Fernández-Suárez et al., 2014), highlighting the therapeutic potential of sustaining eCB tone to counteract neurodegeneration and motor complications in PD.

Regarding the CB2R, its activation appears to be a promising therapeutic strategy, as CB2R-deficient mice exhibit worsened pathology (Gómez-Gálvez et al., 2016; Zhu et al., 2023), while CB2R-overexpressing mice demonstrate increased resilience to parkinsonian neurotoxins (Ternianov et al., 2012). Selective CB2R agonists, including phytocannabinoids like β-caryophyllene and synthetic compounds (HU-308, JWH-133), have consistently shown potential

in reducing neuroinflammation and gliosis across PD models involving mitochondrial dysfunction (Price et al., 2009; Javed et al., 2016; Shi et al., 2017; Viveros-Paredes et al., 2017; Yu et al., 2021; Liu et al., 2024) or proinflammatory insult (García et al., 2011; Gómez-Gálvez et al., 2016). *In vitro* studies implicate the PI3K/Akt/Nrf2 pathway in cannabinoid-induced microglial polarization towards an anti-inflammatory phenotype (Wang et al., 2023b). Additionally, CB2R agonism appears to regulate inflammation by promoting the attenuation of NLRP3 in MPTP-lesioned mice, presumably mediated by facilitating the degradation of this inflammasome component through the autophagy-lysosome pathway (Zhu et al., 2023). CB2R activation has also demonstrated neuroprotection against iron toxicity, reducing behavioural deficits and iron accumulation in 6-OHDA-lesioned mice (Liu et al., 2024), potentially through antioxidant mechanisms (Parlar et al., 2018). Beyond mitigating inflammation, the benefits of CB2R targeting extend to the clearance of pathogenic proteins, by enhancing autophagic and phagocytic processes. HU-308, for instance, reversed motor deficits and improved autophagy in LRRK2^{G2019S} mice (Palomo-Garo et al., 2016). Conversely, CB2R ablation or inverse agonism promotes a phagocytic profile in microglial and peripheral immune cells, contributing to reduce the toxicity of α-syn aggregates by facilitating their removal (Feng et al., 2023; Joers et al., 2024). These findings need to be reconciled with evidence that CB2R deletion in PD models heightens microglial activation and exacerbates neuropathological and motor deficits (Gómez-Gálvez et al., 2016; Komorowska-Müller & Schmöle, 2020). Regarding CB2R localization, a recent study confirmed that the neuroprotective effects of CB2R agonists in experimental parkinsonism are mediated by central receptors (neurons, astrocytes, microglia), rather than peripheral receptors on immune cells (Ayerra et al., 2024).

Lastly, non-canonical cannabinoid receptors have emerged as promising targets for neuroprotection in PD. One notable example is VCE-003.2, a CBG quinone derivative that has demonstrated neuroprotective potential by acting as a partial agonist of PPARγ without engaging CB1R/CB2R (Díaz-Alonso et al., 2016; Aguareles et al., 2019). This synthetic cannabinoid effectively mitigates inflammation, mitochondrial dysfunction, oxidative stress, and α-syn dysregulation across various PD models, targeting critical pathogenic pathways (García et al., 2018; Burgaz et al., 2019; 2021a; 2024). *In vitro* studies confirm its cytoprotective properties, identifying interactions with an alternative site on the PPARγ receptor distinct from the glitazone-binding domain (Burgaz et al., 2021a). Notably, VCE-003.2 demonstrated superior efficacy compared to L-DOPA in preclinical studies and is progressing to clinical trials as EHP-102 (Rodríguez-Carreiro et al., 2023).

Another non-canonical target, GPR55, has also garnered attention in PD research. VCE-006.1, a chromenopyrazole derivative, selectively modulates GPR55 as a biased orthosteric and positive allosteric modulator, with no activity at CB1R/CB2R (Morales et al., 2016; Burgaz et al., 2021c). VCE-006.1 exhibits neuroprotective effects by reducing inflammation and preserving dopaminergic neurons in 6-OHDA-lesioned mice and SH-SY5Y cell models (Burgaz et al., 2021c). Nonetheless, further preclinical studies employing various GPR55-targeting compounds both *in vivo* and *in vitro* PD models have yielded results that are frequently contradictory or inconsistent.

Thus, a considerable number of studies have reported that both agonists and antagonists of GPR55 elicit comparable effects on motor performance, neuroprotection, and anti-inflammatory outcomes across diverse models (Celorrio et al., 2017; Fatemi et al., 2021; Patricio et al., 2022; Sun et al., 2024). This apparent duality underscores the pharmacological complexity of GPR55 in the context of PD. The use of GPR55-deficient mice, as previously employed in other models of neurodegeneration and brain injury (Sisay et al., 2013; Gajghate et al., 2024), might help to more precisely determine the most suitable direction of GPR55 pharmacological modulation for PD.

2.5.6. Clinical evidence of cannabinoid therapeutics in Parkinson's disease

Cannabinoids have also been evaluated in clinical trials as a potential therapy for PD, though most studies have focused on alleviating specific motor and non-motor symptoms rather than affecting disease progression. Overall, these trials indicate that cannabinoids are generally safe and well-tolerated, with only mild adverse effects, but their efficacy remains inconsistent (Varshney et al., 2023; Hafida et al., 2024). For instance, CB1R blockade failed to reduce bradykinesia (Mesnage et al., 2004), while CB1R activation was explored for tremor control with mixed outcomes (Frankel et al., 1990). Also, both CB1R agonists and antagonists have been investigated for mitigating L-DOPA-induced dyskinesia, yielding variable outcomes (Sieradzan et al., 2001; Carroll et al., 2004; Fox et al., 2006; Junior et al., 2020). Other trials have addressed non-motor symptoms using mainly CBD or nabilone (Zuardi et al., 2009; Chagas et al., 2014a; 2014b; Peball et al., 2020; 2022). Additionally, observational studies suggested medicinal cannabis may reduce motor symptoms, like tremor, dystonia, rigidity, and bradykinesia, as well as non-motor issues, such as sleep disturbances, depression, or pain (Lotan et al., 2014; Balash et al., 2017; Kindred et al., 2017; Shohet et al., 2017; Aladeen et al., 2023), without worsening neuropsychiatric symptoms or accelerating disease progression (Goldberg et al., 2003; Domen et al., 2023). However, given the ongoing controversy surrounding these results, future efforts should prioritize larger, long-term randomised clinical trials to enhance study quality and reliability.

HYPOTHESIS AND GOALS

While PD remains without a cure, pharmacological research continues to pursue effective strategies to manage disease progression and improve patients' quality of life. Among the various therapeutic avenues currently under investigation, the ECS, through modulation by cannabinoids, emerges as a particularly attractive option, due to its neuroprotective potential and pleiotropic nature, which may help counteract the complex and multifactorial nature of PD pathophysiology.

The central **hypothesis** of the present doctoral thesis is that the ECS and cannabinoids will provide novel and unexplored strategies that hold promise as neuroprotective and disease-modifying approaches for PD. Although the benefits of cannabinoids have been extensively studied in this and other neurodegenerative disorders, novel approaches have yet to be explored, especially in certain *in vitro* and *in vivo* models of neurodegeneration. This could potentially open new therapeutic opportunities, ultimately leading to meaningful clinical outcomes.

Building upon this initial hypothesis, the **overarching aim** of the present project is to contribute to the development of cannabinoid-based pharmacological therapies for PD by investigating novel strategies involving cannabinoids, the ECS, or related molecular targets through comprehensive preclinical evaluation. This evaluation will focus on assessing the potential neuroprotective and anti-inflammatory effects of the selected approaches, employing a range of *in vitro* and *in vivo* models that collectively recapitulate key pathological features of the disease, including mitochondrial dysfunction, oxidative stress, neuroinflammation, and α-syn dysregulation. Furthermore, the project aims to deepen our understanding of the role of the ECS and related pathways in PD pathophysiology, thereby providing a mechanistic foundation for future therapeutic interventions.

With regard to the cannabinoid-based strategies explored, the overall aim of this project can be divided into three **specific objectives**, each of which will be presented in a separate chapter:

1. To evaluate the CB1R activation and the role of its interactor BiP in a proteinopathic context driven by localised α-syn overexpression *in vivo*.
2. To investigate the role of GPR55 as a novel pharmacological target in PD using two murine models of proteinopathy and mitochondrial dysfunction.
3. To assess the pharmacological potential of the (+)-*trans*-enantiomeric series of CBD and four analogues in inducing anti-inflammatory and neuroprotective effects, both *in vitro* and *in vivo*.

MATERIALS AND METHODS

CHAPTER 1. CANNABINOID TYPE-1 RECEPTOR IN α-SYNUCLEIN DYSREGULATION

1.1. Experimental animals

Male C57BL/6 mice, 6 to 8 months old, were employed throughout the experiments, including WT animals and *Hspa5*$^{+/-}$ heterozygous mice for the chaperone BiP (Jackson Laboratories, Bar Harbor, ME, USA). The BiP-deficient mice were generated by targeted excision of exons 5 to 7, encoding part of the ATPase domain. During breeding and experiments, mice were housed in a room with a controlled 12-hour light/dark photoperiod (lights on from 08:00 to 20:00), temperature (22 ± 1°C) and humidity (60%), with standard rodent chow and water provided *ad libitum*. All experiments were conducted according to national and European guidelines (RD 53/2013 and Directive 2010/63/EU, respectively), as well as following the ARRIVE guidelines and approved by the Animal Welfare Ethics Committee of the Complutense University of Madrid (ref. PROEX 201.8/22). All efforts were made to minimise animal pain and discomfort, and to reduce the number of experimental animals.

1.2. Genotyping

The identification of animal genotypes was achieved through polymerase chain reaction (PCR) using genomic DNA extracted from mouse tail and/or ear biopsies. Genomic DNA extraction and amplification were carried out using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich, Madrid, Spain), according to the manufacturer's instructions. PCR products were separated through electrophoresis on 2% agarose gels and visualised using GelRed (Biotium, Fremont, CA, USA). The sequences of the primer pairs, PCR conditions and the expected band patterns on agarose gel are detailed in **Table 5**.

***Table 5**. Primers sequences and PCR conditions used for the genotyping of BiP-deficient mice.*

Hspa5	**Primer Sequences (5' → 3')**	Common Forward	CTG GCA CTA TTG CTG GAC TG	
		Wild-type Reverse	TCA ATC CTT GCT TGA TGC TG	
		Mutant Reverse	TTT GTT AGG GGT CGT TCA CC	
	PCR conditions	Step	Temperature (°C)	Time
		1	94	2 min
		2	94	20 s
		3	65	15 s
		4	68	10 s
		5	Repeat steps 2-4 for 10 cycles (-0.5°C per cycle at step 3)	
		6	94	15 s
		7	60	15 s
		8	72	10 s
		9	Repeat 6-8 for 28 cycles	
		10	72	5 min
	Expected results	*Hspa5*$^{+/+}$	*Hspa5*$^{+/-}$	*Hspa5*$^{-/-}$
		458 bp band	458 bp and 250 bp bands	Non viable

1.3. Surgical stereotaxic intervention

Mice were anaesthetised by *i.p.* injection of ketamine (40 mg/kg) and xylazine (4 mg/kg) and placed into the stereotaxic apparatus (RWD Life Sciences, Shenzhen, China), in which they remained for the duration of the surgery. Animals were subjected to unilateral injections of viral particles carrying the mutated *Snca*^{A53T} gene (AAV9-α-syn^{A53T}; kindly provided by Dr. Lanciego from CIMA, University of Navarra, Spain), leading to proteostasis dysregulation and local α-syn accumulation. One microlitre of viral suspension (containing 5×10^{12} genome copies (GC)/mL and formulated in 0.1 M phosphate-buffered saline (PBS), pH 7.4, supplemented with 200 mM NaCl and 0.001% pluronic F-68) was injected stereotaxically into the right SN. The suspension was injected at a rate of 0.2 µL/min, using the following coordinates: –2.5 mm (anteroposterior, AP), –1.4 mm (mediolateral, ML), and –4.5 mm (dorsoventral, DV), as previously described (Lastres-Becker et al., 2012). Once injected, the needle was left in place for 5 min before being slowly removed, thus avoiding reflux and a rapid increase in intracranial pressure. Control animals were sham-operated and injected with 1 µL of a viral suspension of AAV9-CMV-null containing the equivalent number of viral particles used in lesioned mice, at the same coordinates.

1.4. Drug preparation and pharmacological treatment

CB1R-selective agonist ACEA (Tocris, Bristol, United Kingdom) was dissolved in absolute ethanol and stored at -20°C under a nitrogen atmosphere to avoid oxidation. Injectable solutions were prepared the day of the experiment by further transferring ACEA from ethanol, which was evaporated under a stream of nitrogen, to a 1:16 Tween-80:saline solution. All plastic tubes employed for drug preparation were previously siliconized with Sigmacote (Sigma-Aldrich, Madrid, Spain) to avoid nonspecific adsorption of the cannabinoid to the plastic surface. ACEA-treated animals received a daily *i.p.* dose of 1.5 mg/kg for two weeks. The first administration was performed approximately 24 h after the stereotaxic surgery, whereas the last injection was administered 24 h before behavioural tests.

1.5. Behavioural assessment

The lesion induction period was extended to two weeks to ensure sufficient neurodegeneration. Following this period, and immediately prior to euthanasia, mice underwent a battery of behavioural tests to assess motor performance. Given that the lesions were unilateral, these tests were designed to evaluate the resulting hemiparesis. Before testing, mice were acclimatised to the testing room for 30 minutes. Efforts were made to maintain low ambient noise levels, and all procedures were conducted under blinded conditions. All equipment was thoroughly cleaned with 70% ethanol between tasks to eliminate residual odours that could affect test performance.

1.5.1. Cylinder Rearing Test

Mice were placed in a transparent methacrylate cylinder (15.5 cm in diameter, 12.7 cm in height) to quantify forepaw preference (ipsilateral, contralateral or both) during wall-contact rearing

movements. A contact was counted whenever the animal placed one or both forepaws on the wall. For a subsequent contact to be recorded, the animal had to return its forepaw(s) to the ground. Forepaw preference was assessed using the following formula: (number of ipsilateral contacts – number of contralateral contacts)/total number of contacts, with the latter including those in which both paws contacted the wall simultaneously. Each score was derived from a 3-minute trial. Data were normalised to the control, sham-operated group and expressed as mean ± standard error of the mean (SEM).

1.5.2. Elevated-body Swing Test

Mice were suspended by the tail in a head-downward position, approximately 15 cm above the surface, along a vertical axis to assess side preference (ipsilateral or contralateral) during swing movements. A swing was recorded whenever the animal moved its head laterally away from the vertical axis. For a subsequent swing to be counted, the animal had to return to the vertical position. Preference was expressed as a score calculated using the following formula: (number of swings to the ipsilateral side – number to the contralateral side)/total number of swings. Each score was obtained from a 1-minute trial. Data were expressed as mean ± SEM.

1.6. Sacrifice and animal sampling

Following behavioural testing, animals were humanely euthanised by rapid decapitation, and their brains were promptly extracted and bisected coronally using a slicer (Ted Pella, Redding, CA, USA). The posterior portion, containing the SN, was fixed in freshly prepared 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 24 h at 4°C, followed by cryoprotection in 30% sucrose solution for at least 48 hours at 4°C. Tissues were subsequently frozen in dry ice–cooled isopentane and stored at –80°C for later immunohistochemical analysis. The anterior portion, containing the caudate-putamen nuclei, was rapidly dissected to isolate the striatum from both hemispheres. The striata were immediately frozen in dry ice–cooled isopentane and stored at –80°C for subsequent reverse transcription quantitative PCR (RT-qPCR) analysis.

1.7. Immunohistochemical proceedings

The fixed-frozen brain halves were sectioned coronally into 30 µm-thick slices encompassing the SN using a cryostat (Leica CM3050, Leica, Wetzlar, Germany). Sections were collected in an antifreeze solution (glycerol/ethylene glycol/PBS; 2:3:5) and stored at –20 °C until required.

1.7.1. 3,3'-Diaminobenzidine immunostaining

Sections were mounted on gelatin-coated slides and, once adhered, washed with 0.1 M potassium phosphate-buffered saline (KPBS), pH 7.4, and permeabilised with KPBS containing 0.1% Triton X-100. Endogenous peroxidase activity was then blocked by incubation for 30 min at room temperature with a peroxidase blocking solution (Dako Cytomation, Glostrup, Denmark). After several washes with KPBS, sections were incubated overnight at 4 °C with the primary antibody

(**Table 6**), prepared in KPBS containing 0.1% Triton X-100 and 2% bovine serum albumin (BSA; Sigma-Aldrich, Madrid, Spain). Subsequently, sections were washed in KPBS and incubated for 1 h at room temperature with the appropriate biotinylated secondary antibody (**Table 6**). The avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine substrate–chromogen system (Dako Cytomation) were used to produce the visible reaction product. Finally, sections were dehydrated and mounted using a synthetic non-aqueous mounting medium (DPX new; Sigma-Aldrich, Madrid, Spain). Negative control sections were processed in parallel using the same protocol, omitting the primary antibody. A Leica DMRB microscope equipped with a DFC300FX camera (Leica, Wetzlar, Germany) was used for image acquisition. Quantification of immunostaining intensity was performed using the NIH ImageJ software (NIH, Bethesda, MD, USA), analysing 4–5 sections per animal, spaced approximately 200 µm apart and examined using a 10× objective. In all cases, the region of interest was the SN. Data were expressed as mean ± SEM.

1.7.2. Fluorescence immunostaining

Sections were mounted on gelatin-coated slides and, once adhered, were washed with 0.25 M Tris-buffered saline (TBS) or phosphate-buffered saline (PBS), pH 7.5, and permeabilised with TBS/PBS containing 0.1% Triton X-100. After additional washes with TBS/PBS, sections were incubated overnight at 4 °C with the primary antibody (**Table 6**), prepared in TBS/PBS containing 0.1% Triton X-100 and 2% BSA (Sigma Chem., Madrid, Spain). Subsequently, sections were washed again with TBS/PBS and incubated with the corresponding fluorophore-conjugated secondary antibody (**Table 6**) for 2 h at 37 °C. The nuclear stain Hoechst (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to facilitate the identification of cell bodies under the microscope. Finally, preparations were mounted in an aqueous fluorescent mounting medium (Dako, Carpinteria, CA, USA). Negative control sections were processed in parallel using the same protocol, omitting the primary antibody. An Imager.M2 upright microscope and an Axiocam 705 mono camera (Zeiss, Oberkochen, Germany) were used for slide observation and image acquisition. Quantification of immunostaining was performed as described in the previous section. Data were expressed as mean ± SEM.

1.8. Reverse transcription quantitative polymerase chain reaction

Fresh-frozen striata were processed to perform RT-qPCR assays for the analysis of gene expression. Total RNA was extracted and purified from the biological samples using TRIzol® reagent (Sigma-Aldrich, Madrid, Spain). The RNA concentration was quantified spectrophotometrically at 260 nm, and its purity assessed by calculating the absorbance ratio at 260/280 nm. To eliminate genomic DNA contamination, total RNA was treated with a DNA removal step prior to reverse transcription. Complementary DNA (cDNA) was synthesised from 0.5–1 µg of total RNA using a commercial reverse transcription kit (Qiagen, Venlo, The Netherlands). The resulting cDNA was stored at −20 °C until PCR amplification.

***Table 6**. Primary and secondary antibodies used in immunohistochemical assays (Chapter 1).*

Primary antibodies				
Antibody	Host	Dilution	Company	Reference
Anti-GFAP	rabbit	1:200	Dako	Z0334
Anti-Iba1	rabbit	1:500	Wako Chemicals	019-19741
Anti-TH	rabbit	1:200	MilliporeSigma	AB152
Anti-CD68	rat	1:200	AbD Serotec	MCA1957
Anti-α-syn	rabbit	1:2000	Santa Cruz Biotechnology	sc-53955
Anti-LAMP1	rat	1:200	Developmental Studies Hybridoma Bank	1D4B
Secondary antibodies				
Antibody	Host	Dilution	Company	Reference
Biotinylated anti-rabbit	goat	1:200	MilliporeSigma	B8895
Biotinylated anti-rat	donkey	1:200	MilliporeSigma	AP189B
AlexaFluor 488 anti-rabbit	donkey	1:200	Invitrogen	A21206
AlexaFluor 564 anti-rat	goat	1:200	Invitrogen	A11081

RT-qPCR assays were conducted using the FastStart Universal Probe Master (ROX) kit (Roche, Basel, Switzerland) to quantify gene expression, employing gene-specific TaqMan probes (Applied Biosystems, Foster City, CA, USA) as listed in **Table 7**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control gene for normalisation. PCR amplification was performed on a 7300 Fast Real-Time PCR System, and the threshold cycle (Ct) values were obtained using the accompanying software. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Data were normalised to the sham-operated group, expressed as mean ± SEM, and visualised using a heatmap.

***Table 7**. TaqMan primers used in real-time qRT-PCR assays (Chapter 1).*

Gene	Protein	Applied Biosystems reference
Ifng	IFN-γ	Mm01168134_m1
Il1b	IL-1β	Mm00434228_m1
Cnr1	CB1R	Mm00432621_s1
Gpr55	GPR55	Mm02621622_s1
Drd1	D1 dopamine receptor	Mm01353211_m1
Drd2	D2 dopamine receptor	Mm00438545_m1
Ppp1r1b	DARRP32	Mm00454892_m1
Atg7	Atg7	Mm00512209_m1
Sqstm1	p62	Mm00448091_m1
Becn1	Beclin1	Mm01265461_m
Hspa5	BiP	Mm00517691_m1
Hsp90b1	Hsp90b1	Mm00441926_m1
Ddit3	Ddit3	Mm00492097_m1
Gapdh	GAPDH	Mm99999915_g1

1.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism® software (version 8.0 for Windows; GraphPad Software Inc., San Diego, CA, USA). Data were assessed for normality using the Shapiro-Wilk test and analysed accordingly with one-way or two-way ANOVA, followed by Tukey's multiple comparison test where appropriate (see figure legends for specific tests). Statistical significance was set at *p*-value lower than 0.05, with significance levels indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

CHAPTER 2. G PROTEIN-COUPLED RECEPTOR 55 AND PARKINSON'S DISEASE

2.1. *Post-mortem* human samples

Freshly-frozen *post-mortem* brain tissue samples from healthy controls and patients with PD, provided by the Biobank of Hospital Universitario Fundación Alcorcón (Madrid, Spain) and the Banco de Tejidos Neurológicos del Instituto de Neurociencias de Castilla y León (BTN-INCYL; Madrid, Spain), were used to evaluate GPR55 gene expression in the midbrain and caudate-putamen nuclei. Relevant patient data are summarised in **Table 8**.

***Table 8**. Human samples for GPR55 expression analyses.*

ID	Age (years)	Sex	*Postmortem* interval (h)	Diagnosis	Source
FHA10415	64	Male	10	PD	Biobanco H.U.F. Alcorcón
FHA10421	71	Male	4		Biobanco H.U.F. Alcorcón
FHA10604	74	Female	4		Biobanco H.U.F. Alcorcón
FHA10769	70	Male	15		Biobanco H.U.F. Alcorcón
FHA10775	73	Male	6		Biobanco H.U.F. Alcorcón
FHA10807	55	Male	6		Biobanco H.U.F. Alcorcón
FHA10850	85	Female	3		Biobanco H.U.F. Alcorcón
FHA10892	74	Male	6		Biobanco H.U.F. Alcorcón
NC001	71	Male	3		BTN-INCYL
NC301	83	Male	4		BTN-INCYL
FHA10202	65	Female	16	Control	Biobanco H.U.F. Alcorcón
FHA10209	57	Female	4		Biobanco H.U.F. Alcorcón
FHA10224	62	Female	8		Biobanco H.U.F. Alcorcón
FHA10312	69	Male	12		Biobanco H.U.F. Alcorcón
FHA10357	81	Male	10		Biobanco H.U.F. Alcorcón
FHA10462	62	Male	14		Biobanco H.U.F. Alcorcón
FHA10589	52	Female	15		Biobanco H.U.F. Alcorcón
FHA10766	76	Female	7		Biobanco H.U.F. Alcorcón
NC198	87	Male	10		BTN-INCYL
NC278	86	Male	6		BTN-INCYL
NC366	82	Female	8		BTN-INCYL

All procedures involving human-derived samples were conducted in accordance with the ethical guidelines established by RD 1716/2011 and approved by the institution's research committee. In all cases, informed consent was obtained from the donor or their legal guardian.

2.2. Experimental animals

Female and male B6;129S5 mice aged 4–8 months were used throughout the experiments, including WT animals and those homozygous for GPR55 deficiency ($Gpr55^{-/-}$) (Taconic Biosciences, Rensselaer, NY, USA). The GPR55-deficient mice were generated by deletion of exon 2, which contains the entire coding sequence. The same housing conditions and legislation (ref. PROEX 201.8/22) described in **section 1.1 of the MATERIALS AND METHODS** were followed.

2.3. Genotyping

The identification of animal genotypes was performed by PCR using genomic DNA extracted from mouse tail and/or ear biopsies. Genomic DNA extraction and amplification were conducted using

the Speedy NZY Direct Genotyping Kit (NZYtech, Lisbon, Portugal), following the manufacturer's instructions. PCR products were separated by electrophoresis on 2% agarose gels and visualised with GelRed (Biotium, Fremont, CA, USA). Primer pair sequences, PCR conditions, and expected results are detailed in **Table 9**.

***Table 9**. Primers sequences and PCR conditions used for the genotyping of GPR55-deficient mice.*

Gpr55	**Primer Sequences (5' → 3')**	Wild-type Forward	GCC ATC CAG TAC CCG ATC C	
		Wild-type Reverse	GTC CAA GAT AAA GCG GTT CC	
		Mutant Forward	GCA GCG CAT CGC CTT CTA TC	
		Mutant Reverse	TCA AGC TAC GTT TTG GGT T	
	PCR conditions	Step	Temperature (°C)	Time
		1	94	15 s
		2	65	30 s
		3	72	40s
		4	Repeat steps 1-3 for 10 cycles (-1°C per cycle at step 2)	
		5	94	15 s
		6	55	30 s
		7	72	40 s
		8	Repeat steps 5-7 for 30 cycles	
	Expected results	*Gpr55*$^{+/+}$	*Gpr55*$^{+/-}$	*Gpr55*$^{-/-}$
		441 bp band	441 bp and 301 bp bands	301 bp band

2.4. Surgical stereotaxic intervention

Mice were anaesthetised by inhalation of 5% isoflurane and placed into the stereotaxic apparatus, with anaesthesia maintained at 2% throughout the entire surgery. For those animals lesioned with AAV9-α-syn^{A53T} (3.33×10^{12} GC/mL), the procedure was the same as described in **section 1.3 of MATERIALS AND METHODS**. The protocol for the 6-OHDA-based lesion was carried out as follows: after anaesthetising the animals, they were pre-treated with desipramine (25 mg/kg, *i.p.*) to achieve specificity for damage to dopaminergic neurons without impacting noradrenergic neurons. Thirty minutes later, 2 µL of 6-OHDA (Tocris, Bristol, UK) free base (2 µg/µL in saline supplemented with 0.02% ascorbate to avoid oxidation) were injected stereotaxically into the right striatum. The 6-OHDA solution was administered at a rate of 0.5 µL/min, using the following coordinates: +0.4 mm AP, -1.8 mm ML and -3.5 mm DV, as previously described (Alvarez-Fischer et al., 2008). Once injected, the needle was left in place for 5 min before being slowly withdrawn, thus avoiding reflux and a rapid increase in intracranial pressure. Control animals were also pre-treated with desipramine but underwent a sham lesion, receiving an injection of 2 µL of 0.02% ascorbate-supplemented saline at the same coordinates.

2.5. Behavioural assessment

Before performing the lesions described previously, the rotarod test and automated actimeter test were conducted to characterise the motor performance of GPR55-deficient mice, while the pole test, cylinder rearing test (CRT) and elevated-body swing test (EBST) were carried out two weeks

after the lesions and just prior to animal sacrifice. Environmental and procedural conditions were the same as described in **section 1.5 of MATERIALS AND METHODS**.

2.5.1. Rotarod Test

Mice were placed on the LE8200 Rotarod (Panlab, Barcelona, Spain), a device featuring an elevated cylinder aligned parallel to the ground and divided into five individual lanes. The cylinder rotated at a predefined speed. Each animal was positioned on the cylinder, one per lane, facing opposite to the direction of rotation, which compelled them to move forward to maintain balance on the rotating surface. Each lane was equipped with a pressure-sensitive plate that automatically stopped the timer when the animal fell from the cylinder.

The protocol for this test, as described by the International Mouse Phenotyping Resource of Standardised Screens (IMPReSS), began with a training phase that included an initial 1-min stationary period, followed by three consecutive 1-min trials at a constant speed of 4 rpm, with 10-min rest intervals between trials. After a minimum resting period of 30 min, the testing phase was conducted. This phase consisted of three trials in which the speed was gradually accelerated from 4 to 40 rpm. The latency for each mouse was automatically recorded until the animal fell and triggered the pressure-sensitive plates. A minimum resting period of 15 min was allowed between trials, and the maximum test duration was limited to 5 min. Data were expressed as mean ± SEM.

2.5.2. Computer-aided Actimeter Test

Mice were placed in the ActiTrack automated actimetry system (Panlab, Barcelona, Spain), an apparatus consisting of a 45 cm x 45 cm arena equipped with evenly spaced infrared beams (2.5 cm apart) connected to a computerised control unit. This system automatically analysed several parameters, including: (i) horizontal activity, (ii) locomotion, (iii) maximum velocity, (iv) mean velocity, (v) distance travelled, (vi) vertical activity (reflecting rearing movements), (vii) slow (<5 cm/s) and (viii) fast (>5 cm/s) movements, and (ix) rearing events. Animals were placed in the test arena for a total duration of 10 min. The first 5 min were considered a period of acclimatisation, and motor performance was assessed based on data collected during the final 5 min. Final values were expressed as mean ± SEM.

2.5.3. Pole Test

Mice were placed in a head-up position at the top of a vertical rough-surfaced pole (8 mm in diameter, 55 cm in height) to record the latency to descend to the floor. A maximum test duration of 90 s was set. If the mouse was unable to turn downward and instead fell from the pole, a default latency of 90 s was recorded. Three consecutive trials were performed for each animal, with a minimum resting period of 10 min between trials. Data were expressed as mean ± SEM.

2.5.4. Cylinder Rearing Test

The protocol detailed in section **1.5.1 of MATERIALS AND METHODS** was followed.

2.5.5. Elevated-body Swing Test

The protocol detailed in **section 1.5.2 of MATERIALS AND METHODS** was followed.

2.6. Sacrifice and animal sampling

Mice were anaesthetised with ketamine (40 mg/kg, *i.p.*) and xylazine (4 mg/kg, *i.p.*) and transcardially perfused with 0.1 M PBS, pH 7.4, at a flow rate of 12 mL/min for 5 min. Following perfusion, brains were promptly extracted and coronally sectioned into two portions using a coronal brain slicer. Subsequently, brain samples were processed according to the protocol detailed in **section 1.6 of MATERIALS AND METHODS**.

2.7. Immunohistochemical proceedings

The protocol detailed in **section 1.7 of MATERIALS AND METHODS** was followed. The primary and secondary antibodies used in the immunohistochemical assays corresponding to this chapter are listed in **Table 10**.

***Table 10**. Primary and secondary antibodies used in immunohistochemical assays (Chapter 2).*

Primary antibodies				
Antibody	Host	Dilution	Company	Reference
Anti-GFAP	rabbit	1:200	Dako	Z0334
Anti-Iba1	rabbit	1:500	Wako Chemicals	019-19741
Anti-TH	chicken	1:200	MilliporeSigma	AB9702
Anti-C3	rat	1:50	Hycult Biotech	HM1045
Anti- α-syn^{S129P}	rabbit	1:200	Abcam	AB515253
Secondary antibodies				
Antibody	Host	Dilution	Company	Reference
Biotinylated anti-rabbit	goat	1:200	MilliporeSigma	B8895
AlexaFluor 488 anti-rabbit	donkey	1:200	Invitrogen	A21206
AlexaFluor 594 anti-rat	goat	1:200	Invitrogen	A11007
AlexaFluor 594 anti-chicken	goat	1:200	Invitrogen	A11042

Additional images of Iba1-positive cells were acquired using a 63× objective in z-stack imaging mode to capture the full three-dimensional morphology. Image stacks were processed and analysed using ImageJ software (NIH, Bethesda, MD, USA). For each animal, 7-8 cells were analysed, with 5-7 animals per experimental group. For each cell, the surface area of the cell body and the mean length of cellular processes were measured. Moreover, the ratio between cell body surface area and mean process length was calculated to provide an index of morphological state, reflecting the spectrum from amoeboid to ramified microglial morphology.

2.8. Reverse transcription quantitative polymerase chain reaction

The protocol detailed in **section 1.8 of MATERIALS AND METHODS** was followed, except that RNA isolation from human samples was carried out using the RNeasy Lipid Tissue Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions and including the optional DNase I treatment step. The TaqMan probes used in the gene expression assays are listed in **Table 11**.

***Table 11**. TaqMan primers used in real-time qRT-PCR assays (Chapter 2).*

Species	Gene	Protein	Applied Biosystems reference
Mus musculus	*Ifng*	IFN-γ	Mm01168134_m1
	Il1b	IL-1β	Mm00434228_m1
	Gpr55	GPR55	Mm02621622_s1
	Gapdh	GAPDH	Mm99999915_g1
Homo sapiens	*GPR55*	GPR55	Hs00995276_m1
	GAPDH	GAPDH	Hs02786624_g1

2.9. Statistical analysis

Statistical analysis was conducted using GraphPad Prism® software (version 8.0 for Windows; GraphPad Software Inc., San Diego, CA, USA). Data were tested for normality using the Shapiro-Wilk test and subsequently analysed using Student's *t*-test or two-way ANOVA, followed by Tukey's post hoc test, as appropriate (see figure legends for details). Statistical significance was set at *p*-value lower than 0.05, with significance levels denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

CHAPTER 3. (+)-*TRANS*-ENANTIOMER OF CANNABIDIOL AND DERIVATIVES AGAINST NEUROINFLAMMATION

3.1. Chemical structures of compounds under study

The compounds evaluated in the final chapter of this thesis include two enantiomeric forms of CBD, used as reference compounds, alongside a selection of structural analogues. This set includes CBDV, which occurs naturally in the cannabis plant in its (–)-*trans*-enantiomeric form, and three novel compounds resulting from ester conjugation at carbon 3 of the benzene ring: cannabidiol-methyl ester (CBDA-Me), cannabidiol-glycol ester (CBDA-Gly), and cannabidiol-hydroxypentyl ester (CBDA-Hyp). Both enantiomeric series correspond to the stereoisomers *trans*-(3R,4R) and *trans*-(3S,4S), referred to as (–) and (+), respectively. These enantiomers differ in the spatial configuration of the isopentenyl and benzyl groups attached to positions 1 and 6, respectively, within the cyclohexene ring (**Figure 16**). Consequently, the chemical structure of the (–)-enantiomer of CBD, referred to as (–)-CBD, is represented as 2-[(1R,6R)-6-isopropenyl-3-methyl-cyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol. Conversely, the (+)-enantiomer of CBD, known as (+)-CBD, is described as 2-[(1S,6S)-6-isopropenyl-3-methyl-cyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol. Due to their hydrophobic nature, stock solutions of these compounds were prepared in dimethylsulfoxide (DMSO).

3.2. *In vitro* proceedings

Initial pharmacological characterization of the aforementioned compounds involves evaluating their affinity for, and intrinsic activity at, CB1R and CB2R, as well as assessing their potential anti-inflammatory and neuroprotective properties *in vitro* in microglial and neuronal cell lines.

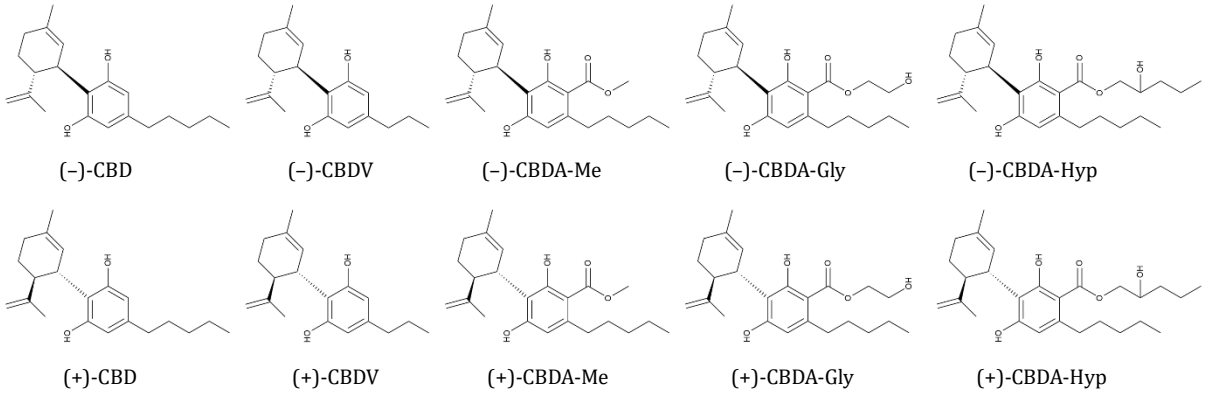


Figure 16. Chemical structures of the compounds evaluated in this study*: CBD, cannabidiol; CBDV, cannabidivarin; CBDA-Me, cannbidiol-methyl ester; CBDA-Gly, cannabidiol-glycol ester; and CBDA-Hyp, cannabidiol-hydroxypentyl ester.*

3.2.1. Radioligand binding assays

Binding assays were conducted to evaluate the affinity of the compounds for CB1R and CB2R. These assays involved a competitive binding approach, in which the test compounds competed with the non-selective agonist CP 55,940, radiolabelled with tritium ([^{3}H]-CP 55,940; PerkinElmer, Boston, MA, USA) for binding to the cannabinoid receptor. The affinity of each compound was

The measurements were performed using commercially available membranes purified from cells transfected with human CB1R or CB2R (PerkinElmer, Boston, MA, USA). Protein concentrations were 8 µg/well for CB1R membranes and 4 µg/well for CB2R membranes. The binding buffer for CB1R contained 50 mM Tris-Cl, 5 mM $MgCl_2$, 2.5 mM EDTA, 0.5 mg/mL BSA, pH 7.4; for CB2R, it contained 50 mM Tris-Cl, 5 mM $MgCl_2$, 2.5 mM EGTA, 1 mg/mL BSA, pH 7.5. The radioligand [^{3}H]-CP55,940 was used at a concentration calculated as K_D × 0.8 nM, with final incubation volumes of 200 µL for CB1R and 600 µL for CB2R. Ninety-six-well plates and tubes used in the experiment were pre-silanised with Sigmacote® (Sigma-Aldrich, Madrid, Spain) to prevent cannabinoid adsorption to plastic surfaces. Membranes were resuspended in the corresponding buffer and incubated for 90 min at 30°C with the radioligand and the compound under study, assaying a complete competition curve (from 10^{-11} M to 10^{-4} M) to determine K_i values (Gómez-Cañas et al., 2016). Filtration was performed using a Harvester® FilterMate (PerkinElmer, Boston, MA, USA) equipped with Filtermat A GF/C filters pretreated with 0.05% polyethylenimine. After filtration, filters were washed nine times with binding buffer and dried, then a melt-on scintillation sheet (Meltilex™ A, PerkinElmer, Boston, MA, USA) was applied. Radioactivity was quantified by a liquid scintillation spectrophotometer (Wallac MicroBeta Trilux, PerkinElmer, Boston, MA, USA). Non-specific binding was determined using 10 µM WIN 55,212-2 (Sigma-Aldrich, Madrid, Spain), and total radioligand binding was measured by incubating membranes in the absence of evaluated compound. Final K_i values were calculated using an iterative curve-fitting procedure and expressed as mean ± SEM from three experiments performed in triplicate.

3.2.2. [^{35}S]-GTPγS binding assays

Functionality assays were conducted to assess the intrinsic activity of the compounds on those receptors for which they displayed relevant affinities (K_i < 40 nM), determining whether the compounds act as agonists, antagonists, or inverse agonists. These assays involved the non-covalent association of the non-hydrolysable guanosine triphosphate analogue [^{35}S]-GTPγS to the α-subunit of the G protein of the receptor when incubated in the presence of a ligand. The effects of the compounds were therefore determined based on their capacity to increase, maintain, or reduce [^{35}S]-GTPγS binding compared to basal activity.

The measurements were performed using CB2R/CB1R-containing membranes (Eurofins Discovery Services, St. Charles, MI, USA). Membranes (5 µg/well) were permeabilised by the addition of saponin (Sigma-Aldrich, Madrid, Spain), then mixed with 0.3 nM [^{35}S]-GTPγS (PerkinElmer, Boston, MA, USA) and 10 µM GDP (Sigma-Aldrich, Madrid, Spain) in 20 mM HEPES (Sigma-Aldrich, Madrid, Spain) buffer containing 100 mM NaCl and 10 mM $MgCl_2$, pH 7.4. A concentration curve of the compound under study (10^{-11} M to 10^{-4} M) was tested in a final volume of 100 µL and incubated for 30 min at 30°C (Gómez-Cañas et al., 2016). Ninety-six-well plates and the tubes necessary for the experiment were previously siliconised with Sigmacote® (Sigma-Aldrich, Madrid, Spain). The reaction was terminated by rapid vacuum filtration using a Harvester® FilterMate (PerkinElmer, Boston, MA, USA) through Filtermat A GF/C filters. Filters were washed nine times with ice-cold filtration buffer (10 mM sodium phosphate, pH 7.4) and dried, and a melt-on scintillation sheet was applied. Radioactivity was then quantified using a liquid scintillation spectrophotometer. Non-specific signal was determined using 10 µM GTPγS (Sigma-Aldrich, Madrid, Spain). Final EC_{50}/IC_{50} and Emax values were established using an iterative curve-fitting procedure and are expressed as mean ± SEM from three experiments performed in triplicate.

3.2.3. Immortalised cell lines

Two immortalised cell lines were used for *in vitro* bioassays: the iMG microglial line and the M213-2O neuronal line. iMG cells (kindly provided by Dr Lastres-Becker, Institute of Biomedical Research 'Alberto Sols', CSIC-UAM, Madrid, Spain) were derived from adult murine brain and immortalised by infection with the v-raf/v-myc retrovirus (McCarthy et al., 2016). The M213-2O cell line (generously donated by Dr. Freed, National Institute on Drug Abuse, Bethesda, MD, USA) was derived from rat striatum (Giordano et al., 1993). Cells were grown and maintained in Dulbecco's Modified Eagle's Medium (Lonza, Verviers, Belgium), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, Madrid, Spain), 2 mM Ultraglutamine (Lonza, Verviers, Belgium) and an antibiotic/antimycotic solution (100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B), in a humidified atmosphere at 37°C with 5% CO_2.

3.2.4. Compounds incubation

For compound screening, iMG microglial cells were seeded in 1% FBS-deprived medium at a density of 2.25×10^5 cells/mL for 3 h. Afterwards, cells were subjected to a series of incubations with compounds that included: (i) the CB2R antagonist SR144528 (10 μM; Cayman Chemical Company, Ann Arbor, MI, USA); (ii) the PPAR-γ inhibitor T0070907 (10 μM; Cayman Chemical Company, Ann Arbor, MI, USA), to investigate these receptors as potential pharmacological targets; (iii) the test compounds at the selected concentrations; and (iv) LPS from *Escherichia coli* O55:B5 (Sigma-Aldrich, Madrid, Spain) at 100 ng/mL as a pro-inflammatory challenge. The treatments were administered with a 1-hour interval between each, and final DMSO (Sigma-Aldrich, Madrid, Spain) concentration did not exceed 0.2% within the well. Sixteen hours after LPS addition, both the cells and the secretome (conditioned culture medium) were collected for the analysis of inflammatory gene expression and neuronal cell viability, respectively (**Figure 17**).

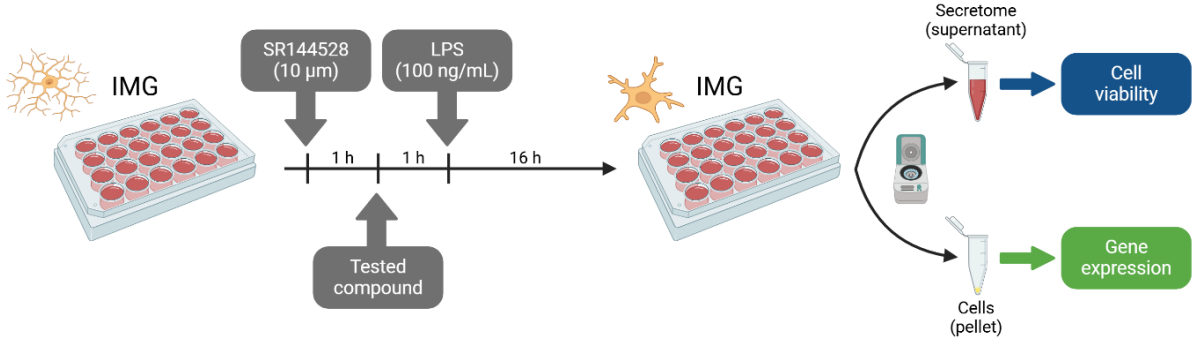


Figure 17. Schematic sequence of compound incubations and stimulation of microglial iMG cell line. *One hour before and after the administration of the tested compound, cells were treated with the CB2R antagonist SR144528 (10 μM), the PPAR-γ inhibitor T0070907 (10 μM), and LPS (100 ng/mL), respectively. Sixteen hours after LPS stimulation, both the secretome and the cells were collected for subsequent analysis. Depending on the experimental group, the compounds were replaced with the vehicle (DMSO).*

3.2.5. Reverse transcription quantitative polymerase chain reaction

Gene expression of various inflammatory markers expressed by PBS-washed microglial iMG cells after the different treatments described in the previous section was analysed through RT-qPCR. Technical details for this experimental procedure were provided in **section 1.8 of MATERIALS AND METHODS**. The Taqman probes used in the gene expression assays corresponding to this chapter are listed in **Table 12**.

Table 12. *TaqMan primers used in real-time qRT-PCR assays (Chapter 3).*

Gene	Protein	Applied Biosystems reference
Tnf	TNFα	Mm99999068_m1
Il1b	IL-1β	Mm00434228_m1
Ptgs2	COX-2	Mm00478372_m1
Nos2	iNOS	Mm01309902_m1
Gapdh	GAPDH	Mm99999915_g1

3.2.6. Methylthiazolyldiphenyl-tetrazolium bromide assay

After pharmacological treatments on microglial cell lines, microglial secretome was also collected to evaluate the effect of potential toxic factors presumably present in conditioned media by microglia on neuronal viability (Gómez-Cañas et al., 2016). To do so, neuronal M213-2O cells were seeded at 1.25×10^5 cells/mL, and their media were replaced by iMG-derived conditioned media for 40 h. Afterwards, methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, Madrid, Spain) assay was performed as an indirect measure of cell viability (Lin et al., 2007) (**Figure 18**). This assay relies on the ability of metabolically active (viable) cells to reduce the yellow tetrazolium salt into purple formazan crystals through the action of functional mitochondrial dehydrogenases. Thus, neuronal M213-2O cells were exposed to MTT at a final concentration of 0.5 mg/mL for a 1.5 h incubation. Then, media were carefully removed and the resulting purple, insoluble formazan crystals were dissolved in 4 mM HCl in isopropanol. Final absorbance at 595 nm was measured using a spectrophotometer (Synergy HTX multimode reader, BioTek, Agilent Technologies, Winooski, VT, USA). Data were normalised to the control group exposed to fresh 1% FBS-deprived medium and expressed as the mean ± SEM of four independent experiments performed in tetraplicate for each point.

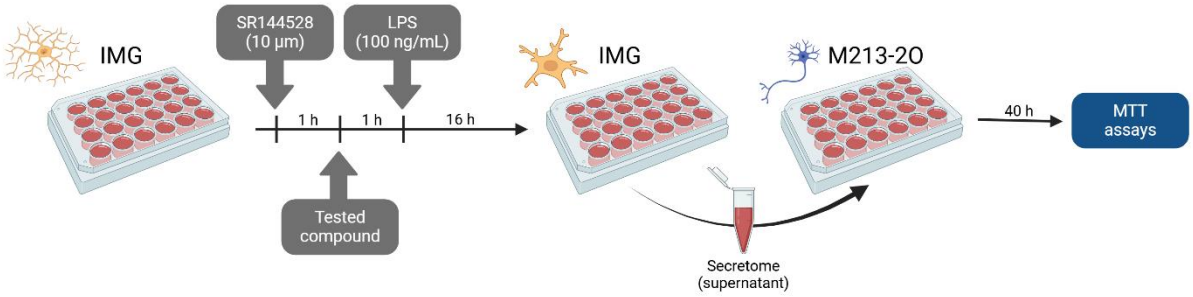


Figure 18. Schematic sequence of compounds incubation and stimulation of microglial IMG cell line prior to assessing neuronal M213-2O cell viability*. Once microglial secretome was collected, it was used to replace the neuronal culture media, followed by a 40-h incubation. After this period, MTT assay was carried out to assess cell viability by measuring the formation of purple crystals within viable cells (with reductive capability) via spectrophotometry.*

3.3. *In silico* prediction of absorption, distribution, metabolism, excretion and toxicity properties

A set of 51 physicochemical descriptors was calculated using QikProp version 3.5, integrated within Maestro (Schrödinger, LLC, NY, USA). These descriptors are detailed in **Table 13**. The 3D conformations employed for the QikProp calculations were generated using Spartan '08 (Wavefunction, Inc., Irvine, CA, USA) following these steps: (i) the structure of each molecule was constructed using the fragment library available in Spartan; (ii) *ab initio* energy minimisations were performed for each structure at the Hartree–Fock 6-31G* level; (iii) a conformational search was conducted using Molecular Mechanics, followed by energy minimisation of each conformer at the Hartree–Fock 6-31G* level; and (iv) the global minimum energy conformer of each compound was selected as the input for ADMET studies with QikProp.

***Table 13**. QikProp properties and descriptors. Range is for 95% of known drugs.*

Property or Descriptor	Description	Range or recommended values
#stars	Number of property or descriptor values that fall outside the 95% range of similar values for known drugs. A large number of stars suggests that a molecule is less drug-like than molecules with few stars.	0 – 5
#amine	Number of non-conjugated amine groups.	0 – 1
#amidine	Number of amidine and guanidine groups.	0
#acid	Number of carboxylic acid groups.	0 – 1
#amide	Number of non-conjugated amide groups.	0 – 1
#rotor	Number of non-trivial (not CX3), non-hindered (not alkene, amide, small ring) rotatable bonds.	0 – 15
#rtvFG	Number of reactive functional groups.	0 – 2
CNS	Predicted central nervous system activity on a –2 (inactive) to +2 (active) scale.	-2 to +2
mol_MW	Molecular weight of the molecule.	130.0 – 725.0
dipole	Computed dipole moment of the molecule.	1.0 – 12.5
SABA	Total solvent accessible surface area (SASA) in square angstroms using a probe with a 1.4 Å radius.	300.0 – 1000.0
FOSA	Hydrophobic component of the SASA (saturated carbon and attached hydrogen).	0.0 – 750.0
FISA	Hydrophilic component of the SASA (SASA on N, O, and H on heteroatoms).	7.0 – 330.0
PISA	π (carbon and attached hydrogen) component of the SASA.	0.0 – 450.0
WPSA	Weakly polar component of the SASA (halogens, P, and S).	0.0 – 175.0
volume	Total solvent-accessible volume in cubic angstroms using a probe with a 1.4 Å radius.	500.0 – 2000.0
donorHB	Estimated number of hydrogen bonds that would be donated by the solute to water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can be non-integer.	0.0 – 6.0
accptHB	Estimated number of hydrogen bonds that would be accepted by the solute from water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can be non-integer.	2.0 – 20.0
dip^2/V	Square of the dipole moment divided by the molecular volume. This is the key term in the Kirkwood-Onsager equation for the free energy of solvation of a dipole with volume V.	0.00 – 0.13
ACxDN^.5/SA	Index of cohesive interaction in solids. This term represents the relationship $(accptHB(\sqrt{donorHB}))/(SA)$	0.00 – 0.05
glob	Globularity descriptor, $(4\pi r^2)/(SASA)$, where r is the radius of a sphere with a volume equal to the molecular volume. Globularity is 1.0 for a spherical molecule.	0.75 – 0.95
Qppolrz	Predicted polarizability in cubic angstroms.	13.0 – 70.0
QplogPC16	Predicted hexadecane/gas partition coefficient.	4.0 – 18.0
QplogPoct	Predicted octanol/gas partition coefficient.	8.0 – 35.0
QplogPw	Predicted water/gas partition coefficient.	4.0 – 45.0
QplogPo/w	Predicted octanol/water partition coefficient.	-2.0 – 6.5
QPlogS	Predicted aqueous solubility, log S. S in mol dm–3 is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid.	-6.5 – 0.5
CIQPlogS	Conformation-independent predicted aqueous solubility, log S. S in mol dm–3 is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid.	-6.5 – 0.5
QPlogHERG	Predicted IC50 value for blockage of HERG K+ channels.	concern below -5
QPPCaco	Predicted IC50 value for blockage of HERG K+ channels.	<25 poor, >500 great

***Table 13**. QikProp properties and descriptors. Range is for 95% of known drugs. (Continued).*

Property or Descriptor	Description	Range or recommended values
QPlogBB	Predicted brain/blood partition coefficient. Note: QikProp predictions are for orally delivered drugs so, for example, dopamine and serotonin are CNS negative because they are too polar to cross the blood-brain barrier	–3.0 – 1.2
QPPMDCK	Predicted apparent MDCK cell permeability in nm/sec. MDCK cells are considered to be a good mimic for the blood-brain barrier. QikProp predictions are for non-active transport.	<25 poor, >500 great
QPlogKp	Predicted skin permeability, log K_p.	–8.0 – –1.0
IP(eV)	PM3 calculated ionization potential.	7.9 – 10.5
EA(eV)	PM3 calculated electron affinity.	–0.9 – 1.7
#metab	Number of likely metabolic reactions.	1 – 8
QPlogKhsa	Prediction of binding to human serum albumin.	–1.5 – 1.5
HumanOral-Absorption	Predicted qualitative human oral absorption: 1, 2, or 3 for low, medium, or high. The text version is reported in the output. The assessment uses a knowledge-based set of rules, including checking for suitable values of PercentHumanOralAbsorption, number of metabolites, number of rotatable bonds, logP, solubility and cell permeability.	-
PercentHuman-OralAbsorption	Predicted human oral absorption on 0 to 100% scale. The prediction is based on a quantitative multiple linear regression model. This property usually correlates well with HumanOralAbsorption, as both measure the same property.	>80% is high <25% is poor
SAFluorine	Solvent-accessible surface area of fluorine atoms.	0.0 – 100.0
SAamideO	Solvent-accessible surface area of amide oxygen atoms.	0.0 – 35.0
PSA	Van der Waals surface area of polar nitrogen and oxygen atoms.	7.0 – 200.0
#NandO	Number of nitrogen and oxygen atoms.	2 – 15
RuleOfFive	Number of violations of Lipinski's rule of five. The rules are: mol_MW < 500, QPlogPo/w < 5, donorHB ≤ 5, accptHB ≤ 10. Compunds that satisfy these rules are considered drug-like. (The "five" refers to the limits, which are multiples of 5.)	maximum is 4
#ringatoms	Number of atoms in rings.	maximum is 3
#in34	Number of atoms in 3- or 4-membered rings.	-
#in56	Number of atoms in 5- or 6-membered rings.	-
#noncon	Number of ring atoms not able to form conjugated aromatic systems (e.g. sp^3 C).	-
#nonHatm	Number of heavy atoms (nonhydrogen atoms).	-
RuleOfThree	Number of violations of Jorgensen's rule of three. The three rules are: QPlogS > -5.7, QPPCaco > 22 nm/s, # Primary Metabolites < 7. Compounds with fewer (and preferably no) violations of these rules are more likely to be orally available.	-
Jm	Predicted maximum transdermal transport rate, $K_p \times MW \times S$ ($\mu g\ cm^{-2}\ hr^{-1}$). K_p and S are obtained from the aqueous solubility and skin permeability, QPlogKp and QPlogS.	-

3.4. *In vivo* proceedings

After *in vitro* and *in silico* assessments, some of the (+)-enantiomers were administered in a murine model of PD with a pronounced inflammatory component, in order to investigate their potential anti-inflammatory and neuroprotective effects *in vivo*.

3.4.1. Experimental animals

WT male C57BL/6 mice aged 4-6 months were used for this experiment. The same housing conditions and legislation (ref. PROEX 056/19) described in **section 1.1 of MATERIALS AND METHODS** were adhered to throughout breeding and experimentation.

3.4.2. Surgical stereotaxic intervention

Mice were anaesthetised by *i.p.* injection of ketamine (40 mg/kg) and xylazine (4 mg/kg) and placed into the stereotaxic apparatus, where they remained for the duration of the surgery. Animals were subjected to unilateral injections of LPS from *Salmonella minnesota* (Sigma-Aldrich, Madrid, Spain), inducing an extensive inflammatory response. One microlitre of LPS (5 μg/μL in saline) was stereotaxically injected into the right striatum at two different coordinates: +1.1 mm AP, -1.8 mm ML and -3.5 mm DV, as well as -0.3 mm AP, -2,5 mm ML and -3.2 mm DV, as previously described (Hunter et al., 2009). Once injected at a rate of 0.5 μL/min, the needle was left in place for 5 min before being slowly withdrawn, thus avoiding reflux and a rapid increase in intracranial pressure. Control animals were sham-operated and injected with 1 μL of saline at the same coordinates.

3.4.3. Drug preparation and pharmacological treatment

The compounds with CB2R-agonistic activity and superior pharmacokinetic properties, (+)-CBD and (+)-CBDV (synthesised by Dr. Caprioglio, Università degli Study del Piamonte Orientale, Novara, Italy), were dissolved in absolute ethanol and stored at –20 °C under a nitrogen atmosphere to prevent oxidation. Injectable solutions were prepared on the day of the experiment by further transferring (+)-CBD and (+)-CBDV from ethanol, eliminated under nitrogen flux, to a 1:16 Tween-20:saline solution. All plastic tubes employed for drug preparation were previously siliconised with Sigmacote® (Sigma-Aldrich, Madrid, Spain). (+)-CBD- and (+)-CBDV-treated animals received a daily *i.p.* dose of 3 mg/kg for three weeks. The first administration was performed approximately 24 h after stereotaxic surgery, while the final injection was administered 24 h before the behavioural tests.

3.4.4. Cylinder Rearing Test

Brain lesions were allowed to progress for three weeks to achieve an optimal neurodegeneration and inflammation. After this period, and immediately before sacrifice, mice were subjected to the CRT to evaluate motor performance. The protocol detailed in **section 1.5.1 of MATERIALS AND METHODS** was followed as previously described.

3.4.5. 3,3'-Diaminobenzidine immunohistochemical procedure

Immediately after the behavioural tests, the animals were swiftly decapitated, and their brains promptly extracted. The protocols outlined in **section 1.6 of MATERIALS AND METHODS** were

followed to process the samples for immunohistochemical analysis, which was performed as described in **section 1.7**.

3.5. Statistical analysis

Statistical analysis was conducted using GraphPad Prism® software (version 8.0 for Windows; GraphPad Software Inc., San Diego, CA, USA). Nonlinear regression analysis employing the Levenberg–Marquardt algorithm to minimise the sum of squared residuals was performed to fit the data to dose–response equations with a fixed Hill slope of –1.0 for affinity assays, and either –1.0 or 1.0 for functionality assays, depending on the intrinsic activity. K_i values for each compound were calculated from the $logIC_{50}$ values estimated from the resulting sigmoidal curves of semilogarithmic plots, using the Cheng–Prusoff equation (Cheng & Prusoff, 1973). Emax values were similarly calculated from $logIC_{50}$ or $logEC_50$, while pharmacological profiles were qualitatively identified by analysing the trajectories of the semilogarithmic dose–response curves.

Data obtained from the *in vitro* bioassays and *in vivo* treatments were tested for normal distribution using the Shapiro-Wilk normality test and analysed accordingly, employing one-way ANOVA followed by Dunnett's or Tukey's multiple comparison tests as appropriate (refer to figure legends for specific tests used). Statistical significance was set at a *p*-value lower than 0.05, with the following notation: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

RESULTS

CHAPTER 1

CANNABINOID TYPE-1 RECEPTOR IN α-SYNUCLEIN DYSREGULATION

CB1R has been extensively investigated as a pharmacological target for alleviating certain motor symptoms and complications associated with PD. Notably, CB1R antagonists have proven effective in reducing hypokinetic signs (Cao et al., 2007) and in ameliorating dyskinetic states (Gutiérrez-Valdez et al., 2013). Paradoxically, CB1R agonists have also exhibited anti-dyskinetic activity (Morgese et al., 2007) and have shown benefits in counteracting tremors (Sañudo-Peña et al., 1998). However, CB1R-mediated neuroprotection has received comparatively less attention, with most studies instead focusing on alternative targets such as CB2R (García et al., 2011; Gómez-Gálvez et al., 2016; Liu et al., 2024) or enzymes responsible for eCB inactivation (Maccarrone et al., 2003; Fernández-Suárez et al., 2014; Celorrio et al., 2016).

Various neuroprotective mechanisms have been attributed to CB1R across different models and pathologies, including the attenuation of glutamate-induced excitotoxicity (Marsicano et al., 2003), activation of pro-survival pathways (Van der Stelt & Di Marzo, 2005), and induction of autophagy to facilitate the clearance of protein aggregates (Costa et al., 2016). However, evidence supporting CB1R-mediated neuroprotection in the context of PD remains limited, partly due to the widely held view that CB1R activation may, under certain circumstances and in acute settings, exacerbate akinetic symptoms. Notably, Pérez-Rial and colleagues reported that CB1R-deficient mice displayed increased susceptibility to 6-OHDA-induced lesions (Pérez-Rial et al., 2011), thereby suggesting a potential neuroprotective role for this receptor.

A key pathological hallmark of PD is the accumulation and aggregation of α-syn (Spillantini et al., 1997). These protein aggregates disrupt homeostasis, compromise cellular integrity, and impair the survival of both neurons and glial cells in the affected regions (see **section 1.4.2 of INTRODUCTION**), rendering their clearance, or the prevention of their formation, a major therapeutical goal. Unfortunately, current strategies, including immunotherapy and the use of autophagy enhancers, have yet to yield the expected results (Minakawa & Nagai, 2021; Zhang et al., 2021). For this reason, we sought to explore CB1R as an alternative modulator of proteostasis. While its pro-autophagic role is well established in cancer, where it contributes to tumour cell death (Velasco et al., 2016), its ability to restore proteostatic balance in the context of neurodegeneration has been less extensively investigated (Costa et al., 2016). Specifically in PD, *in vitro* studies have shown that CB1R activation can induce autophagy and restore α-syn levels (Erustes et al., 2021). However, the translation of these findings to *in vivo* models remains an essential and unmet need.

1.1. Pharmacological activation of cannabinoid type-1 receptor in adeno-associated viral α-synuclein^{A53T} vector model

Recent data from our group indicate that pharmacological activation of CB1R with ACEA, a synthetic AEA analogue and selective CB1R agonist, enhances autophagic flux, reduces aggregation markers, and restores TDP-43 protein levels in a mouse model of frontotemporal dementia (Gonzalo-Consuegra et al., 2024), in line with previous findings (Costa et al., 2016). Building upon these observations, we sought to evaluate the effects of CB1R activation in a PD mouse model based on the localised overexpression of mutated α-syn^{A53T} in the SN using AAV technology (Castro-Sánchez et al., 2018). This model recapitulates dopaminergic neurodegeneration, inflammatory responses, and motor impairments resulting from exacerbated α-syn expression (Dovonou et al., 2023). ACEA was administered at the established effective dose of 1.5 mg/kg (Aso et al., 2012) to AAV9-α-syn^{A53T}-lesioned male mice. Non-lesioned animals were not treated with ACEA, as previous work by Aso and colleagues had already demonstrated that 1.5 mg/kg ACEA produces no significant effects in control mice (Aso et al., 2012). This approach allowed us to reduce the number of experimental animals used, in accordance with the principles of the 3Rs.

1.1.1. Activation of cannabinoid type-1 receptor exacerbated lysosomal LAMP-1, astrocytic GFAP, and α-synuclein immunoreactivity in the substantia nigra of lesioned mice

Immunohistochemical analysis of the SN revealed that direct inoculation of AAV9-α-syn^{A53T} resulted in the accumulation of α-syn in this brain region, as evidenced by a marked increase in immunoreactivity for exogenous human α-syn in the lesioned group compared with sham-operated animals (**Figure 19A**). However, this approach does not permit the determination of whether the protein is present in an aggregated form. A significant increase in immunoreactivity for the lysosomal marker LAMP-1 was also observed in lesioned animals (**Figure 19B**), likely reflecting a cellular response to proteotoxic stress. This may indicate an attempt to degrade the accumulated protein via the lysosomal pathway, although it could alternatively suggest a pathological condition characterised by lysosomal overload. In parallel, a pronounced neuroinflammatory response was detected, with significant increases in the astroglial marker GFAP (**Figure 19C**) and the microglial/infiltrating macrophage marker CD68 (**Figure 19D**).

Notably, *i.p.* administration of ACEA further increased LAMP-1 (**Figure 19B**) and GFAP (**Figure 19C**) immunoreactivity. These findings suggest that this CB1R-selective agonist may exert its effects through astroglial receptors and potentially enhance lysosome-associated degradative processes. However, this response does not appear to resolve the pathological condition, as human α-syn levels remained unchanged in the treated group, instead exhibiting a non-significant upward trend (**Figure 19A**), which may reflect further accumulation of α-syn within lysosomes. Finally, ACEA treatment did not significantly modify the increase in CD68 labelling induced by the lesion (**Figure 19D**).

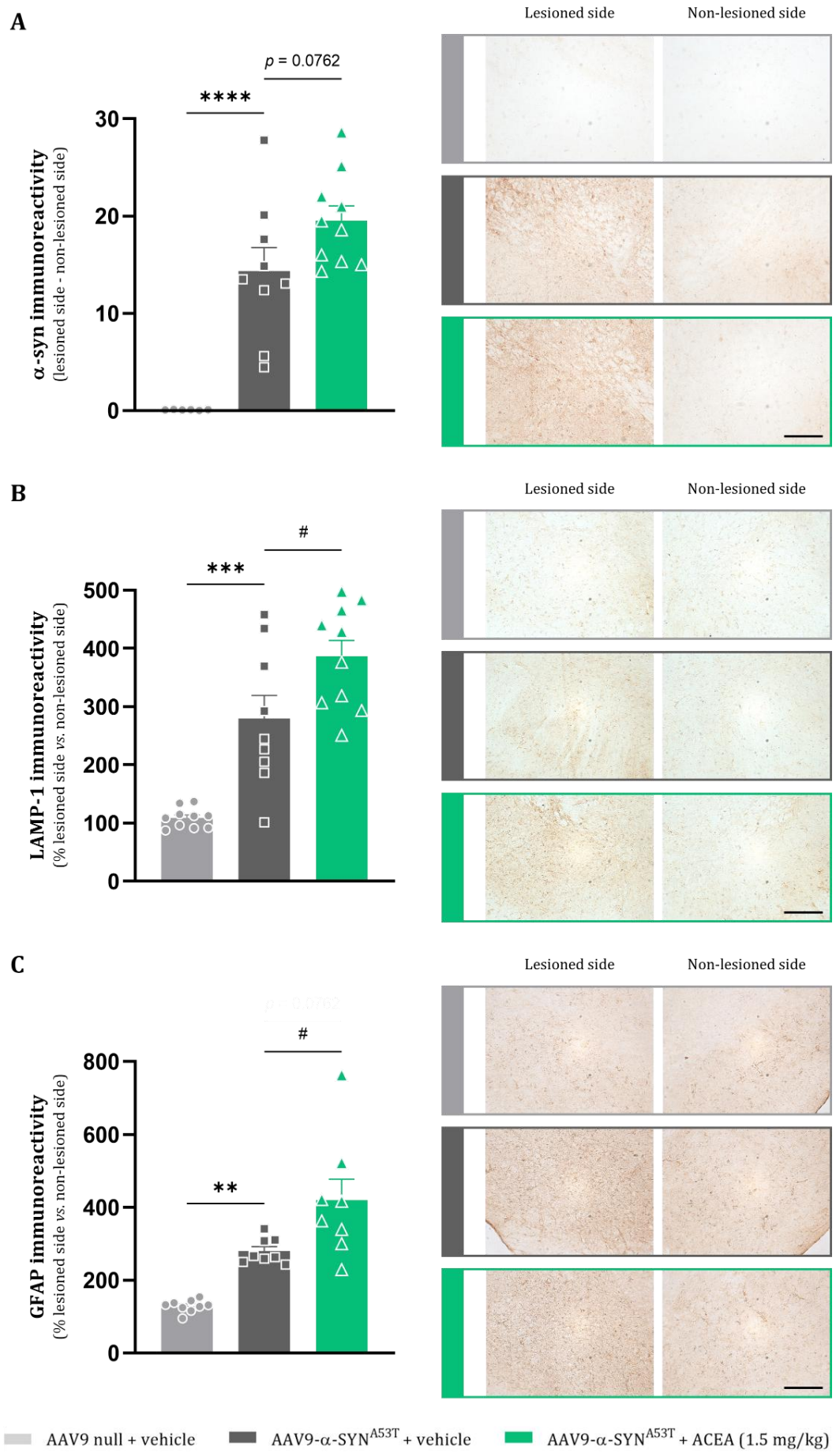


Figure 19. Effect of ACEA treatment on α-synuclein accumulation, proteotoxic stress, and glial reactivity, in the SNpc of AAV9-α-syn^{A53T}-lesioned mice. *Continued on the next page.*

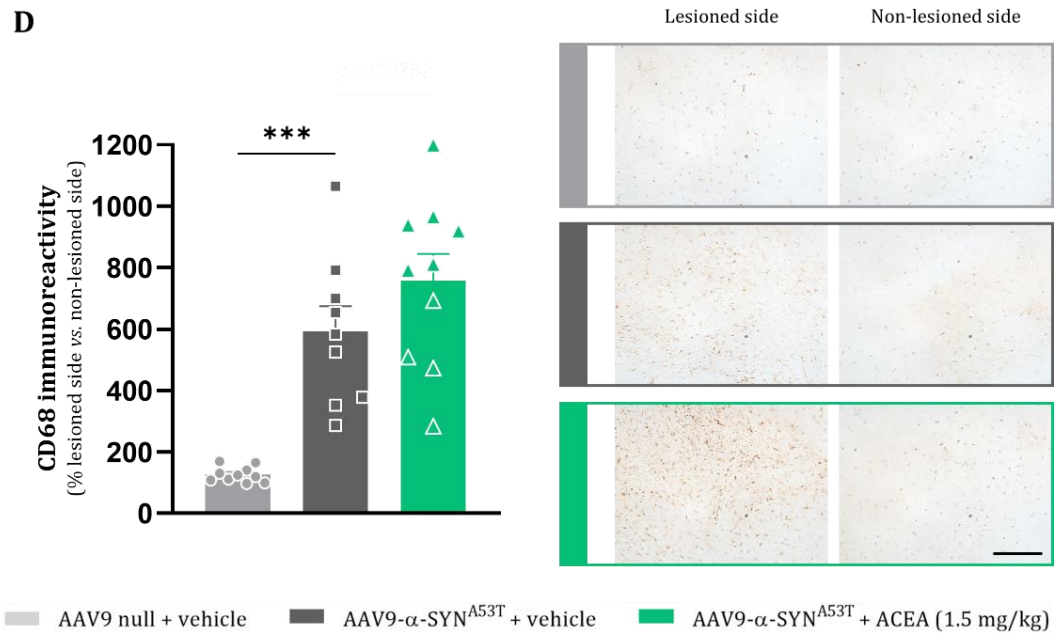


Figure 19. Effect of ACEA treatment on α-synuclein accumulation, proteotoxic stress, and glial reactivity, in the SNpc of AAV9-α-syn^{A53T}-lesioned mice. *Immunohistochemical assays were performed to evaluate four markers: (A) α-synuclein (α-syn), (B) lysosomal-associated membrane protein 1 (LAMP-1), (C) glia fibrillary acidic protein (GFAP), and (D) cluster of differentiation 68 (CD68). Immunoreactivity values were quantified from 3-5 images per animal and calculated as the difference or percentage of the lesioned side relative to the non-lesioned side. Final values are expressed as mean ± SEM of 7-10 animals per group. Data analysis was conducted using one-way ANOVA followed by Tukey's multiple comparison test (** p < 0.01, *** p < 0.001, **** p < 0.0001 vs. Sham-operated vehicle group; # p < 0.05 vs. AAV9-α-syn^{A53T}-lesioned vehicle group). Representative immunostaining images for each experimental group are shown to the right of the corresponding graph (scale bar = 200 µm).*

1.1.2. ACEA treatment slightly preserved dopaminergic neurons in the substantia nigra, but it was insufficient to prevent motor impairments provoked by lesion

As expected from previous studies (Burgaz et al., 2024), local overexpression of mutated α-syn^{A53T} in the SN led to significant damage in TH-positive neurons, with lesioned animals exhibiting an approximately 60% reduction in TH immunoreactivity compared to the sham-operated group (**Figure 20**). This loss of nigral dopaminergic neurons clearly affected the motor performance of the animals, as assessed by two distinct behavioural tests (**Figure 21**). Given that the parkinsonian lesion was performed unilaterally, we focused on specific motor tasks to quantify lateralised motor behaviour. To this end, we employed the CRT (Fleming et al., 2013) and the EBST (Bentea et al., 2015). In both CRT and EBST, the AAV9-α-syn^{A53T} nigral lesion induced motor impairments, as shown by an increased preference for using the ipsilateral forepaw in CRT (**Figure 21A**) and a higher number of contralateral swings in EBST (**Figure 21B**), in the vehicle-treated lesioned group compared to sham-operated animals. However, statistical significance was reached only in the latter.

The *i.p.* administration of ACEA in lesioned mice did not significantly restore nigral TH levels compared to the vehicle-treated lesioned group. However, a modest upward trend was observed, suggesting a slight neuroprotective effect of this CB1R-selective agonist (**Figure 20**). This finding indicates that the previously described effects of ACEA on lysosomal content and astroglial staining may be, at the very least, non-detrimental to this neuronal population. Nevertheless, this mild preservation of TH immunoreactivity was insufficient to elicit a meaningful

improvement in motor performance, as ACEA treatment failed to markedly ameliorate motor deficits in either behavioural test (**Figures 21A** and **21B**). Notably, no significant differences were observed between groups in the total number of paw contacts in the CRT or swing events in the EBST (**Figures 21C** and **21D**).

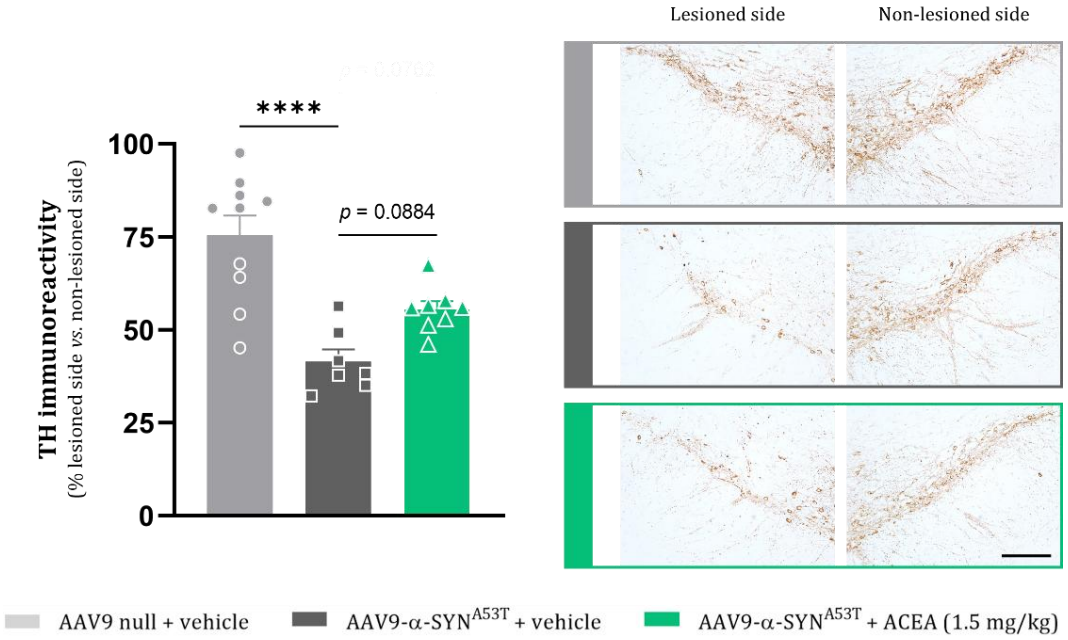


Figure 20. Effect of ACEA treatment on neuronal integrity in the SNpc of AAV9-α-syn^{A53T}-lesioned mice. *Immunohistochemical analysis was performed to assess dopaminergic neuronal integrity using tyrosine hydroxylase (TH) as a marker. Immunoreactivity was quantified from 3-5 images per animal and expressed as a percentage of the lesioned side relative to the non-lesioned side. Data are presented as mean ± SEM from 7-10 animals per group. Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparison test (**** $p < 0.0001$ vs. Sham-operated vehicle group). Representative immunostaining images for each experimental group are shown to the right of the graph (scale bar = 200 µm).*

1.1.3. Nigral overexpression of α-synuclein^{A53T} altered striatal unfolded protein response-related gene expression, mildly corrected by ACEA treatment

In addition to the SN, the striatum is another brain region profoundly affected by PD pathology. These two structures are interconnected via the nigrostriatal dopaminergic pathway, whereby neuronal loss in the SN leads to DA denervation in the striatum, ultimately resulting in diminished dopaminergic neurotransmission. This denervation may influence the expression of specific molecular markers in striatal neurons that are regulated by nigrostriatal dopaminergic input. For this reason, the striatum was also examined, with particular attention given to markers associated with dopaminergic signalling, such as DA receptors. If ACEA exerts beneficial effects on dopaminergic neurons, such neuroprotection would be expected to be reflected in the regulation of these downstream striatal markers.

Striatal gene expression, visualised through a heatmap (**Figure 22**), revealed that the AAV9-α-syn^{A53T} lesion exerted a limited impact on key components of dopaminergic signalling. No major alterations were detected in the expression of DA D1 (*Drd1*) and D2 (*Drd2*) receptors, and only a downward trend (purple hue) was observed for DARRP32 (*Ppp1r1b*), a downstream signalling molecule. Interestingly, the expression of DARRP32 appeared to recover following treatment with ACEA (orange hue), although this change did not reach statistical significance. Beyond

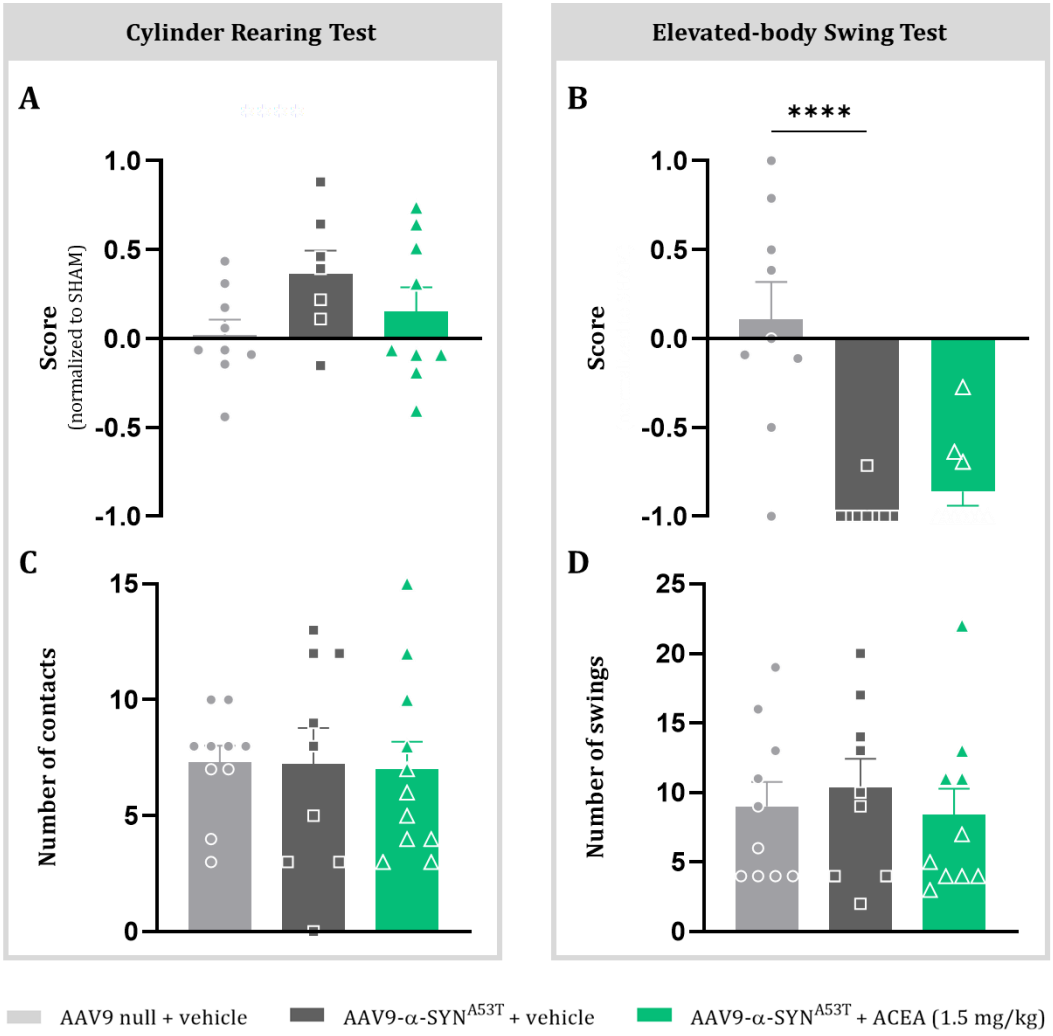


Figure 21. Effect of ACEA treatment on the motor performance of AAV9-α-syn^{A53T}-lesioned mice assessed by the cylinder rearing test (CRT) and the elevated-body swing test (EBST). *In the CRT, (A) lateralization was quantified by subtracting the number of contacts made with the ipsilateral paw from those made with the contralateral paw and dividing the result by (C) the total number of contacts. In the EBST, (B) the lateral preference was calculated similarly, by subtracting the number of swings to the ipsilateral side from those to the contralateral side and dividing the result by (D) the total number of swings. Values are presented as mean ± SEM of 7-11 animals per group. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (**** $p < 0.0001$ vs. Sham-operated vehicle group).*

dopaminergic signalling, we also explored the influence of the lesion on regulatory degradative pathways, focusing on genes involved in autophagy and the UPR. Autophagy-related markers did not exhibit substantial alterations in expression following either mutant α-syn overexpression or ACEA administration. In contrast, the lesion had a more pronounced effect on UPR-associated genes. The misfolded protein chaperone BiP (*Hspa5*) showed the most notable reduction in expression, followed by the ER stress-induced transcription factor CHOP (*Ddit3*), and to a lesser extent, the ER chaperone GRP94 (*Hsp90b1*), which displayed a milder decreasing trend. Although ACEA treatment did not significantly reverse these lesion-induced changes, it appeared to counteract the trend by increasing the expression of these markers, as reflected by a shift towards orange hues in the heatmap (**Figure 22**). This suggests that CB1R activation by ACEA may elicit a modest biological response, warranting further investigation. Notably, CB1R (*Cnr1*) expression itself remained largely unaffected by either the AAV9-α-syn^{A53T} lesion or ACEA treatment.

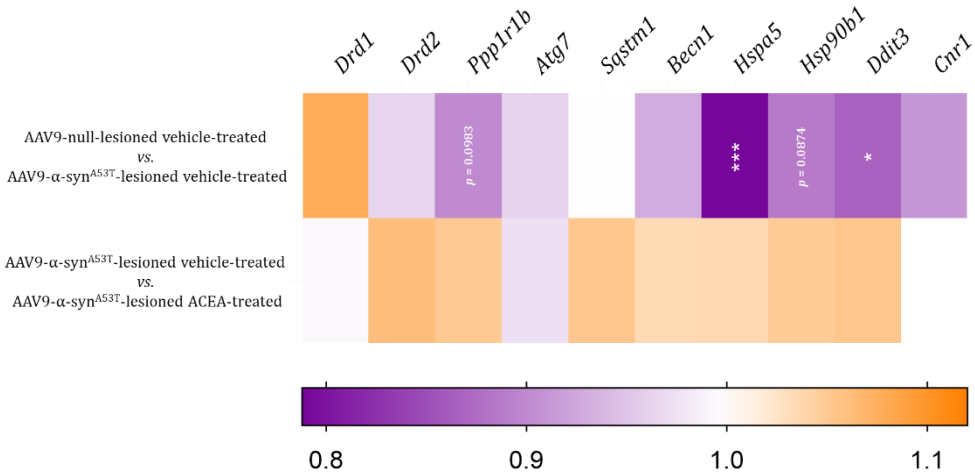


Figure 22. Effect of AAV9-α-synA53T lesion and ACEA treatment on gene expression profiles in the striatum. *mRNA levels were measured by qPCR and visualised as a heatmap to evaluate the expression of genes involved in dopaminergic signalling, including Drd1 (dopamine D1 receptor), Drd2 (dopamine D2 receptor), and Ppp1r1b (dopamine- and cAMP-regulated phosphoprotein of 32 kDa, DARRP32); autophagy, such as Atg7 (autophagy-related 7, ATG7), Sqstm1 (sequestosome 1, also known as p62), and Becn1 (beclin-1); and the UPR, including Hspa5 (binding immunoglobulin protein, BiP), Ddit3 (C/EBP homologous protein, CHOP), and Hsp90b1 (glucose-regulated protein 94, GRP94). The cannabinoid receptor gene Cnr1 (CB1R) was also included. Gapdh was used as the housekeeping gene. The colour scale below the heatmap indicates relative expression levels. Each experimental group comprised 8-11 animals. Data were analysed using one-way ANOVA followed by Tukey's multiple comparison test (* p < 0.05, *** p < 0.001 vs. Sham-operated vehicle group).*

1.2. Effect of local α-synuclein^{A53T} overexpression in BiP-deficient mice

Despite its limited neuroprotective effect, ACEA may influence degradative pathways, as evidenced by increased LAMP-1 immunoreactivity and a modest elevation in the expression of genes related to degradative processes in the striatum. Although CB1R has been widely reported to regulate proteostasis through autophagy induction, our data suggest that an alternative mechanism, namely, the UPR, may also contribute to protein dysregulation in our model. Notably, recent studies have demonstrated that key components of the UPR, such as the chaperone BiP and the ubiquitin E3 ligase cereblon, interact strongly with the intracellular domain of CB1R, thereby modulating its signalling (Costas-Insua et al., 2021; 2024). These interactions may bias CB1R activation towards the regulation of protein homeostasis. Interestingly, BiP has been implicated in α-syn toxicity in PD patients (Baek et al., 2016), and its upregulation has been shown to ameliorate α-syn-induced toxicity in a rat model of PD (Gorbatyuk et al., 2012). This is in line with our previous observation of reduced BiP expression, alongside decreases in other UPR-related markers (**Figure 22**). Given the potential link between CB1R, BiP and α-syn, we sought to further investigate the role of this CB1R-interacting chaperone in PD pathology. To this end, BiP-deficient male mice and their WT littermates were subjected to the same lesion procedure described in the previous section (AAV9-α-syn^{A53T} inoculation into the SN), a model in which protein dysregulation represents the primary cytotoxic insult. As complete BiP deficiency results in embryonic lethality, only heterozygous animals could be employed in our study.

1.2.1. BiP-deficient and wild-type mice showed similar neurodegenerative patterns and motor impairments after nigral lesion

Assessment of the AAV9-α-syn^{A53T}-induced lesion via TH immunostaining revealed a marked reduction in TH immunoreactivity in lesioned WT animals, indicative of dopaminergic neuronal

dysfunction. A comparable reduction was observed in lesioned BiP$^{+/-}$ mice (**Figure 23**), suggesting no significant genotype-related differences in the extent of the lesion. In light of this, similar levels of motor impairment were anticipated in both genotypes across behavioural tests, a hypothesis that was subsequently confirmed (**Figure 24**). Both lesioned groups displayed comparable preference scores for the ipsilateral paw (relative to the lesion side) during rearing in the CRT (**Figure 24A**), as well as similar contralateral swing bias in the EBST (**Figure 24B**). Notably, analysis of the total number of contacts and swings revealed no major differences between groups in either the CRT (**Figure 24C**) or EBST (**Figure 24D**), with the exception of a reduced number of swings in lesioned heterozygous mice compared to their WT counterparts. This finding may suggest a subtle motor effect of BiP deficiency, albeit not directly attributable to the parkinsonian lesion itself.

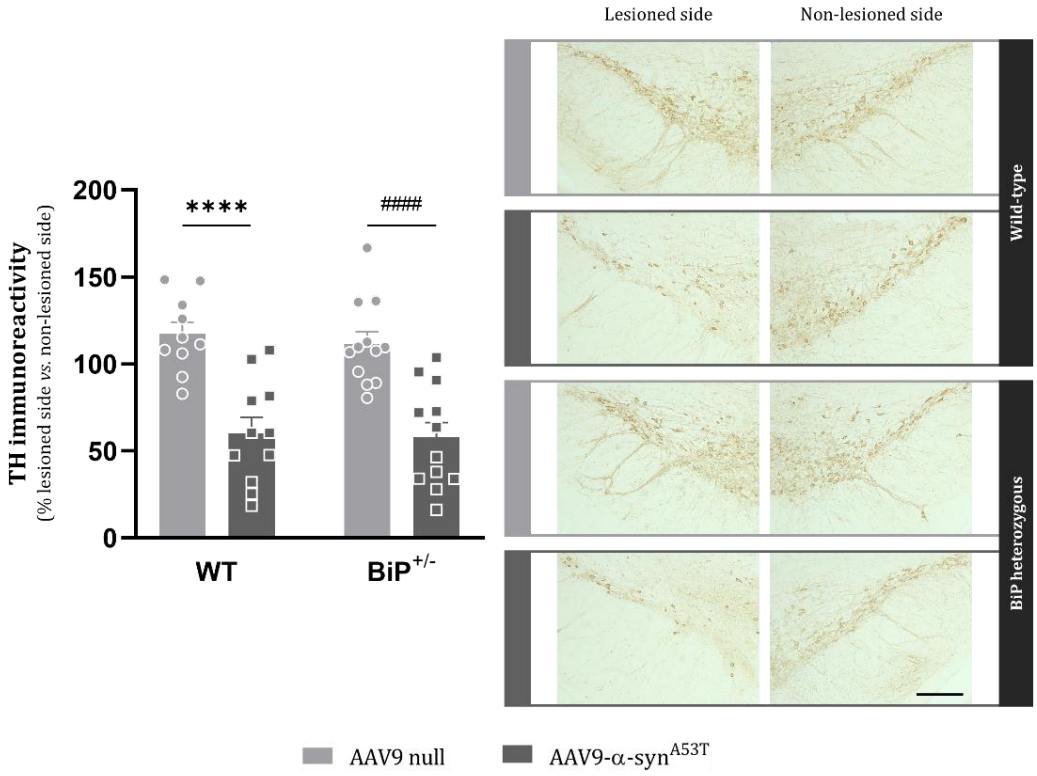


Figure 23. Effect of partial BiP deficiency on neuronal integrity in the AAV9-α-syn^{A53T}-lesioned SNpc*. Immunohistochemical assays were performed to evaluate the dopaminergic neuronal marker tyrosine hydroxylase (TH). Immunoreactivity values were quantified from 3-5 images per animal and expressed as the percentage of the lesioned side relative to the non-lesioned side. Final values are presented as mean ± SEM of 8-12 animals per group. Data were analysed using two-way ANOVA followed by Tukey's multiple comparison test (**** p < 0.0001 vs. WT Sham-operated group; #### p < 0.05 vs. BiP$^{+/-}$ Sham-operated group). Representative immunostaining images for each experimental group are shown to the right of the graph (scale bar = 200 µm).*

1.2.2. BiP deficiency tended to modify the microglial response in the substantia nigra of lesioned mice, without affecting astrocytic immunoreactivity

In addition to evaluating dopaminergic neurons via TH immunostaining, we also assessed glial responses by measuring microglial and astrocytic markers using Iba1 and GFAP immunostaining, respectively. Notably, Iba1 was selected over CD68 as a microglial marker, given that previous work in our laboratory demonstrated a more pronounced effect of BiP heterozygosity on Iba1 immunoreactivity, whereas no differences were observed with CD68 (Gómez-Almería et al., 2021). As expected, lesioned animals exhibited a marked increase in both microglial (**Figure 25A**) and

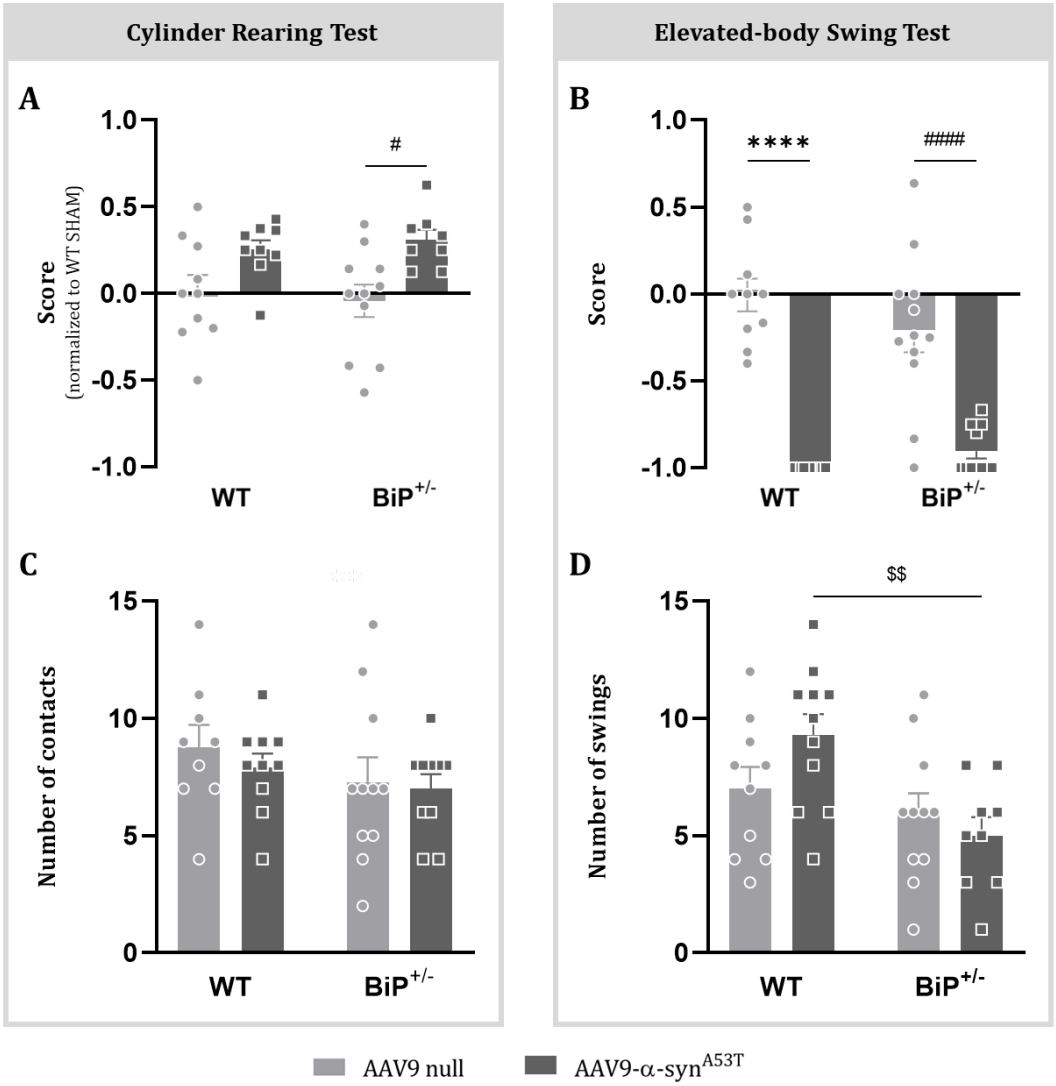


Figure 24. Effect of partial BiP deficiency on motor performance in AAV9-α-syn^{A53T}-lesioned mice assessed by the cylinder rearing test (CRT) and elevated-body swing test (EBST). *In the CRT, (A) the score of motor asymmetry was calculated by subtracting the number of contacts made with the ipsilateral paw from the number of contacts made with the contralateral paw, and dividing the result by (C) the total number of contacts. In the EBST, (B) the asymmetry score was calculated similarly, subtracting the turns to the ipsilateral side from the turns to the contralateral side, and dividing the result by (D) the total number of turns. Data are expressed as mean ± SEM of 8-12 animals per experimental group. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test (**** p < 0.0001 vs. WT Sham-operated group; # p < 0.05, #### p < 0.0001 vs. BiP$^{+/-}$ Sham-operated group; $$ p < 0.01 vs. WT AAV9-α-syn^{A53T}-lesioned group).*

astrocytic (**Figure 25B**) immunoreactivities, indicative of glial activation. However, no significant genotype-related differences were observed for either marker. The only exception was a trend towards increased Iba1 immunoreactivity, and thus enhanced microglial activation, in BiP$^{+/-}$ mice following lesion, in line with previous findings (**Figure 25A**).

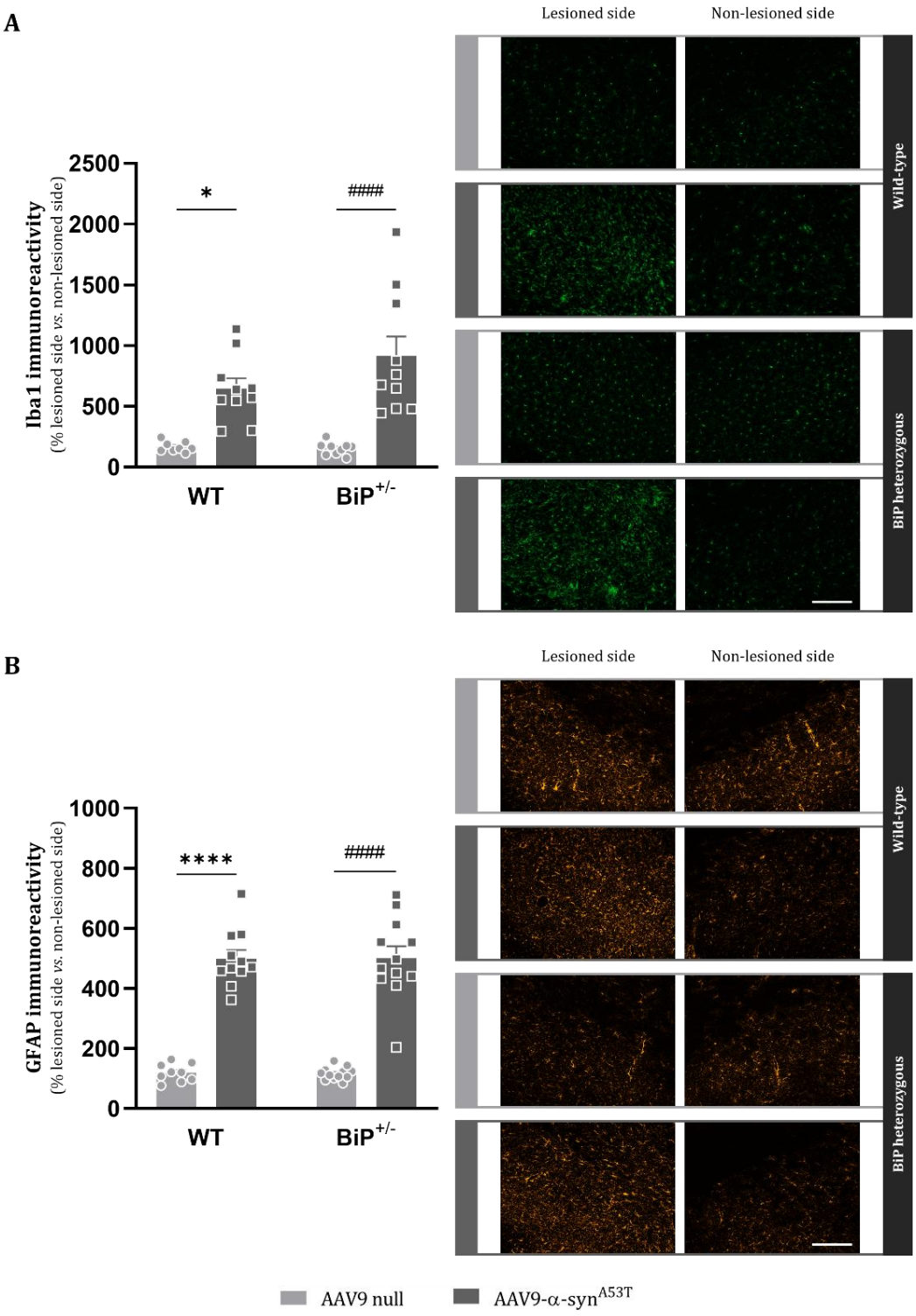


Figure 25. Effect of partial BiP deficiency on glial reactivity in the AAV9-α-syn^{A53T}-lesioned SNpc*. Immunohistochemical assays were performed to evaluate glial activation using two markers: (A) ionised calcium-binding adapter molecule 1 (Iba1), and (B) glial fibrillary acidic protein (GFAP). Immunoreactivity was quantified from 3–5 images per animal and expressed as the percentage of the lesioned side relative to the non-lesioned side. Data are presented as mean ± SEM of 8–12 animals per group. Statistical analysis was conducted using two-way ANOVA followed by Tukey's multiple comparison test (* $p < 0.05$, **** $p < 0.0001$ vs. WT Sham-operated group; #### $p < 0.0001$ vs. BiP$^{+/-}$ Sham-operated group). Representative immunostaining images for each experimental group are shown to the right of the corresponding graph (scale bar = 200 µm).*

CHAPTER 2

G PROTEIN-COUPLED RECEPTOR 55 AND PARKINSON'S DISEASE

Beyond the canonical cannabinoid receptors CB1R and CB2R, cannabinoids may exert their effects through interactions with so-called atypical receptors, such as PPARγ and GPR55. The latter has been extensively studied in various pathological conditions due to its widespread expression across multiple tissues and organs (Shore & Reggio, 2015), where it is implicated in a wide range of physiological and pathological processes. Notably, GPR55 activity has attracted growing interest within the CNS, where it is expressed in brain regions including the striatum, hippocampus, cerebral cortex, cerebellum, GP, and SNpc (Wu et al., 2013; Patricio et al., 2022; Sánchez-Zavaleta et al., 2022). Indeed, studies employing GPR55-deficient models have highlighted its involvement in motor coordination (Wu et al., 2013; Hurst et al., 2017; Marichal-Cancino et al., 2017), neurogenesis (Cherif et al., 2015; Hill et al., 2018; 2019), immune and inflammatory regulation (Staton et al., 2008; Hill et al., 2019), axonal guidance (Cherif et al., 2015), and neuroprotection (Sisay et al., 2013; Gajghate et al., 2024).

Various GPR55-interacting compounds have been tested in PD models. In MPTP-lesioned mice, abnormal-CBD, a GPR18 agonist that also exhibits activity at GPR55, prevented motor impairments, protected dopaminergic cell bodies in the SN, though not in striatal dopaminergic terminals, and induced microglial morphological changes consistent with a resolutive phenotype (Celorrio et al., 2017). Interestingly, in the same study, CBD, acting as a GPR55 antagonist, induced similar microglial alterations, yet had no effect on motor behaviour or neuronal preservation. In 6-OHDA-lesioned rats, findings have been inconsistent. Fatemi and colleagues reported similar motor improvements with both the GPR55 agonist LPI and the antagonist ML-193 (Fatemi et al., 2021). However, another study documented slight differences between the agonist (LPI) and antagonists (CBD, CID16020046), with better motor performance observed following antagonist administration (Patricio et al., 2022). Additionally, neuroprotective effects have been reported for the GPR55 agonist O-1602 in models of cognitive impairment (Wang et al., 2022b; Xiang et al., 2022a; 2022b).

Despite the conflicting findings regarding GPR55 in neurodegenerative contexts, it is evident that this receptor mediates actions that may counteract neurodegeneration. However, given its complex pharmacology, likely shaped by biased agonism and cell-specific functional selectivity, we opted to investigate GPR55 genetic ablation as a means to further elucidate its role in PD. To date, no studies have examined parkinsonian models in the context of GPR55 deficiency. To address this gap, we employed two distinct lesion models: nigral AAV9-α-syn^{A53T} inoculation and striatal 6-OHDA administration, in both male and female mice, allowing for a more comprehensive analysis of the contribution of GPR55 to PD pathogenesis under different cytotoxic conditions. Additionally, we assessed potential alterations in GPR55 expression using these same models, as well as in *post-*

mortem human samples, given the limited data on its dysregulation in PD (Celorrio et al., 2017; Martínez-Pinilla et al., 2020).

2.1. GPR55 expression was largely unchanged in Parkinson's disease patients and in two murine models of parkinsonism

Before evaluating the potential impact of GPR55 on the pathogenesis of PD, it is essential to determine how GPR55 expression is affected by the disease itself. To this end, we first analysed GPR55 expression in *post-mortem* human samples, focusing on midbrain regions containing the SN and the caudate-putamen nuclei. These samples are of high value, as they provide direct insight into disease-associated alterations. It is worth noting that, for these human analyses, data from both sexes were pooled to increase statistical power and minimise the impact of data dispersion and missing values in specific subgroups. No significant changes in GPR55 expression were observed in either the midbrain (**Figure 26A**) or the striatum (**Figure 26B**). Nonetheless, if any trends can be discerned from these data, they indicate an upregulation in the midbrain and a downregulation in the striatum.

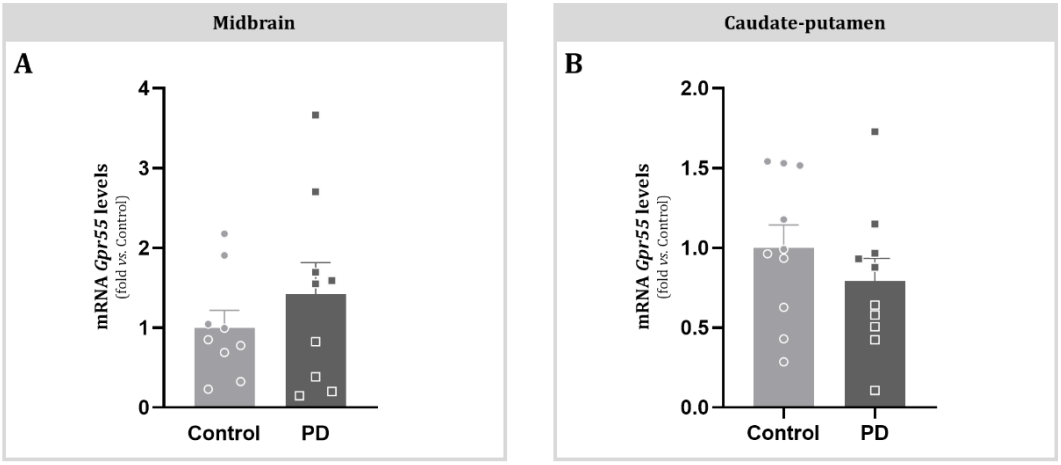


Figure 26. Impact of PD on GPR55 expression in post-mortem human samples. *Samples analysed included (A) midbrain regions containing the SNpc and (B) caudate-putamen nuclei of the striatum. Both healthy controls and PD patients, as well as samples from both sexes, were included in these analyses. GAPDH was used as the housekeeping gene, and expression values were normalised to the mean of the control group. Final values are presented as mean ± SEM for 9-12 samples per experimental group. Data were analysed using Student's t-test.*

We then extended our analysis of GPR55 expression to the striatum, the principal brain region expressing this receptor, in mouse models of PD induced via two distinct approaches: nigral stereotaxic injection of AAV9-α-syn^{A53T} and striatal stereotaxic injection of 6-OHDA. In both models, no significant lesion-induced differences were observed in either sex, nor were any sex-related differences detected in sham-operated or lesioned animals (**Figures 27A** and **27B**). Notably, a significant overall increase in receptor expression following lesion, irrespective of sex, was observed in the AAV9-α-syn^{A53T} model (p = 0.0448) (**Figure 27A**). By contrast, no detectable changes or subtle trends were evident in the mitochondrial 6-OHDA model (**Figure 27B**).

It is worth noting that, despite numerous attempts to directly assess GPR55 protein levels via Western blotting, immunohistochemistry, or proteomics, we were unable to obtain reliable or conclusive results. This was primarily due to the lack of effective experimental tools, such as high-

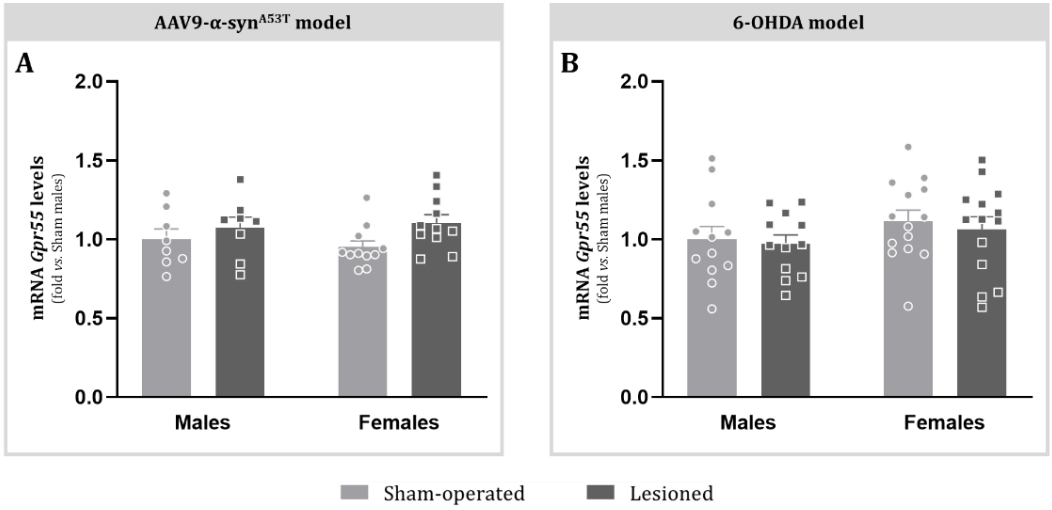


Figure 27. Impact of two distinct parkinsonian lesions on striatal GPR55 expression in male and female mice. *mRNA levels od Gpr55 were quantified by qPCR in mice subjected to either (A) nigral inoculation of AAV9-α-syn^{A53T} or (B) striatal injection of 6-OHDA. Gapdh was used as the housekeeping gene, and values were normalised to the mean of the male sham-operated group. Final values are presented as mean ± SEM for 12-14 animals per experimental group. Data were analyzed using two-way ANOVA followed by Tukey's multiple comparison test.*

quality anti-GPR55 antibodies. To circumvent these limitations, alternative methods, including *in situ* hybridisation and single-nucleus RNA sequencing, are currently being considered, as they may allow for a more accurate and cell type–specific characterisation of GPR55 expression. Although results from these ongoing experiments are not yet available, we anticipate that they will significantly enhance our understanding of how GPR55 expression is regulated in the context of PD.

2.2. GPR55-deficient mice showed impaired rotarod performance, with no changes in other motor parameters or overall nigral histology

Although the lack of marked changes in GPR55 expression in both human samples and murine models might suggest a limited role in PD pathogenesis, it does not preclude the possibility that GPR55 may still represent a viable therapeutic target. For this reason, we next explored the functional relevance of GPR55 in PD by employing KO mice. These GPR55 KO mice have been primarily used to investigate the physiological roles of this receptor *in vivo*. As part of the necessary characterisation of this colony in our laboratory, and in light of the previously reported phenotypic alterations (Staton et al., 2008; Wu et al., 2013), we first performed a brief pre-lesion assessment of motor behaviour, alongside nigral immunohistochemical analyses of dopaminergic neurons, microglia, and astrocytes.

Since the CRT and EBST are specifically designed for assessing unilateral lesions, the rotarod and actimeter tests were employed to evaluate the overall motor performance of GPR55 KO animals in comparison to their WT counterparts prior to lesion induction. In the rotarod test, GPR55 deficiency significantly reduced the latency to fall (p = 0.0004; **Figure 28A**). When analysing the sexes separately, this effect was more pronounced in females, where it reached statistical significance, whereas in males the reduction was minimal and did not reach significance (**Figure 28A**). The genotype-associated effect was less marked across the various motor

parameters evaluated using the actimeter, with statistical significance observed only for resting time, which was longer in GPR55-deficient animals (p = 0.0335; **Figure 28B**). Nonetheless, downward trends were evident in several parameters, including horizontal activity (p = 0.0916; **Figure 28C**), locomotion (p = 0.0697; **Figure 28D**), maximum velocity (p = 0.0764; **Figure 28E**), mean velocity (p = 0.0608; **Figure 28F**), and total distance travelled (p = 0.0535; **Figure 28G**). No significant differences or observable trends were found in slow or fast movement counts (**Figures 28H** and **28I**), or in rearing events (**Figure 28J**). Notably, when analysed separately by sex, none of these parameters reached statistical significance in any experimental group (**Figures 28B–J**).

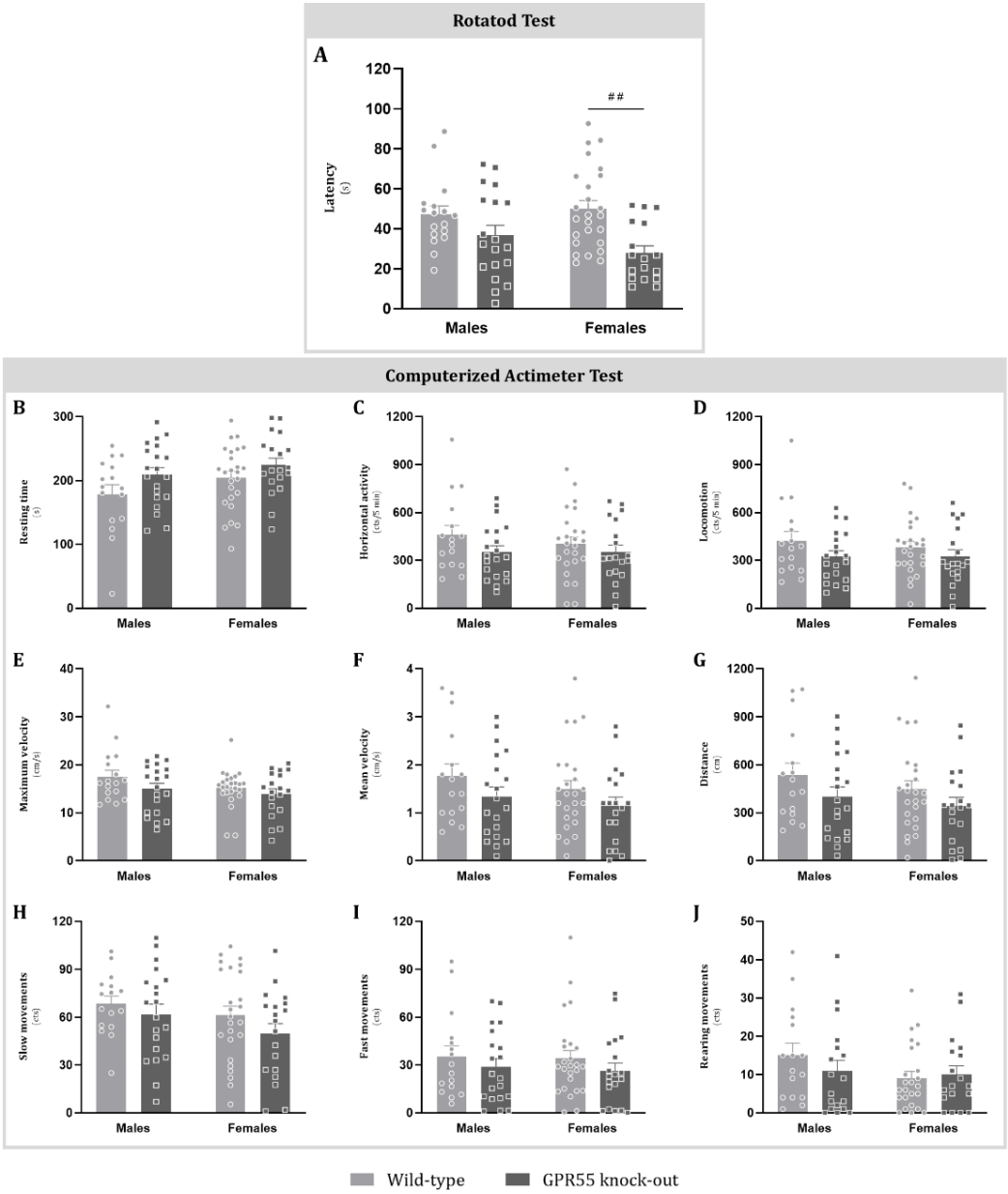


Figure 28. Motor characterisation of GPR55-deficient mice. *(A) Latency on rotarod test, as well as various parameters derived from the computerised actimeter, including (B) resting time, (C) horizontal activity, (D) locomotion, (E) maximum velocity, (F) mean velocity, (G) distance traveled, (H) slow movements, (I) fast movements, and (J) rearing movements, were assessed in both male and female WT and GPR55 KO mice. For the rotarod test, latency values represent the mean of three trials. Data are expressed as mean ± SEM of 14-25 animals per group. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test (## p < 0.01 vs. WT female group).*

In the SN, no significant differences in immunoreactivity were observed between genotypes in either sex for TH (**Figure 29A**), Iba1 (**Figure 29B**), or GFAP (**Figure 29C**). However, for the

microglial marker Iba1, a significant overall effect of GPR55 deficiency was detected, with reduced immunoreactivity observed in GPR55-deficient mice (p = 0.0448). This finding is consistent with previous reports indicating a microglial dependency in the anti-inflammatory actions mediated by GPR55 ligands (Kallendrusch et al., 2013).

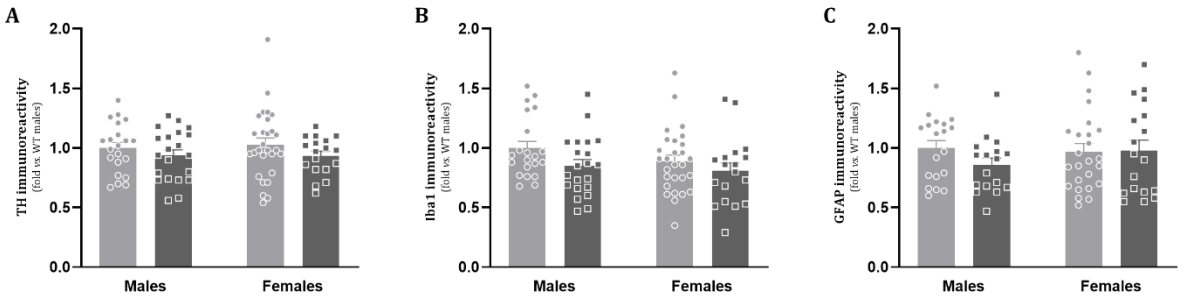


Figure 29. Effect of genetic GPR55 ablation on neuronal and glial cell populations within SNpc. *Immunohistochemical assays were performed to evaluate (A) tyrosine hydroxylase (TH) as a marker of dopaminergic neurons, (B) ionised calcium-binding adapter molecule 1 (Iba1) for microglia and (C) glial fibrillary acidic protein (GFAP) for astrocytes. Immunoreactivity values were quantified from 3-5 images per animal and normalised to the mean of the male WT group. Final values are expressed as mean ± SEM of 17-28 animals per group. Data were analysed by two-way ANOVA followed by Tukey's multiple comparison test.*

2.3. G protein-coupled receptor 55 deficiency in adeno-associated α-synuclein^{A53T} viral vector model

After evaluating whether GPR55 expression was altered by parkinsonian brain lesions and assessing the impact of GPR55 deficiency on motor performance and the cellular status of the SN and striatum, the next step was to analyse how this genetic ablation might influence the pathological phenotypic progression in both PD-like murine models. We first investigated this in the AAV9-α-syn^{A53T} model, which recapitulates key features of PD pathology, including dopaminergic neuronal death, disruptions in proteostasis, α-synuclein accumulation, and a robust inflammatory response (Castro-Sánchez et al., 2018).

2.3.1. GPR55-deficient mice showed less unilateral preference after nigral α-synuclein^{A53T} overexpression in the CRT, but not in the EBST

Once again, the CRT and EBST were employed to evaluate the motor performance of WT and GPR55 KO mice. In both tests, the nigral lesion induced by AAV9-α-syn^{A53T} elicited motor impairments, resulting in a marked preference for one paw (**Figure 30B**) or one side (**Figures 30D** and **30E**). This effect reached statistical significance in both sexes, except in males in the CRT, where the enhanced lateralised preference was less pronounced (**Figure 30A**). Regarding GPR55-deficient mice, these animals appeared less sensitive to the lesion in the CRT, exhibiting a weaker preference in females (**Figure 30B**), and no preference at all in males (**Figure 30A**). However, this increased resistance was not replicated in the EBST, where GPR55 KO mice displayed a similar degree of motor impairment as their WT counterparts following the unilateral lesion (**Figures 30D** and **30E**). Interestingly, even in the absence of a lesion, GPR55-deficient mice showed a slight increase in the preference for swinging towards the ipsilateral side compared to WT animals. Finally, when analysing the number of wall contacts or swing events in each experimental group,

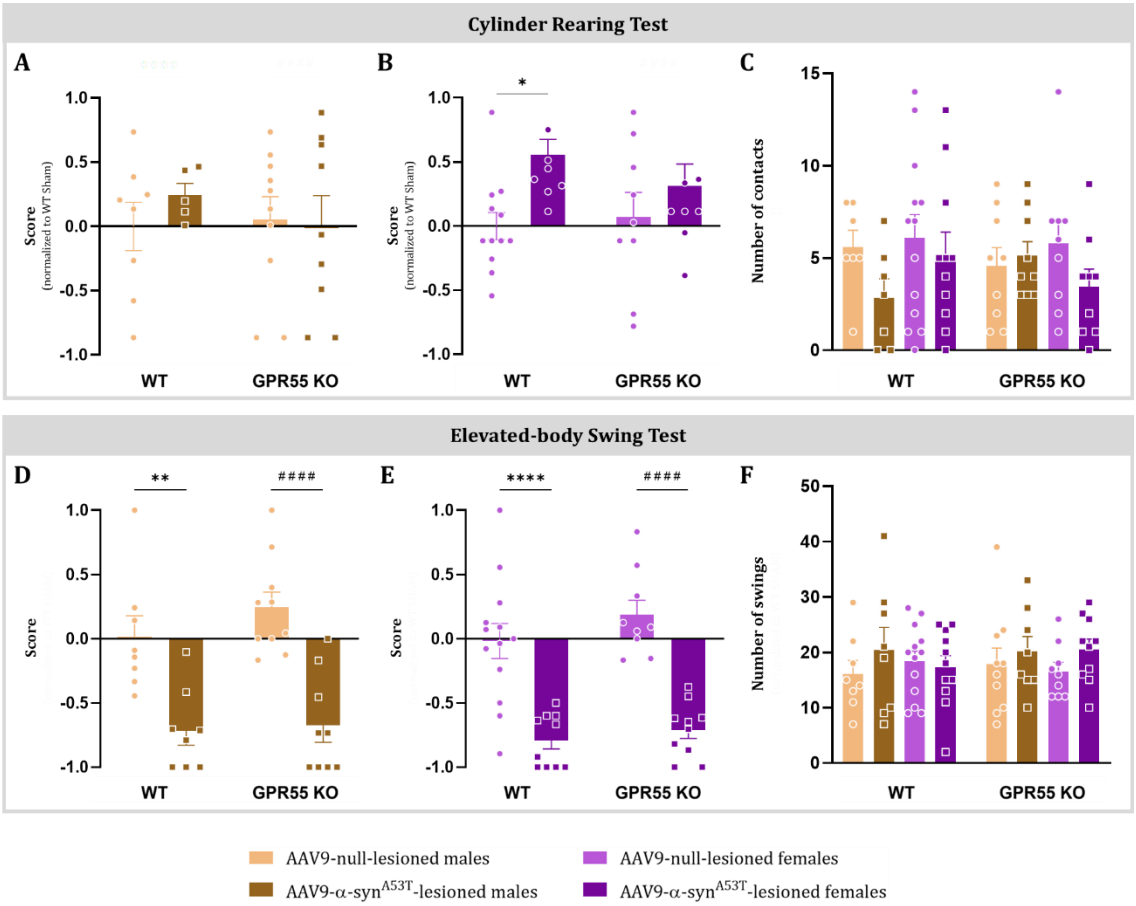


Figure 30. Effect of GPR55 depletion on motor performance of AAV9-α-syn^{A53T}-lesioned mice*. In the CRT, performed in (A) male and (B) female mice, the score was calculated by subtracting the number of contacts made with the ipsilateral paw from the number of contacts made with the contralateral paw, and dividing the result by (C) the total number of contacts. In the EBST, also carried out in (D) male and (E) female animals, the score was calculated similarly, subtracting the turns to the ipsilateral side from the turns to the contralateral side, and dividing the result by (F) the total number of turns. Data are expressed as mean ± SEM of 8-13 animals per group. Statistical analysis was performed using (A, B, D, E) two-way ANOVA or (C, F) three-way ANOVA followed by Tukey's multiple comparison test (* p < 0.05, ** p < 0.01, **** p < 0.0001 vs. WT Sham-operated group; #### p < 0.0001 vs. GPR55 KO Sham-operated group).*

no significant differences were detected between groups or in the overall effects of lesion, sex, or genotype (**Figures 30C** and **30F**).

2.3.2. GPR55 deficiency reduced dopaminergic neuronal loss and altered glial response after nigral lesion, with sex-specific effects

After the behavioural characterisation, we examined the histopathological status of the animals following AAV9-α-syn^{A53T} inoculation. All experiments were conducted in both males and females; however, representative images are shown only for females to avoid redundancy. As previously reported, nigral inoculation with AAV9-α-syn^{A53T} resulted in a marked increase in α-synuclein protein levels within this brain region, to a similar extent across sexes and genotypes (**Figure 31A**). This was accompanied by a striking 60% reduction in TH immunostaining in WT animals (**Figure 31B**). However, the loss of this dopaminergic marker was less pronounced in GPR55-deficient animals, resulting in a lower statistical significance between sham-operated and lesioned groups in both males and females (**Figure 31B**). Notably, in females, lesioned GPR55 KO animals exhibited a statistically significant increase in TH immunostaining compared to their lesioned WT counterparts, while in males, this increase was observed as a non-significant trend (**Figure 31B**).

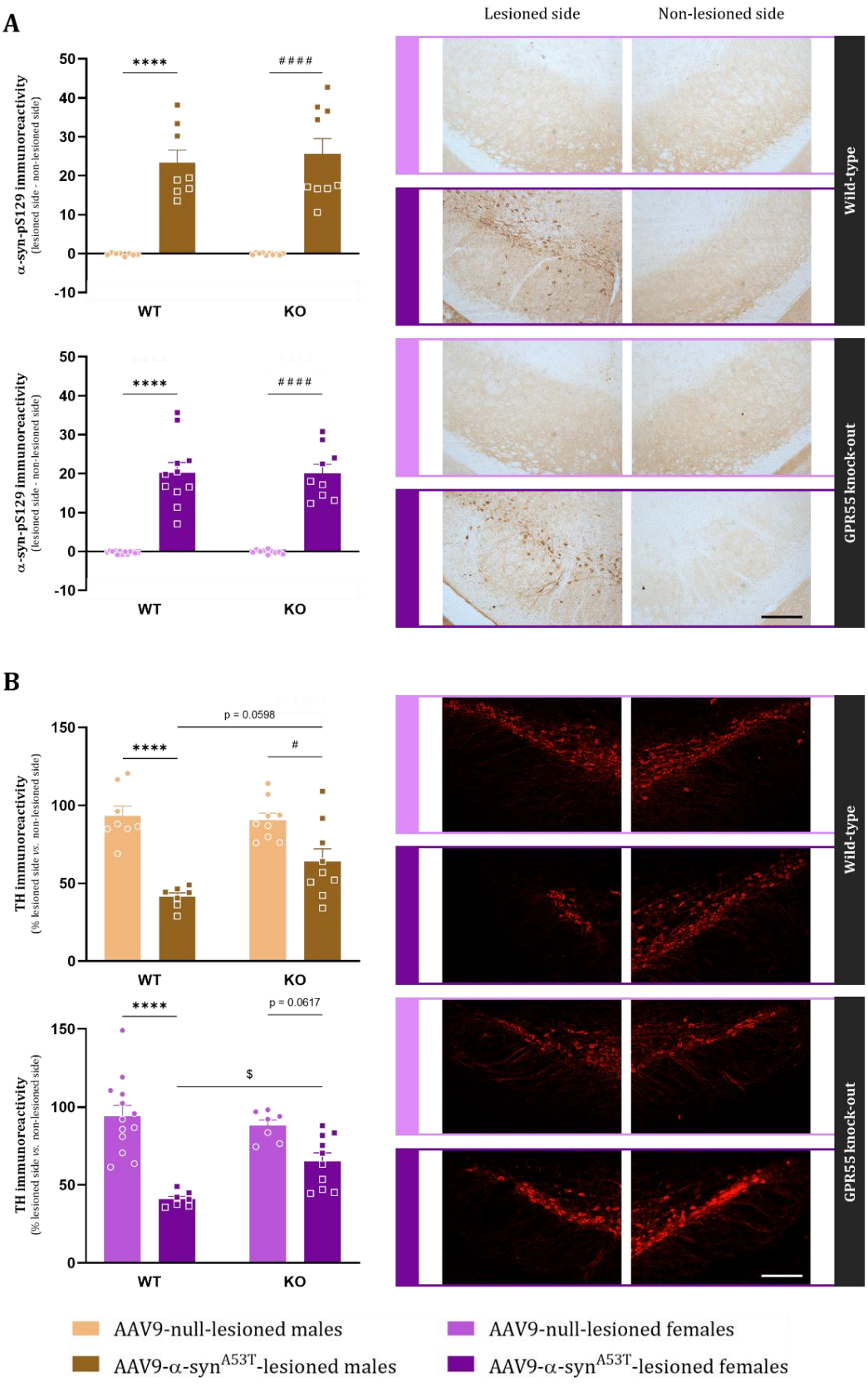


Figure 31. Effect of GPR55 depletion on protein dysregulation and neuronal integrity in the AAV9-α-syn^{A53T}-lesioned SNpc. *Immunohistochemical assays were performed to evaluate two markers: (A) α-synuclein (α-syn), and (B) tyrosine hydroxylase (TH). Immunoreactivity values were quantified from 3-5 images per animal and calculated as the difference or percentage of the lesioned side relative to the non-lesioned side. Final values are expressed as mean ± SEM of 7-15 animals per group. Data were analysed using two-way ANOVA followed by Tukey's multiple comparison test (**** $p < 0.0001$ vs. WT Sham-operated group; # $p < 0.05$, #### $p < 0.0001$ vs. GPR55 KO Sham-operated group; $ $p < 0.05$ WT AAV9-α-syn^{A53T}-lesioned group). Representative immunostaining images for each experimental group are shown as right panels and only for females (scale bar = 200 μm).*

Analysis of glial cells via Iba1 immunostaining revealed a marked increase in response to the

parkinsonian lesion, similarly observed in both sexes, with no significant differences between genotypes (**Figure 32A**). Given previous evidence indicating a significant role for this receptor in microglial activity and response, we further investigated morphological changes exhibited by microglia across experimental groups. As expected, AAV9-α-syn^{A53T} lesioning led to an increase in cell body area (**Figure 32B**) and a reduction in branch length (**Figure 32C**) in both sexes. These alterations resulted in an elevated cell body area-to-branch length ratio in lesioned WT animals (**Figure 32D**), indicating a shift towards an amoeboid-like morphology. In GPR55-deficient animals, similar lesion-induced morphological changes were observed. Notably, in females, branch length was further reduced in the absence of GPR55 following lesion (**Figure 32C**). This also affected the cell body area-to-branch length ratio, which was significantly higher in GPR55-deficient female mice lesioned with AAVs (**Figure 32D**). These findings suggest that microglia from female GPR55-deficient animals adopt an even more amoeboid-like morphology, which may influence their response to the proteotoxic insult.

This pronounced glial response was also evident in GFAP immunostaining, which revealed a substantial increase in lesioned mice, particularly in WT animals (**Figure 33A**). However, this increase was more moderate in GPR55-deficient animals, resulting in a reduction or even loss of statistical significance between sham-operated and lesioned groups. Nevertheless, no significant differences were observed between lesioned WT and GPR55 KO mice in either sex. This modest trend prompted us to further examine the status of GFAP-positive cells by assessing their colocalisation with complement component 3 (C3), a key mediator of inflammatory activation, which may serve as a preliminary indicator of astroglial reactivity. Through this approach, we identified a more pronounced effect of GPR55 deficiency. In WT animals, the lesion induced a significant increase in C3 expression within GFAP-positive astrocytes (**Figure 33B**). However, in GPR55-deficient females, this increase in colocalisation was completely abolished, resulting in a significant difference between the lesioned groups (**Figure 33B**). By contrast, this effect of GPR55 deficiency was not observed in males, once again highlighting a sex-related bias in the results.

2.3.3. Striatal upregulation of IL-1β and IFNγ in lesioned mice is substantially affected by GPR55 deficiency in females

Striatal gene expression analysis of the pro-inflammatory cytokines IL-1β and IFNγ revealed a general increase in lesioned groups of both sexes ($p = 0.0270$, **Figure 34A**; $p = 0.0047$, **Figure 34B**; $p = 0.0082$, **Figure 34C**; $p = 0.0170$, **Figure 34D**), indicating that α-syn-induced inflammation extends beyond the SN. GPR55 deletion did not significantly alter mRNA levels between lesioned and sham-operated groups in male mice. In females, although no statistically significant differences were observed between lesioned WT and KO animals for either cytokine, a subtle genotype-dependent effect could be identified: the increase in cytokine expression observed between sham and lesioned WT animals was not present in their GPR55 KO counterparts (**Figures 34B** and **34D**). This suggests a mild modulatory role of GPR55 in the striatal inflammatory response as well.

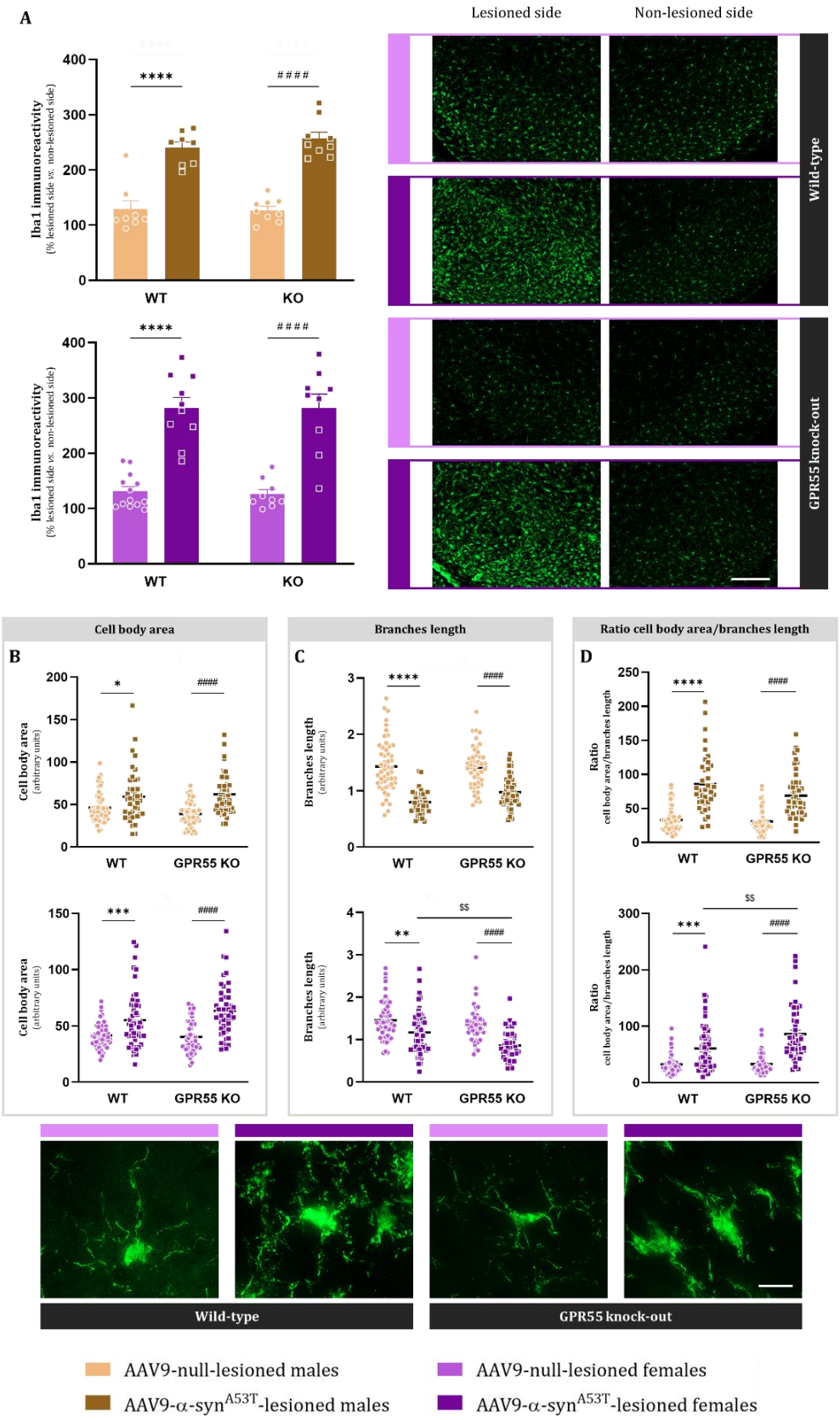


Figure 32. Effect of GPR55 depletion on microglial reactivity in the AAV9-α-syn^{A53T}-lesioned SNpc. *Immunohistochemical assays were performed to evaluate the microglial marker ionised calcium-binding adapter molecule 1 (Iba1). (A) Immunoreactivity values were quantified from 3-5 images per animal and calculated as the percentage of the lesioned side relative to the non-lesioned side. Final values are expressed as mean ± SEM of 7-15 animals per group. Representative immunostaining images for each experimental group are shown as right panels and only for females (scale bar = 200 µm). Morphological parameters, including (B) cell body area, (C) branch length, and (D) their ratio, were analysed at higher magnification (×63). Final values are expressed as mean ± SEM of 40-56 cells from 6-7 animals, per group. Representative immunostaining images for each experimental group are shown in the lower panels and also correspond to females only (scale bar = 20 µm). Data were analysed using two-way ANOVA followed by Tukey's multiple comparison test (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 vs. WT Sham-operated group; #### p < 0.0001 vs. GPR55 KO Sham-operated group; $ p < 0.05, $$ p < 0.01 vs. WT AAV9-α-syn^{A53T}-lesioned group).*

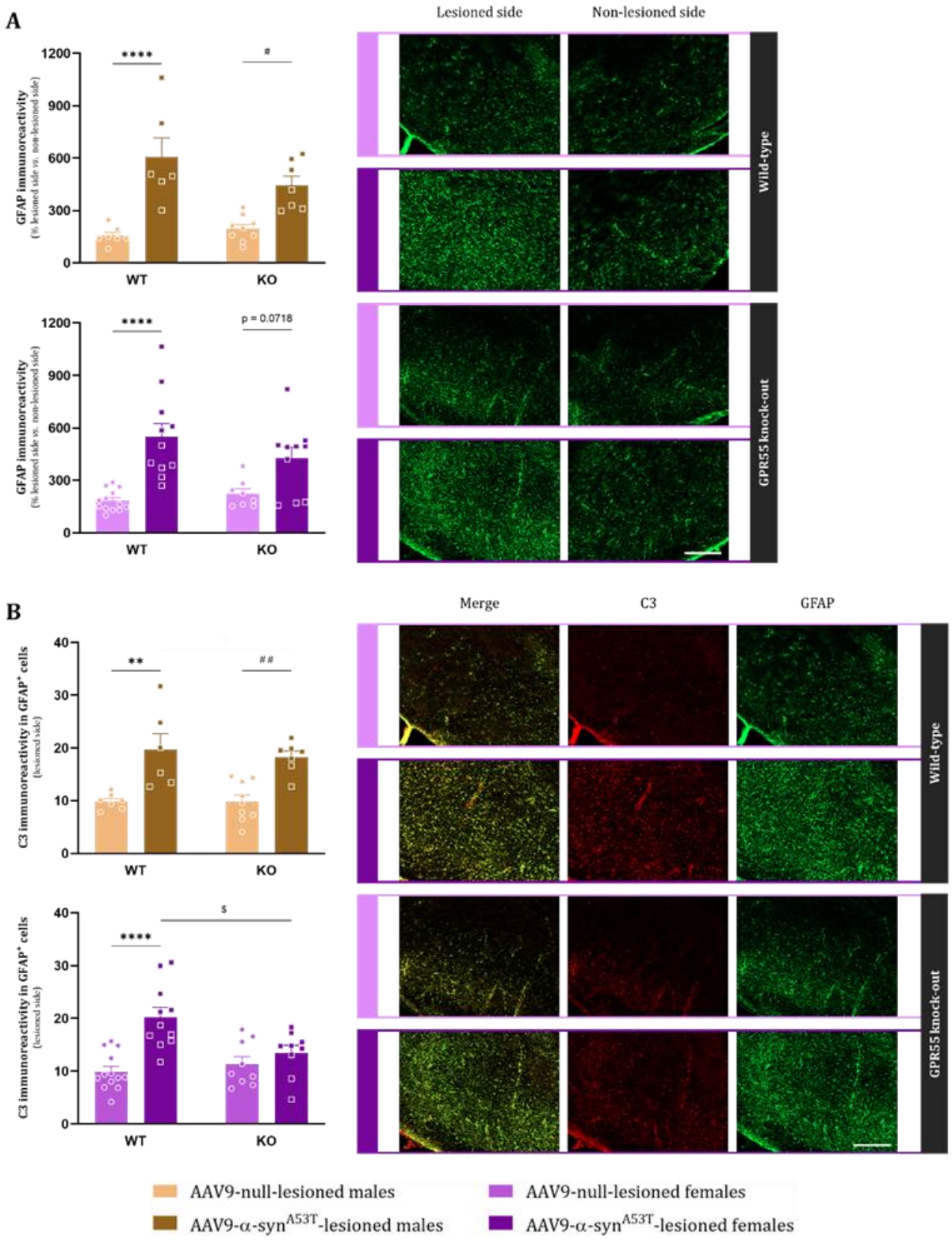


Figure 33. Effect of GPR55 depletion on astroglial reactivity in the AAV9-α-syn^{A53T}-lesioned SNpc*. Immunohistochemical assays were performed to evaluate the astroglial markers (A) glial fibrillary acidic protein (GFAP) and (B) complement component 3 (C3). Immunoreactivity values were quantified from 3–5 images per animal and calculated as follows: (A) for GFAP, as the percentage of the lesioned side relative to the non-lesioned side; and (B) for C3, as the percentage of the C3-positive area overlapping with the GFAP-positive area on the lesioned side. Final values are expressed as mean ± SEM of 6-13 animals per group. Data were analysed using two-way ANOVA followed by Tukey's multiple comparison test (** p < 0.01, **** p < 0.0001 vs. WT Sham-operated group; # p < 0.05, ## p < 0.01 vs. GPR55 KO Sham-operated group; $ p < 0.05 vs. WT AAV9-α-syn^{A53T}-lesioned group). Representative immunostaining images for each experimental group are shown as right panels and only for females (scale bar = 200 µm).*

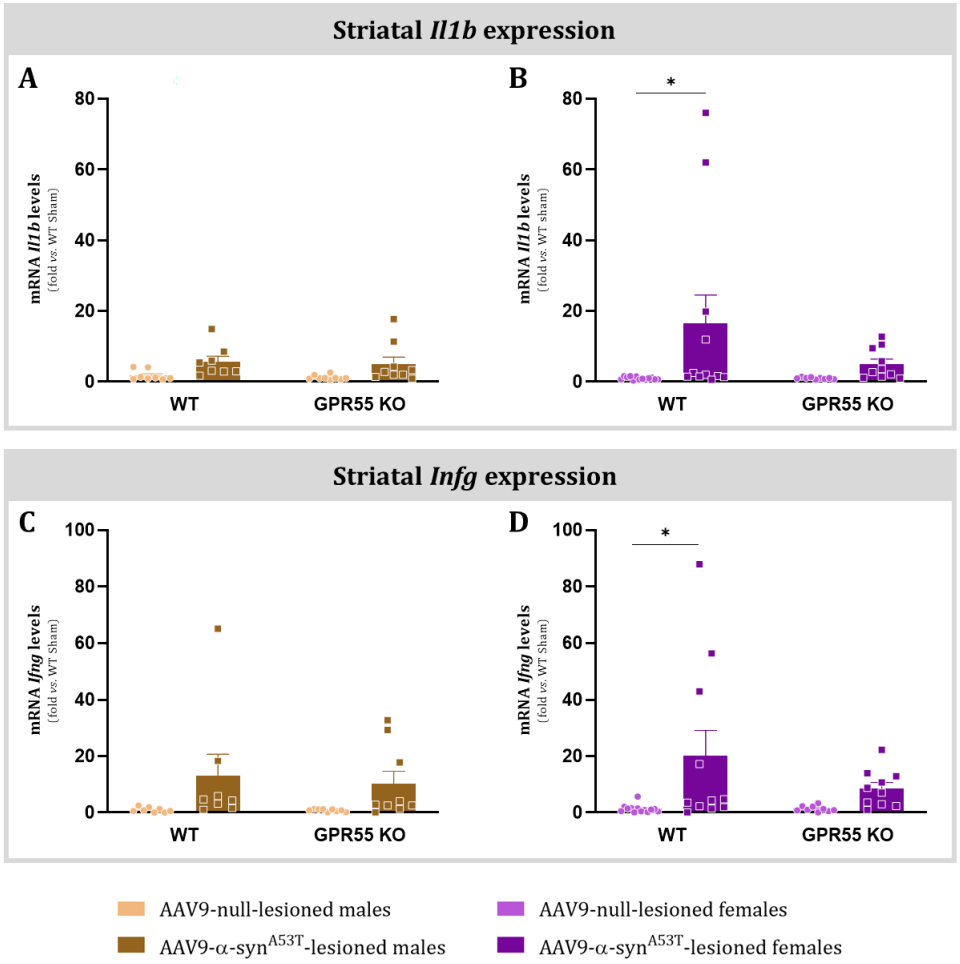


Figure 34. Effect of GPR55 depletion on the gene expression profile in the striatum of AAV9-α-syn^{A53T}-lesioned mice. *mRNA levels were evaluated by qPCR to assess the expression of two proinflammatory cytokine: interleukin 1β, in (A) males and (B) females, and interferon γ, also in (C) males and (D) females. Gapdh was used as a housekeeping gene, and values were normalized to the mean of the WT Sham-operated group. Final values are expressed as mean ± SEM for 10-15 animals per experimental group. Data analysis was conducted using two-way ANOVA followed by Tukey's multiple comparison test (* p < 0.05 vs. WT Sham-operated group).*

2.4. G protein-coupled receptor 55 deficiency in 6-hydoxydopamine model

Once GPR55 deficiency had been assessed in the proteinopathy-related AAV9-α-syn^{A53T} model, we sought to evaluate the impact of the same genetic alteration in a different parkinsonian model, based on the neurotoxin 6-OHDA, which primarily induces mitochondrial dysfunction and oxidative stress. The rationale for conducting experiments in an additional model was to avoid restricting the study to a single primary cytotoxic mechanism, as multiple pathological events occur simultaneously in the human disease. Therefore, when investigating a potential pharmacological target, it is crucial to verify its relevance across different models.

2.4.1. Female GPR55-deficient mice exhibited improved motor performance in the pole test following striatal 6-hydroxydopamine lesion

To assess motor performance in 6-OHDA-lesioned mice, we employed the pole test in addition to the CRT and EBST, as motor impairments resulting from unilateral lesions with this toxin have been shown to be detectable with this test, despite its non-lateralised nature (Burgaz et al., 2021a). In line with previous findings, comparison of descent latency revealed a clear increase in

lesioned WT animals, irrespective of sex (**Figures 35A** and **35B**). A similar prolongation in latency was also observed in lesioned GPR55-deficient males, but notably, this effect was absent in their female counterparts. These results further support the notion that GPR55 deficiency differentially affects motor behaviour, exerting a more pronounced influence in females while having minimal impact in males.

A similar trend was observed in the other motor tests. The lesion-induced side preference seen in WT animals was maintained in GPR55-deficient males (**Figures 35C** and **35F**), whereas in females, it appeared slightly attenuated in the CRT (**Figure 35D**) and almost completely abolished in the EBST (**Figure 35G**). However, it is important to note that statistical significance in these latter two tests was notably limited. Additionally, analysis of wall contacts in the CRT (**Figure 35E**) and swing events in the EBST (**Figure 35H**) revealed a significant effect of the lesion in both tests ($p < 0.0001$ and $p = 0.0005$, respectively). In the CRT, both WT males and WT females exhibited significant reductions in the number of contacts between control and lesioned groups (**Figure 35E**). Similarly, in the EBST, a significant reduction in swing events was observed in KO males (**Figure 35H**). Notably, in the CRT, a global effect of sex and genotype was also detected on the total number of rearing movements (**Figure 35E**). Therefore, these findings should be interpreted with caution, given the potential confounding influence of sex and genotype.

2.4.2. GPR55 deficiency only mildly reduced dopaminergic loss after striatal 6-hydroxydopamine lesion and had no effect on glial responses

The 6-OHDA-induced lesion was confirmed by quantification of TH immunoreactivity in the SN (**Figure 36**), revealing an approximately 60% reduction in dopaminergic neuronal staining on the lesioned side relative to the contralateral hemisphere in both WT females and males. Animals lacking GPR55 exhibited a similar, statistically significant reduction in TH immunoreactivity between sham-operated and lesioned groups. However, it is noteworthy that 6-OHDA-treated KO females showed a near-significant trend towards higher levels of this neuronal marker compared to their lesioned WT counterparts, suggesting a modestly attenuated neuronal degeneration in the absence of GPR55.

In contrast to the findings from the AAV9-α-syn^{A53T} model, neuronal damage induced by 6-OHDA was not accompanied by a significant inflammatory response in the SN. No increase in Iba1 (**Figure 37A**) or GFAP (**Figure 37B**) immunoreactivity was observed in either WT or GPR55 KO animals, irrespective of sex. Only in the case of GFAP immunostaining in males was a significant overall effect of the lesion detected ($p = 0.0074$). Furthermore, the expression of C3 protein in GFAP-positive cells was not elevated in lesioned animals (**Figure 37C**), further supporting the limited glial reactivity observed in the SN in this model.

2.4.3. Neither the 6-OHDA-induced lesion nor GPR55 deletion altered the striatal expression levels of IL-1β and IFN-γ in either sex

Gene expression analysis of IL-1β and IFN-γ in the striatum revealed no lesion-associated changes in the 6-OHDA model (**Figures 38**), consistent with the absence of Iba-1 and GFAP

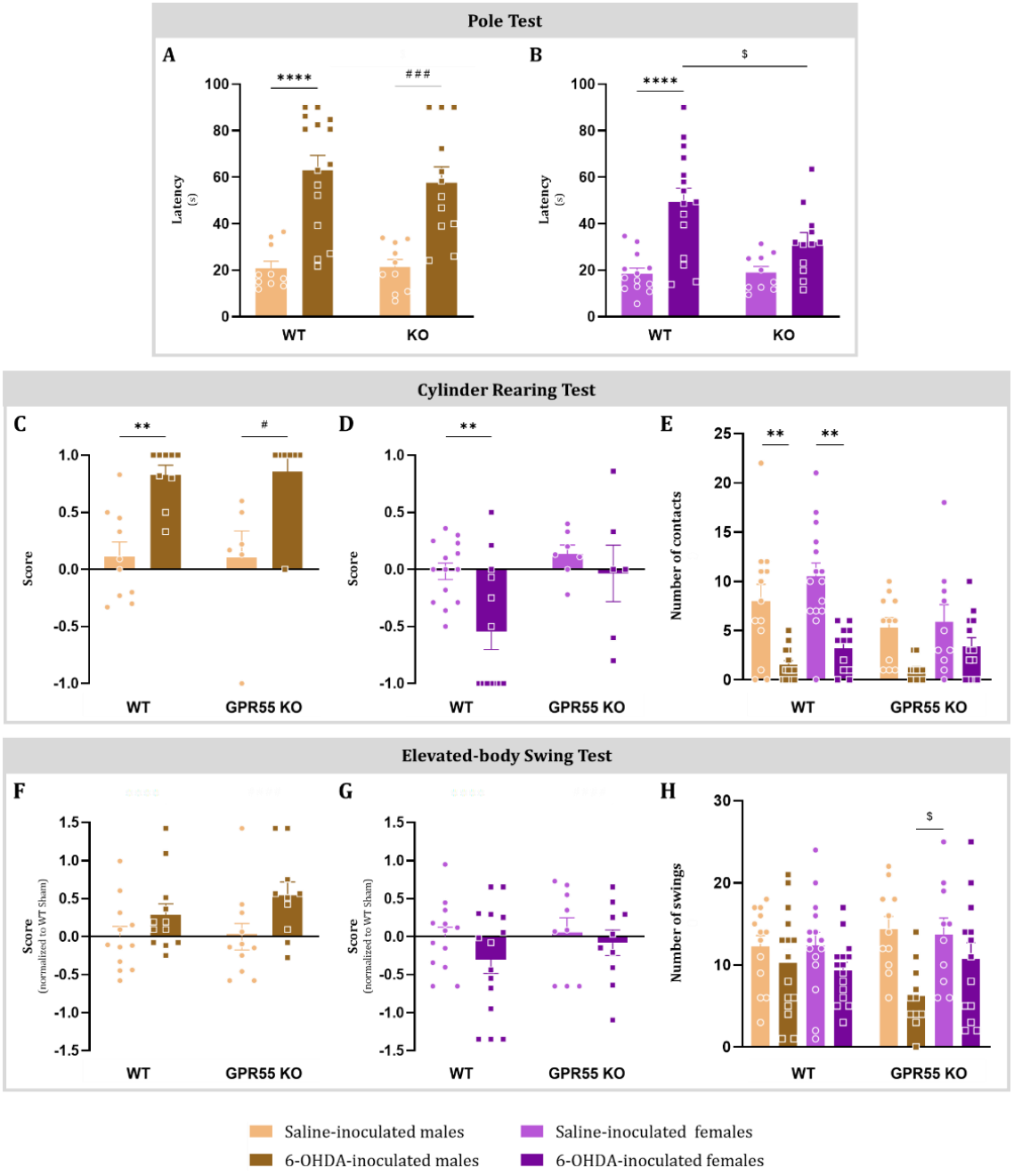


Figure 35. Effect of GPR55 depletion on motor performance of 6-OHDA-lesioned mice. *Pole test, CRT and EBST were employed to evaluate motor impairments induced by the 6-OHDA lesion in both sexes. Latency in the pole test, measured in (A) males and (B) females, was calculated as the mean of three trials. For the CRT, performed in (C) male and (D) female mice, the score was calculated by subtracting the number of contacts made with the ipsilateral paw from the number of contacts made with the contralateral paw, and dividing the result by (E) the total number of contacts. For the EBST, also conducted in (F) male and (G) female animals, the score was calculated similarly, subtracting the turns to the ipsilateral side from the turns to the contralateral side, and dividing the result by (H) the total number of turns. Values are expressed as mean ± SEM of 8-13 animals per group. Data were analysed using (A, B, C, D, F, G) two-way ANOVA or (E, H) three-way ANOVA followed by Tukey's multiple comparison test (** $p < 0.01$, **** $p < 0.0001$ vs. WT Sham-operated group; # $p < 0.05$, ### $p < 0.001$ vs. GPR55 KO Sham-operated group; $ $p < 0.05$ vs. WT 6-OHDA-lesioned group).*

immunoreactivity changes in the SN. No statistically significant differences were observed between groups, and neither upregulation nor downregulation of IL-1β or IFN-γ expression was detected in GPR55-deficient animals compared to their WT counterparts, in either sex. The only exception was a significant genotype effect observed for IL-1β in females ($p = 0.0328$), indicating a slight increase in expression levels in GPR55 KO animals (**Figure 38A**). However, this alteration did not reach biologically meaningful levels when compared with those observed in the AAV9-α-syn^{A53T} model.

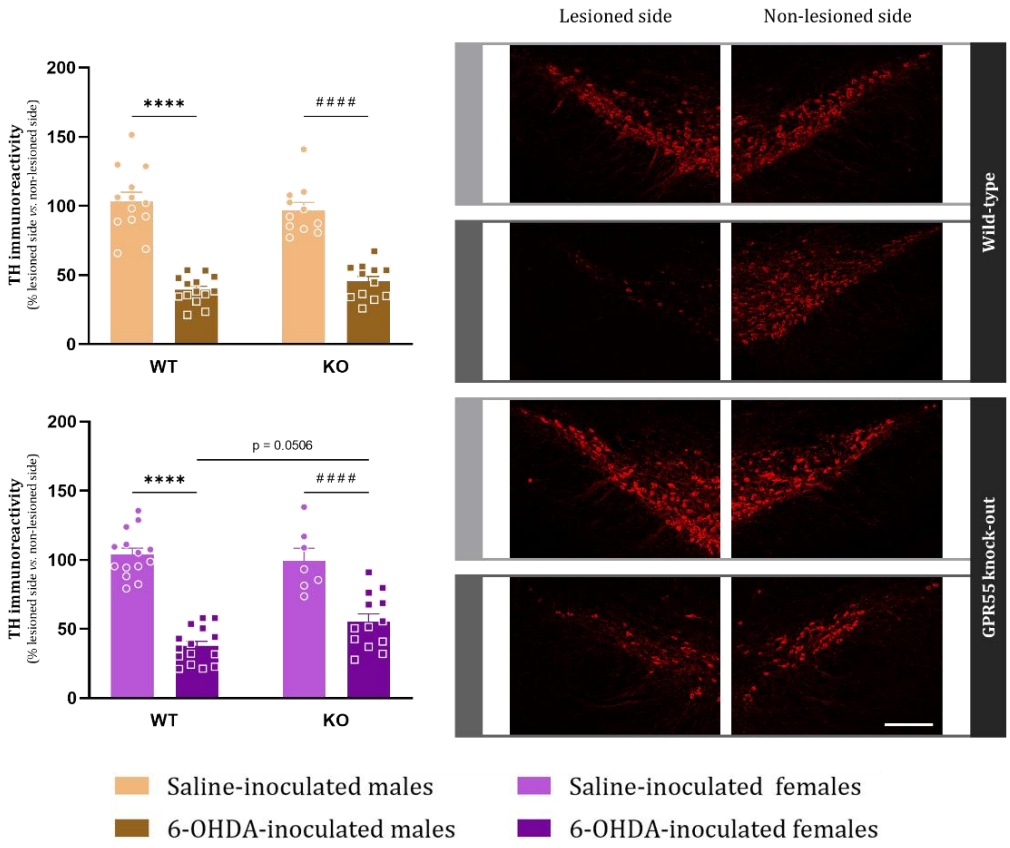


Figure 36. Effect of GPR55 depletion on neuronal integrity in the SNpc of 6-OHDA-lesioned mice. *Immunohistochemical analysis was performed to evaluate tyrosine hydroxylase (TH). Immunoreactivity values were quantified from 3-5 images per animal and calculated as the percentage of the lesioned side relative to the non-lesioned side. Final values are expressed as mean ± SEM of 7-15 animals per group. Data were analysed using two-way ANOVA followed by Tukey's multiple comparison test (**** p < 0.0001 vs. WT Sham-operated group; #### p < 0.0001 vs. KO Sham-operated group). Representative immunostaining images for each experimental group are shown as right panels and only for females (scale bar = 200 µm).*

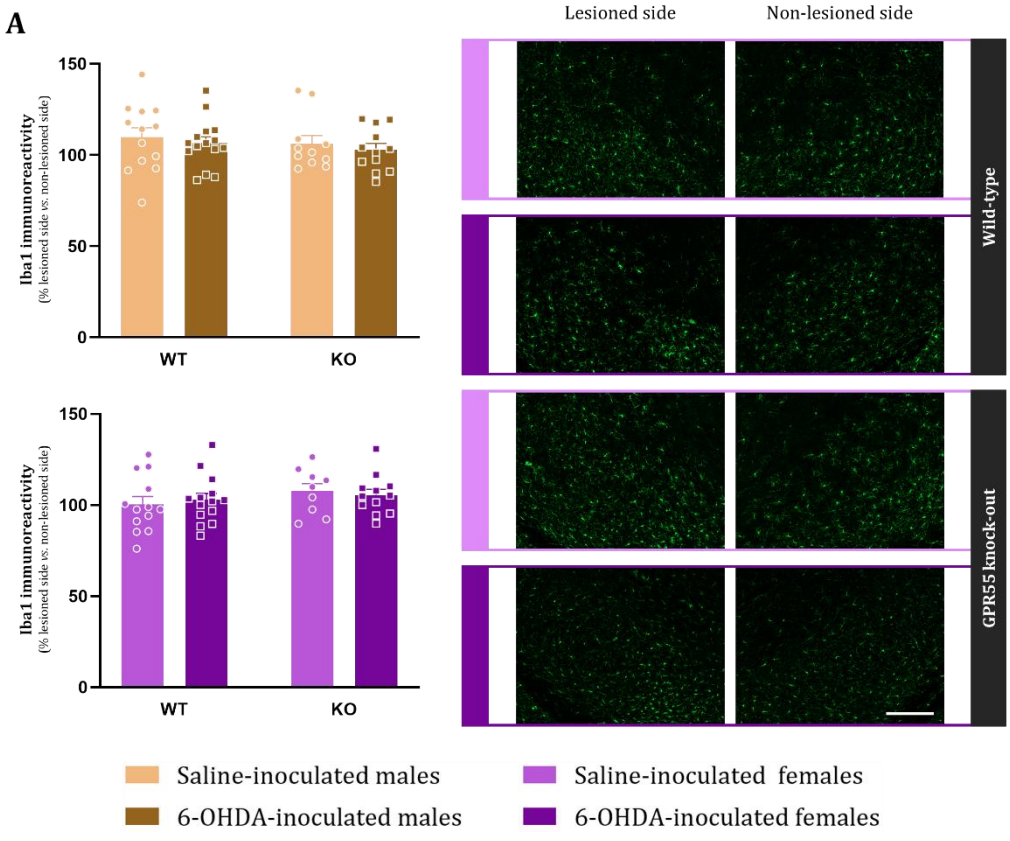


Figure 37. Effect of GPR55 depletion on glial reactivity in the SNpc of 6-OHDA-lesioned mice. *To be continued on the next page.*

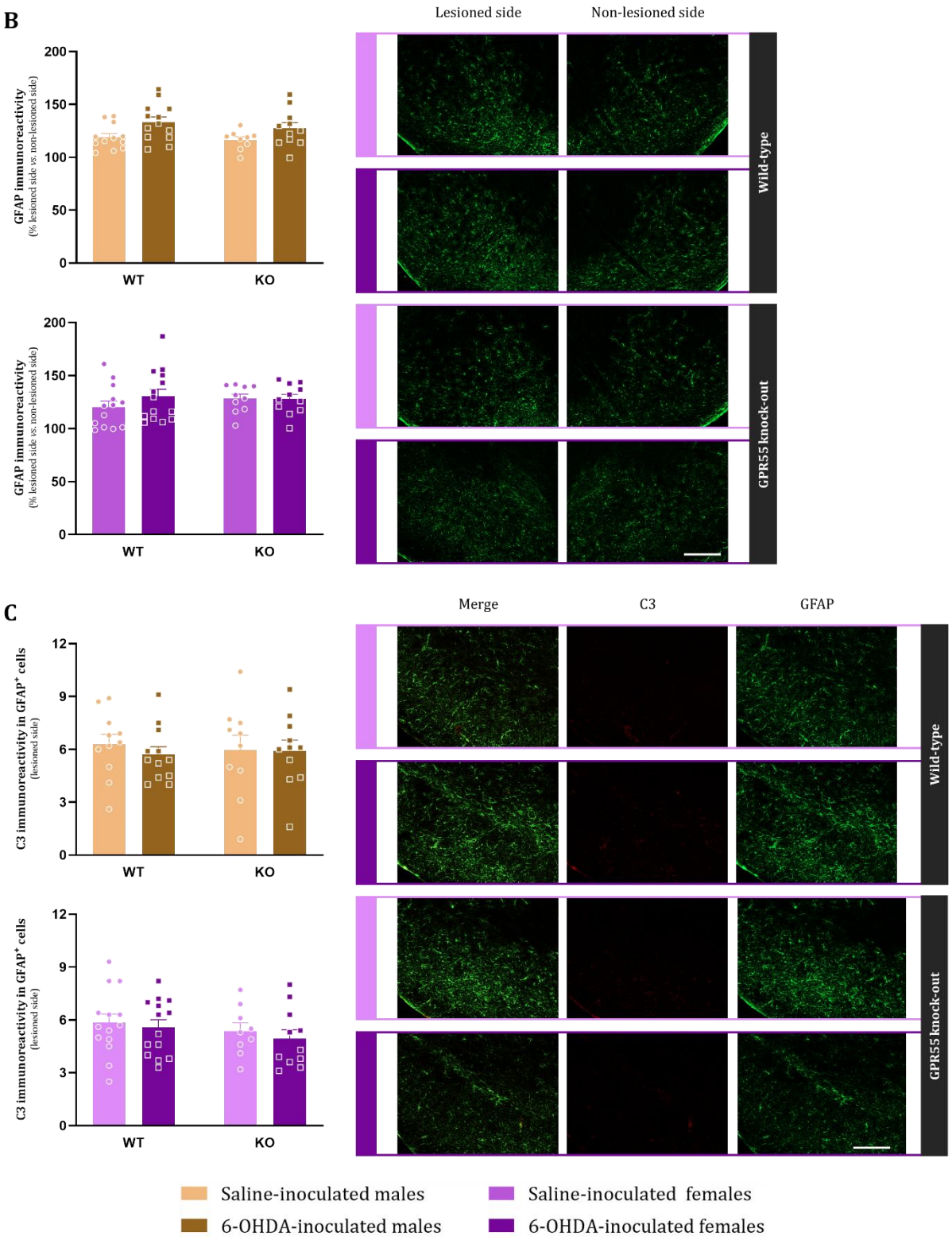


Figure 37. Effect of GPR55 depletion on glial reactivity in the SNpc of 6-OHDA-lesioned mice. *Immunohistochemical assays were performed to evaluate three markers: (A) ionised calcium-binding adapter molecule 1 (Iba1), (B) glial fibrillary acidic protein (GFAP), and (C) complement component 3 (C3). Immunoreactivity values were quantified from 3-5 images per animal and calculated as the percentage of the lesioned side relative to the non-lesioned side. Final values are expressed as mean ± SEM of 9-15 animals per group. Data were analysed using two-way ANOVA followed by Tukey's multiple comparison test. Representative immunostaining images for each experimental group are shown as right panels and only for females (scale bar = 200 μm).*

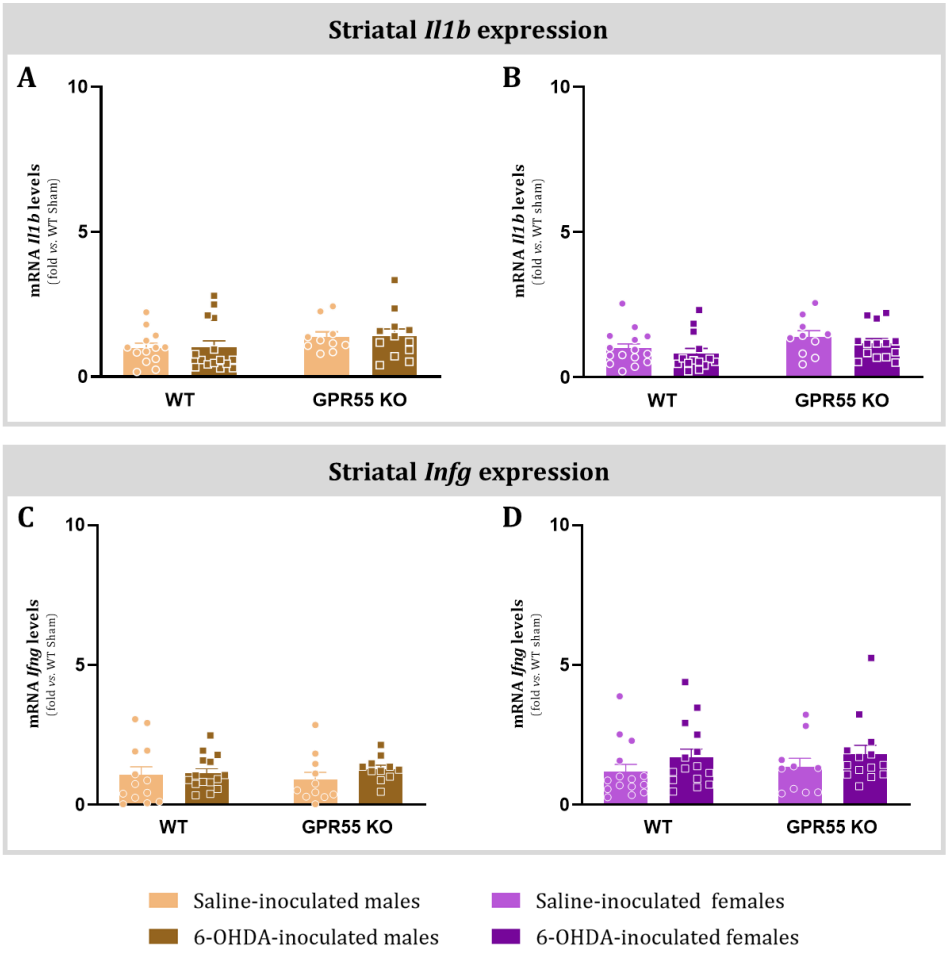


Figure 38. Effect of GPR55 depletion on gene expression profiles in the striatum of 6-OHDA-lesioned mice. *mRNA levels were evaluated by qPCR to assess the expression of two proinflammatory cytokines: interleukin 1β in (A) males and (B) females, and interferon γ in (C) males and (D) females. Gapdh was used as the housekeeping gene, and values were normalised to the mean of the WT sham-operated group. Final values are expressed as mean ± SEM for 10-15 animals per experimental group. Data were analysed using two-way ANOVA followed by Tukey's multiple comparison test.*

CHAPTER 3

(+)-*TRANS*-ENANTIOMER OF CANNABIDIOL AND DERIVATIVES AGAINST NEUROINFLAMMATION

As demonstrated in the two preceding chapters, this thesis project aims to explore novel strategies against PD involving components of the ECS, such as CB1R and GPR55. However, beyond cannabinoid receptors, cannabinoids themselves may offer new therapeutic avenues, particularly through chemical modifications of their structure. A wide range of phytocannabinoid derivatives has been identified in *Cannabis sativa*, such as CBDV, or synthetically developed to enhance their pharmacological properties. For instance, VCE-003.2, an aminoquinone derivative of CBG, has shown therapeutic potential in neurodegenerative diseases, including PD (Burgaz et al., 2019; 2021a; 2024; Rodríguez-Carreiro et al., 2023), amyotrophic lateral sclerosis (Rodríguez-Cueto et al., 2018), and HD (Aguareles et al., 2019). Among phytocannabinoids, CBD has attracted considerable interest due to its clinical potential. In line with this trend, various synthetic derivatives have been developed, including fluorinated CBD, which exhibits greater potency (Breuer et al., 2016); abnormal CBD, which is active at GPR18 (Matouk et al., 2018); and CBD hydroquinones, which act on PPARs (Del Río et al., 2016). Moreover, phytocannabinoids may also be modified in terms of stereoisomerism, opening the possibility that enantiomers of a given compound may differ in their mechanisms of action, for example, in their affinity for classical cannabinoid receptors.

CBD and Δ^9-THC possess two chiral centres, giving rise to four possible stereoisomeric conformations (see **Box 6 in INTRODUCTION**). In plants, these phytocannabinoids occur naturally in the (–)-*trans* enantiomeric form, whereas (+)-*trans* forms can be synthetically produced. Optical stereoisomerism has long been suggested to influence the interaction of Δ^9-THC and CBD with CB1R and CB2R (Mechoulam et al., 1987; Hanus et al., 2005; Rao et al., 2025), although more research to date has focused on Δ^9-THC and its derivatives. In general, (+)-*trans* enantiomers display lower affinity and potency at CB1R and CB2R compared to their (–)-*trans* counterparts (Little et al., 1988). Notably, the (+)-enantiomer of dimethylheptyl-Δ^9-THC, known as HU-211, lacks cannabinoid receptor activity but acts as a non-competitive NMDA receptor antagonist (Feigenbaum et al., 1989). Fewer studies have investigated the effects of CBD stereoisomerism on CB1R and CB2R interactions, likely due to the weak activity of the naturally occurring (–)-enantiomers at these receptors. Nevertheless, it has been reported that stereochemical modifications can confer cannabinoid receptor binding capacity to CBD derivatives (Rao et al., 2025), potentially unlocking novel pharmacodynamic properties and expanding their therapeutic applications.

In this final chapter, we aimed to compare the pharmacological profile of the (–)-*trans* and (+)-*trans* enantiomers of CBD, its natural analogue CBDV, and three synthetic derivatives, CBDA-Me,

CBDA-Gly, and CBDA-Hyp (see **Figure 15 in MATERIALS AND METHODS**), in order to identify potential advantages associated with stereoisomeric modification that may influence their pharmacological efficacy. Our objective was to evaluate potential differences between these enantiomeric pairs in terms of binding affinity and intrinsic activity at CB1R and CB2R, as well as *in silico* ADMET properties. Furthermore, we explored the anti-inflammatory and neuroprotective potential of the (+)-*trans* enantiomers both *in vitro*, using microglial and neuronal cell line models, and in *vivo*, through a murine model of striatal LPS inoculation. Particular attention was given to determining whether the observed effects were receptor-dependent, with a focus on key anti-inflammatory targets sensitive to cannabinoids, namely CB_2R and PPARγ

3.1. Assessment of affinity and intrinsic activity on cannabinoid receptors

Competition binding studies were carried out using commercial membranes transfected with either CB1R or CB2R, employing [^{3}H]-CP55940 as the radioligand, to compare the binding affinities of the (–)-*trans* and (+)-*trans* enantiomers of CBD, CBDV, and the three synthetic derivatives CBDA-Me, CBDA-Gly, and CBDA-Hyp. Compounds were tested across a concentration range of 10^{-4} to 10^{-11} M, enabling the calculation of K_i values as indicators of their affinities for CB1R and CB2R. Overall, the (+)-enantiomers exhibited lower K_i values, and thus higher binding affinities, than their (–)-counterparts at both receptors, with the difference being particularly pronounced at CB_2R (see **Table 14**).

Once affinity assessment was completed, those compounds showing K_i values in the low nanomolar range (<40 nM) were systematically investigated for their intrinsic activity (agonist, antagonist, or inverse agonist) through [^{35}S]-GTPγS binding assays. These assays primarily focused on CB2R, as the highest affinities were generally observed for this receptor (**Table 14**).

3.1.1. (+)-Cannabidiol and derivatives showed higher affinity for the cannabinoid type-1 receptor compared to their (–)-counterparts

When comparing CB1R affinities between enantiomers of the same compound, all tested molecules demonstrated higher affinities in their (+)-enantiomeric forms, as indicated by lower K_i values, relative to their respective (–)-counterparts. For the naturally occurring CBD and CBDV, the (–)-enantiomers exhibited only minimal affinity for CB1R, with K_i values within the micromolar range. However, inversion of stereochemistry resulted in a moderate reduction in K_i values, corresponding to approximately 10-fold (**Figure 39A**) and 40-fold (**Figure 39B**) increases in affinity, respectively. Similarly, (–)-CBDA-Gly displayed only weak affinity for CB_1R, whereas its (+)-enantiomer exhibited a one-order-of-magnitude improvement in binding (**Figure 39C**). Among the compounds tested, the most pronounced enantioselective difference was observed for CBDA-Hyp. While (–)-CBDA-Hyp showed moderate affinity for CB1R, with a K_i value in the high-nanomolar range, its (+)-enantiomer exhibited an approximately 300-fold lower K_i, reaching the low-nanomolar range (**Figure 39D**), thereby representing the highest CB1R affinity among all

Table 14. *Summary of the Ki values for the (-) and (+)-enantiomeric series of the five compounds tested at both CB1R and CB2R, showing the increase in affinity between each pair of stereoisomers and the selectivity of each compound for CB2R. Fold increases were calculated as $Ki_{(-)\text{-compound}}/Ki_{(+)\text{-compound}}$ for the same receptor, while CB2R selectivity was determined as Ki_{CB1R}/Ki_{CB2R} ratio for each compound.*

Compound	Cannabinoid receptor type 1		Cannabinoid receptor type 2		
	Ki (nM)	Fold increase in affinity of the (+)-enantiomer over the (-)-enantiomer	Ki (nM)	Fold increase in affinity of the (+)-enantiomer over the (-)-enantiomer	CB2R Selectivity
(-)-CBD	8854 ± 449	9.6	3883 ± 992	107.3	2.3
(+)-CBD	926 ± 275		36.2 ± 7.1		25.6
(-)-CBDV	12450 ± 989	42.3	3561 ± 398	107.6	3.5
(+)-CBDV	294 ± 46.9		33.1 ± 4.1		8.9
(-)-CBDA-Me	379 ± 197.7	1.1	46.6 ± 12.9	1.7	8.1
(+)-CBDA-Me	345 ± 89.8		28.0 ± 1.9		12.3
(-)-CBDA-Gly	3923 ± 1547	10.9	374 ± 47.7	29.0	10.5
(+)-CBDA-Gly	359 ± 67.1		12.9 ± 2.7		27.8
(-)-CBDA-Hyp	604 ± 50.5	335.6	55.4 ± 10.9	79.1	10.9
(+)-CBDA-Hyp	1.8 ± 0.8		0.7 ± 0.1		2.6

compounds analysed. In contrast, CBDA-Me displayed the most consistent binding profile, with both enantiomers presenting nearly identical K_i values within the mid-nanomolar range (**Figure 39E**). In summary, with the exception of CBDA-Me, all compounds tested exhibited enhanced CB1R affinity in their (+)-*trans* configuration relative to the (–)-*trans* form.

3.1.2. (+)-Cannabidiol and derivatives showed higher affinity and selectivity for the cannabinoid type-2 receptor compared to their (–)-counterparts

Affinity increases observed for (+)-enantiomers relative to their (–)-counterparts were also evident at CB2R, with even greater differences detected at this receptor than at CB1R. Furthermore, all tested compounds, irrespective of their enantiomeric form, exhibited selectivity for CB2R, as determined by the CB1R-K_i/CB2R-K_i ratio. This CB2R preference was generally enhanced in the (+)-enantiomers. For CBD and CBDV, the (+)-enantiomers displayed the most pronounced improvements in CB2R affinity, exhibiting approximately 100-fold higher affinities than their (–)-enantiomeric forms (**Figures 40A** and **40B**). This shift resulted in a transition from weak, low-micromolar affinities to K_i values within the mid-nanomolar range. Additionally, both (+)-CBD and (+)-CBDV demonstrated markedly greater CB2R selectivity, with their selectivity ratios increasing from 2.3 to 25.6 and from 3.5 to 8.9, respectively (**Table 14**). Notably, the highest CB2R selectivity among all compounds tested was observed for (+)-CBDA-Gly, whose ratio rose from 10.5 in its (–)-form to 27.8 in the (+)-enantiomer (**Table 14**), despite a more modest 30-fold increase in CB2R affinity, reaching the nanomolar range (**Figure 40C**). In the case of CBDA-Hyp, the (+)-enantiomer exhibited an approximately 80-fold increase in CB2R affinity relative to its

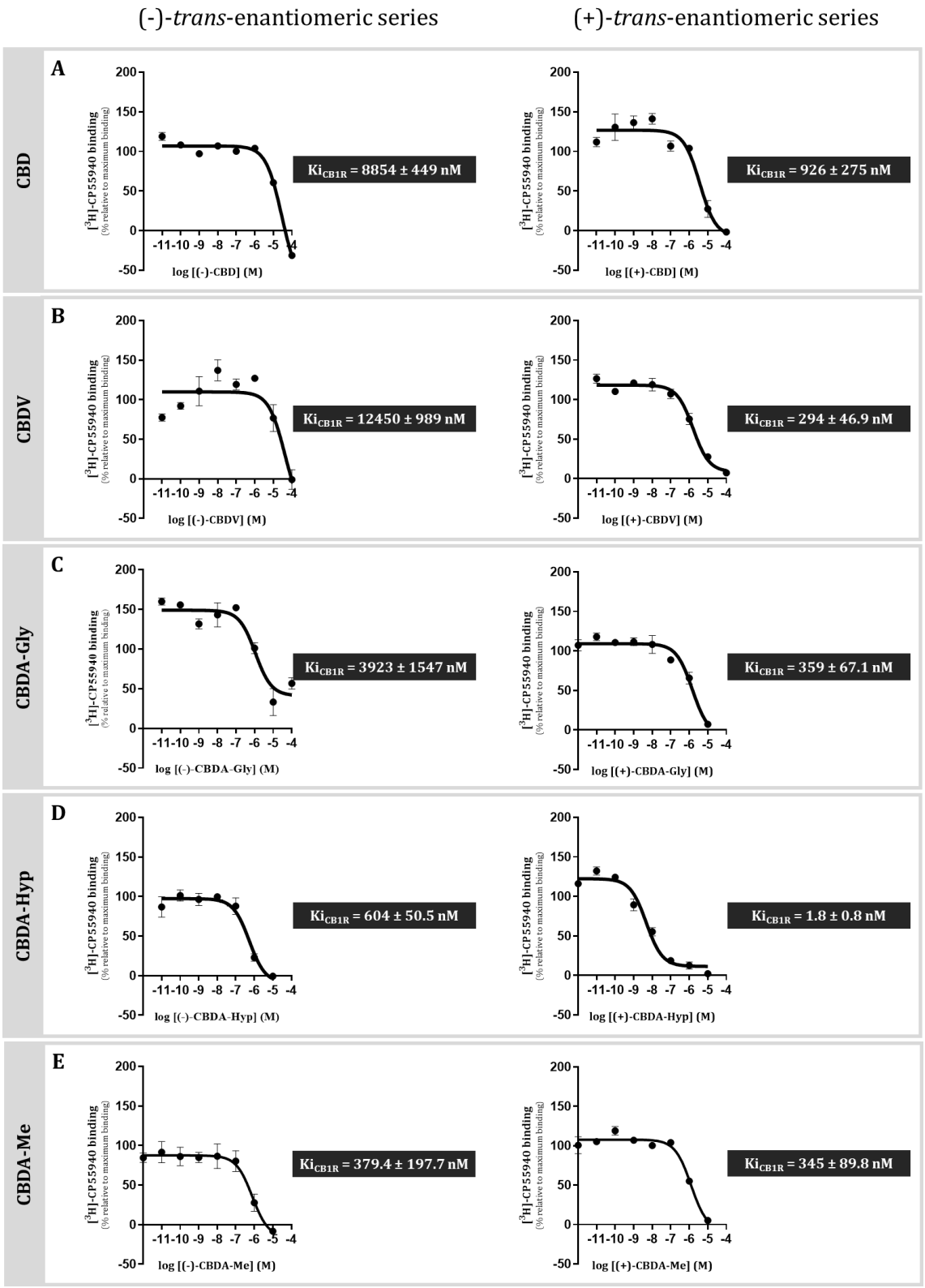


***Figure 39. Representative competition binding curves and K_i values for CB1R of the (–)-trans and (+)-trans enantiomeric series of the tested compounds (A-E)**. Ki values were derived from three independent experiments performed in triplicate. Data are expressed as mean ± SEM.*

(–)-form, yielding the lowest K_i value observed in the entire compound panel and approaching the picomolar range (**Figure 40D**). However, despite this substantial enhancement in affinity, (+)-CBDA-Hyp was the only compound for which CB2R selectivity decreased in the (+)-form, from a ratio of 10.9 to 2.6 (**Table 14**). Finally, CBDA-Me again displayed the most consistent binding behaviour between enantiomers, with both forms showing similar nanomolar affinities and CB1R-

K_i/CB2R-K_i ratios of approximately 10 (**Figure 40E**). In summary, the CB2R binding data closely paralleled the results observed for CB_1R, with all compounds, except CBDA-Me, demonstrating enhanced affinity in the (+)-*trans* configuration. Moreover, the stereoisomeric change increased CB2R selectivity in CBD, CBDV, and CBDA-Gly.

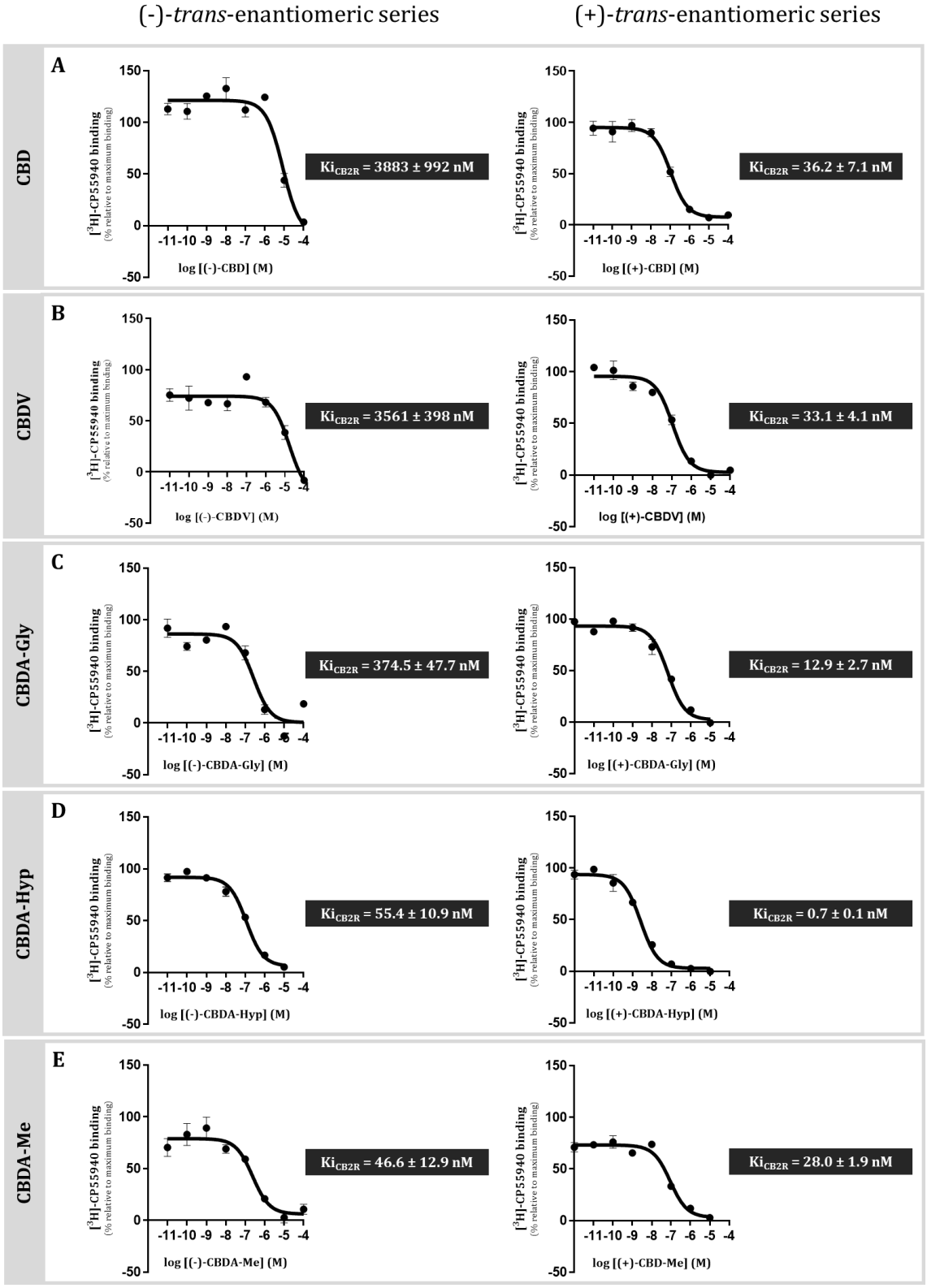
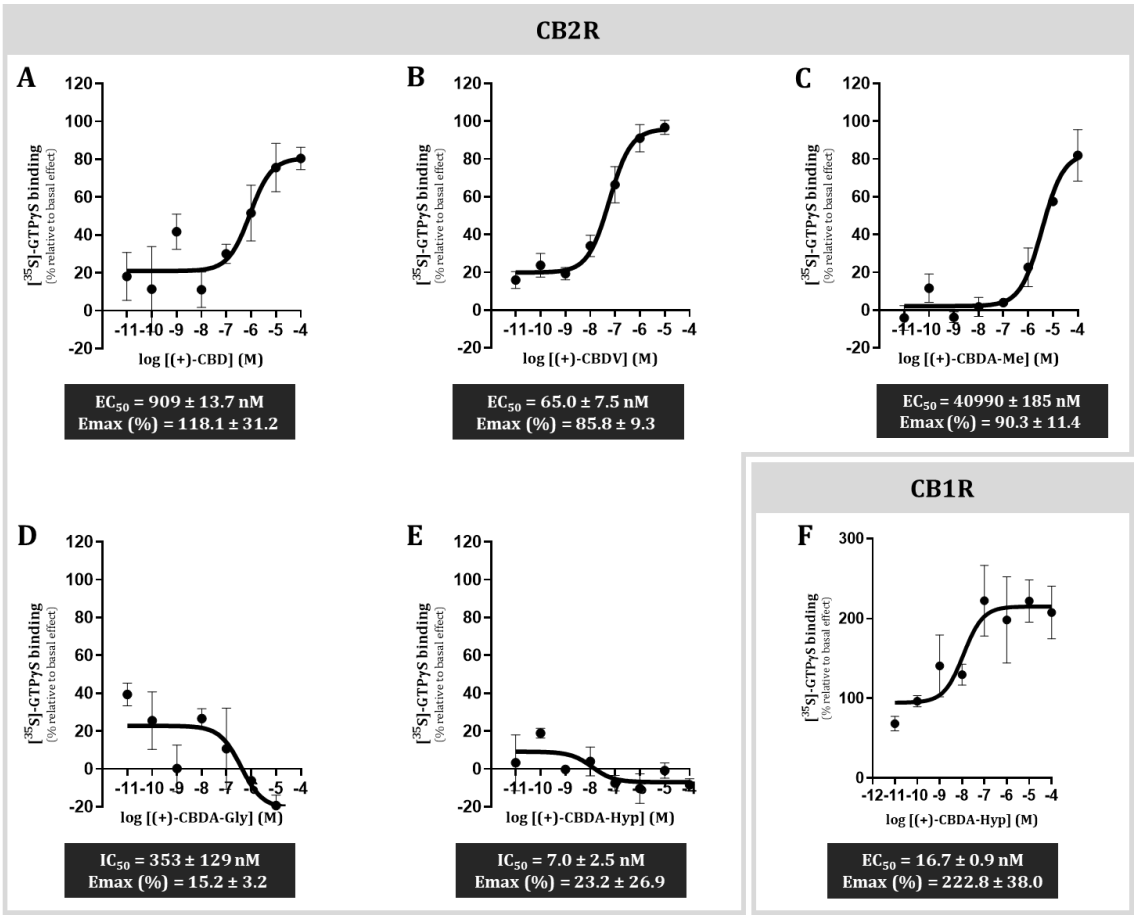


Figure 40. Representative competition binding curves and K_i values for CB2R of the (–)-trans and (+)-trans enantiomeric series of the tested compounds (A-E). *Ki values were derived from three independent experiments performed in triplicate. Data are expressed as mean ± SEM.*

3.1.3. (+)-Cannabidiol, (+)-cannabidivarin, and (+)-cannabidiol-methyl ester acted as agonists at the cannabinoid type-2 receptor, whereas the other two (+)-enantiomers showed inverse agonist activity

[^{35}S]-GTPγS binding assays were performed to evaluate the intrinsic activity at CB2R of (+)-CBD, (+)-CBDV, (+)-CBDA-Me, (+)-CBDA-Gly, and (+)-CBDA-Hyp, i.e., those compounds exhibiting the lowest K_i values in the preceding affinity assays. The results revealed that (+)-CBD (**Figure 41A**), (+)-CBDV (**Figure 41B**), and (+)-CBDA-Me (**Figure 41C**) acted as CB2R agonists. Notably, (+)-CBDV displayed the highest potency (EC_{50} < 100 nM), followed by (+)-CBD (EC_{50} < 1 μM) and (+)-CBDA-Me (EC_{50} < 50 μM). In contrast, (+)-CBDA-Gly and (+)-CBDA-Hyp functioned as inverse agonists at this receptor (**Figures 41D** and **41E**, respectively), with IC_{50} values below 400 nM and 10 nM, respectively. Additionally, the intrinsic activity of (+)-CBDA-Hyp was also assessed at CB1R, due to its high affinity for this receptor, and was found to exhibit agonistic behaviour (**Figure 40F**), with notable potency (EC_{50} < 20 nM).



3.2. *In silico* analysis indicated that (+)-cannabidiol and (+)-cannabidivarin exhibited the most favorable pharmacokinetic properties

In addition to pharmacodynamic profiling, pharmacokinetic parameters were also assessed, as both may mutually influence one another. This analysis aimed to elucidate why certain (+)-CBD

derivatives exhibit CB1R binding in vitro yet fail to elicit the classical cannabinoid tetrad in vivo (Fride et al., 2004). To this end, we evaluated the ADMET properties of the (+)-enantiomers using in silico predictive models, with (−)-CBD serving as the reference compound. The data revealed favourable predicted ADMET profiles for all tested molecules, particularly for (+)-CBD and (+)-CBDV (**Table 15**), with most parameters falling within acceptable ranges. Several parameters were of particular relevance. QPlogS and CIQPlogS, predictors of aqueous solubility, yielded better values for (+)-CBD and (+)-CBDV relative to the other three (+)-CBD derivatives. Another key parameter was potential cardiotoxicity, assessed via QPlogHERG, which raised safety concerns for (+)-CBDA-Gly and, notably, (+)-CBDA-Hyp. With respect to bioavailability, indicators of tissue barrier permeability, such as QPPCaco, QPlogBB and QPPMDCK, as well as predicted human oral absorption (HumanOralAbsorption), supported superior absorption and permeability profiles for (+)-CBD and (+)-CBDV. By contrast, the remaining derivatives, particularly (+)-CBDA-Gly, demonstrated poorer predicted performance in these domains (**Table 15**). It is important to emphasise that these conclusions are based solely on computational predictions and require further validation through *in vitro* and *in vivo* experimentation.

***Table 15**. Data generated by the in-silico analysis of (+)-CBD, (+)-CBDV, (+)-CBDA-Me, (+)-CBDA-Gly, and (+)-CBDA-Hyp, with (-)-CBD as the reference molecule, using QikProp descriptors.*

Property or Descriptor	(-)-CBD	(+)-CBD	(+)-CBDV	(+)-CBDA-Me	(+)-CBDA-Gly	(+)-CBDA-Hyp
#stars	1	1	1	1	1	2
#amine	0	0	0	0	0	0
#amidine	0	0	0	0	0	0
#acid	0	0	0	0	0	0
#amide	0	0	0	0	0	0
#rotor	7	7	5	8	11	13
#rtvFG	0	0	0	1	1	1
CNS	0	0	0	-1	-2	-2
mol_MW	314.467	314.467	286.413	372.503	402.530	444.610
dipole	3.103	2.612	3.140	1.054	3.854	6.001
SABA	631.291	640.278	576.023	705.516	748.368	831.670
FOSA	472.196	486.737	428,953	566.455	536.088	656.364
FISA	64.286	61.749	64.295	78.006	142.868	108.109
PISA	94.809	91.793	82.775	61.055	69.411	67.197
WPSA	0	0	0	0	0	0
volume	1149.979	1158.401	1042.094	1296.946	1385.659	1547.154
donorHB	2	2	2	1	2	2
accptHB	1.5	1.5	1.5	2.5	4.2	4.2
dip^2/V	0.0083735	0.0058899	0.0094584	0.0008558	0.0107165	0.0232750
ACxDN^.5/SA	0.0033603	0.0033131	0.0036827	0.0035435	0.0079369	0.0071419
glob	0.8408699	0.8331098	0.8629715	0.8152168	0.8031946	0.7778632
QPpolrz	36.279	36.587	33.131	41.196	42.900	48.058
QPlogPC16	10.481	10.514	9.435	11.483	13.073	14.420

***Table 15**. Data generated by the in-silico analysis of (+)-CBD, (+)-CBDV, (+)-CBDA-Me, (+)-CBDA-Gly, and (+)-CBDA-Hyp, with (-)-CBD as the reference molecule, using QikProp descriptors. (Continued)*

Property or Descriptor	(-)-CBD	(+)-CBD	(+)-CBDV	(+)-CBDA-Me	(+)-CBDA-Gly	(+)-CBDA-Hyp
QPlogPoct	15.108	15.138	14.110	15.741	19.058	20.728
QPlogPw	5.183	5.167	5.410	4.525	7.604	7.112
QPlogPo/w	5.282	5.349	4.579	5.928	5.135	6.391
QPlogS	-5.788	-5.953	-5.098	-6.928	-6.399	-7.573
CIQPlogS	-5.269	-5.269	-4.689	-6.243	-6.083	-6.943
QPlogHERG	-4.559	-4.670	-4.144	-4.813	-5.048	-5.447
QPPCaco	2433.758	2572.398	2433.276	1803.729	437.603	934.767
QPlogBB	-0.465	-0.451	-0.313	-0.701	-1.576	-1.411
QPPMDCK	1293.809	1373.653	1293.532	935.925	202.488	459.922
QPlogKp	-1.698	-1.662	-1.933	-1.974	-2.852	-2.027
IP(eV)	9.028	9.026	9.035	9.143	9.199	9.066
EA(eV)	-0.250	-0.276	-0.245	0.332	0.205	0.233
#metab	8	8	8	8	9	9
QPlogKhsa	1.034	1.056	0.820	1.343	1.025	1.374
HumanOral-Absorption	3	3	3	1	1	1
PercentHuman-OralAbsorption	100	100	100	100	91.321	100
SAFluorine	0	0	0	0	0	0
SAamideO	0	0	0	0	0	0
PSA	39.063	37.661	39.093	63.839	90.166	85.370
#NandO	2	2	2	4	5	5
RuleOfFive	1	1	0	1	1	1
#ringatoms	12	12	12	12	12	12
#in34	0	0	0	0	0	0
#in56	12	12	12	12	12	12
#noncon	4	4	4	4	4	4
#nonHatm	23	23	21	27	29	32
RuleOfThree	2	2	1	2	2	2
Jm	0.01	0.008	0.027	0	0	0

3.3. *In vitro* analysis of anti-inflammatory and neuroprotective effects

Since three of the (+)-enantiomers displayed acquired or enhanced affinity and agonistic activity towards CB2R, we aimed to assess the capacity of (+)-CBD, (+)-CBDV, and (+)-CBDA-Me to elicit anti-inflammatory and neuroprotective effects via activation of this cannabinoid receptor, using appropriate cell-based models. CB2R agonism has been implicated in the attenuation of inflammation and the promotion of neuroprotection (Gómez-Cañas et al., 2016).

3.3.1. (+)-Cannabidiol, (+)-cannabidivarin, and to a lesser extent (+)-cannabidiol methyl ester, reduced the expression of some proinflammatory factors

Anti-inflammatory effects were evaluated in cultured microglial-like iMG cells stimulated with LPS by assessing the expression of four key pro-inflammatory markers: IL-1β, TNF-α, COX-2, and iNOS. As expected, LPS stimulation led to a significant increase in the expression levels of all four markers (**Figure 42**). Cells were preincubated for 1 h with three different concentrations (0.01 μM, 0.1 μM, and 1 μM) of (+)-CBD, (+)-CBDV, and (+)-CBDA-Me prior to LPS treatment. Administration of (+)-CBD and (+)-CBDV, and to a lesser extent (+)-CBDA-Me, attenuated the LPS-induced upregulation of pro-inflammatory cytokines, reaching statistical significance at the highest concentration (1 μM; **Figures 42A** and **42B**), with the strongest effect observed for IL-1β. No significant reductions were detected in the expression of COX-2 or iNOS, although a trend towards decreased expression was noted, particularly at 1 μM, with the most evident reduction observed in iNOS levels (**Figures 42C** and **42D**). At 0.1 μM, reductions did not reach statistical significance but showed a tendency, especially for IL-1β (**Figure 42A**) and iNOS (**Figure 42D**).

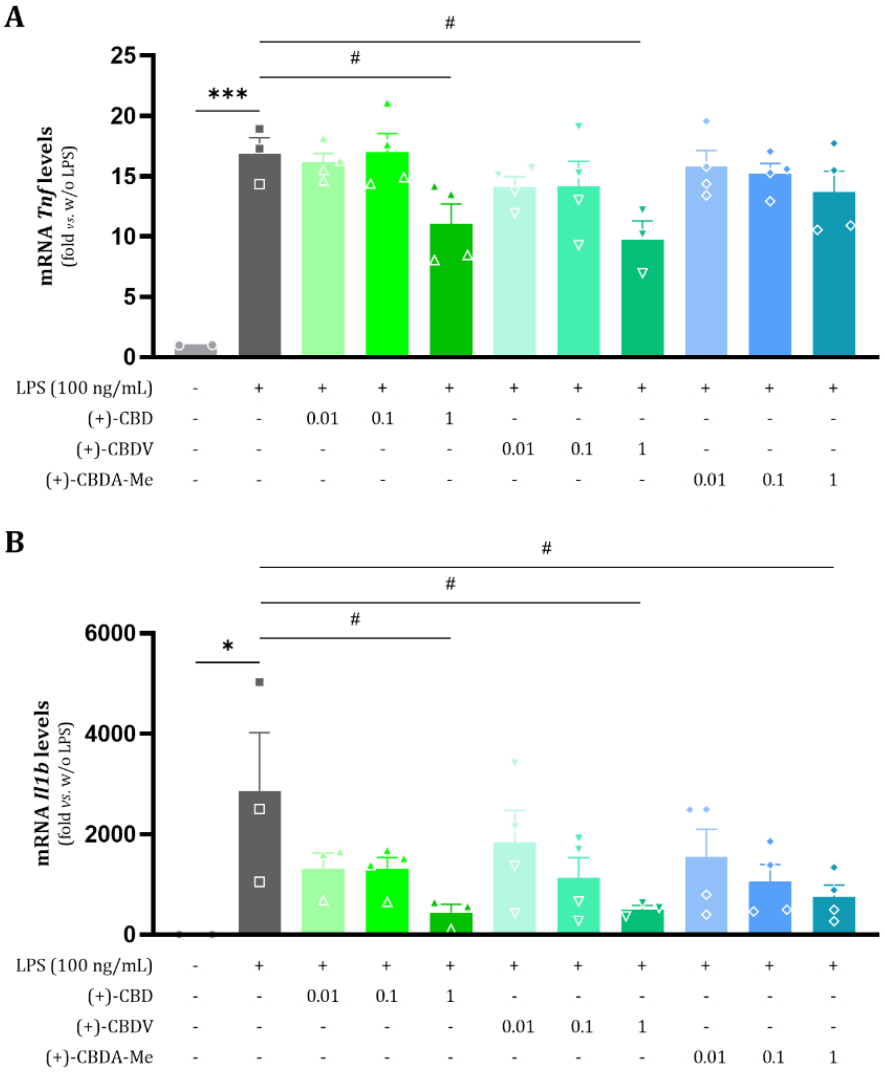


Figure 42. In vitro anti-inflammatory effects of (+)-CBD, (+)-CBDV, and (+)-CBDA-Me. *To be continued on the next page.*

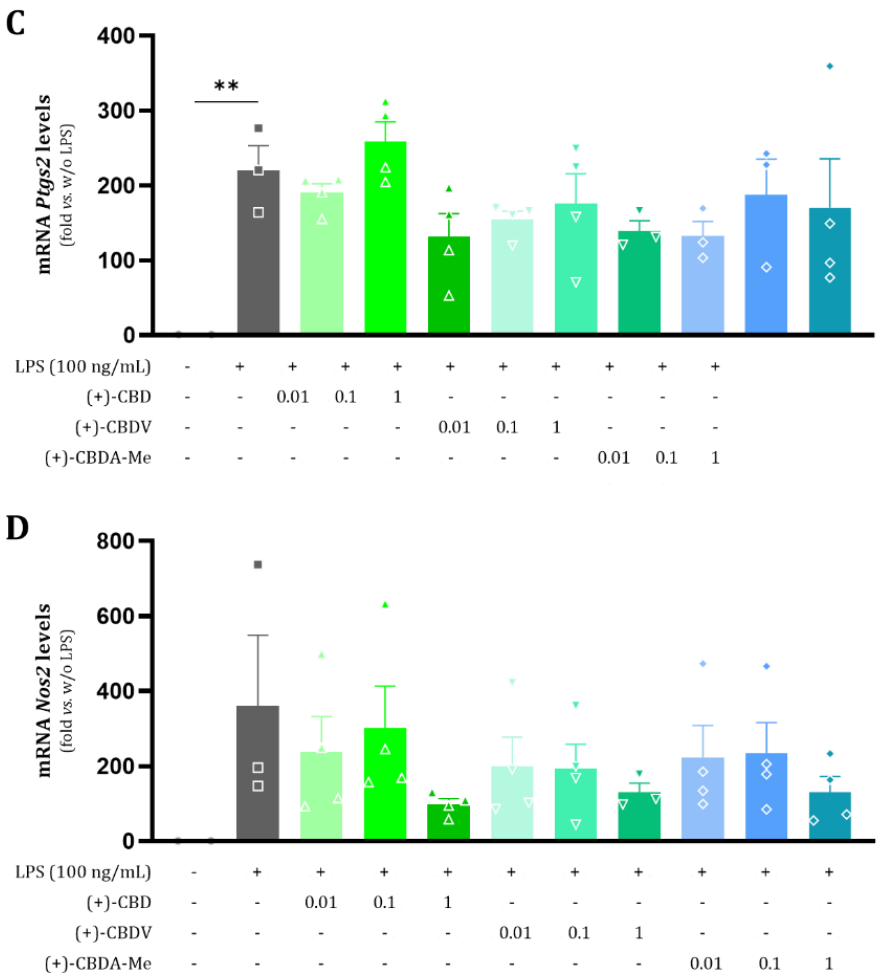


Figure 42. In vitro anti-inflammatory effects of (+)-CBD, (+)-CBDV, and (+)-CBDA-Me. *mRNA levels of the proinflammatory cytokines (A) TNF-α and (B) IL-1β, as well as the enzymes (C) COX-2 and (D) iNOS, were quantified by qPCR in iMG cells stimulated with LPS and treated with three different concentrations (0.01, 0.1, and 1 μM). Gapdh was used as the housekeeping gene, and values were normalized to the mean of the unstimulated group. Final values are expressed as mean ± SEM of four independent experiments, each performed in triplicate. Statistical analysis was conducted using one-way ANOVA followed by Dunnett's multiple comparison test (* p < 0.05, ** p < 0.01, *** p < 0.001 vs. unstimulated group; # p < 0.05 vs. LPS-treated group).*

3.3.2. (+)-Cannabidivarin, (+)-cannabidiol methyl ester, and, to a lesser extent, (+)-cannabidiol produced moderate effects on neuronal metabolism that depended on the cannabinoid type-2 receptor

After confirming the anti-inflammatory action of the (+)-enantiomers and identifying 1 μM as the most active concentration, the next objective was to determine whether the observed reduction in inflammatory marker expression could translate into neuroprotective effects. To this end, conditioned media were collected from iMG cell cultures treated with vehicle, LPS, and/or (+)-CBD, (+)-CBDV, or (+)-CBDA-Me, and subsequently applied to neuronal-like M213-2O cells to evaluate their impact on metabolic activity and, indirectly, cell viability. Our results confirmed that conditioned media from LPS-stimulated iMG cells reduced M213-2O metabolic activity, as measured by the MTT assay, by approximately 40% (**Figure 43**). Treatment with (+)-CBD, (+)-CBDV, and (+)-CBDA-Me partially reversed this reduction, leading to a loss of statistical significance when compared to the LPS group, particularly in the cases of (+)-CBDV and (+)-CBDA-Me (**Figure 43**). This partial neuroprotection exerted by the (+)-enantiomers appears to be mediated through CB2R, as co-incubation with the CB2R antagonist SR144528 abolished the

protective effects in all three cases. Notably, inhibition of PPARγ with T0070907 had no impact on the neuroprotective effects of (+)-CBDV and (+)-CBDA-Me, but significantly enhanced the effect of (+)-CBD (**Figure 43**).

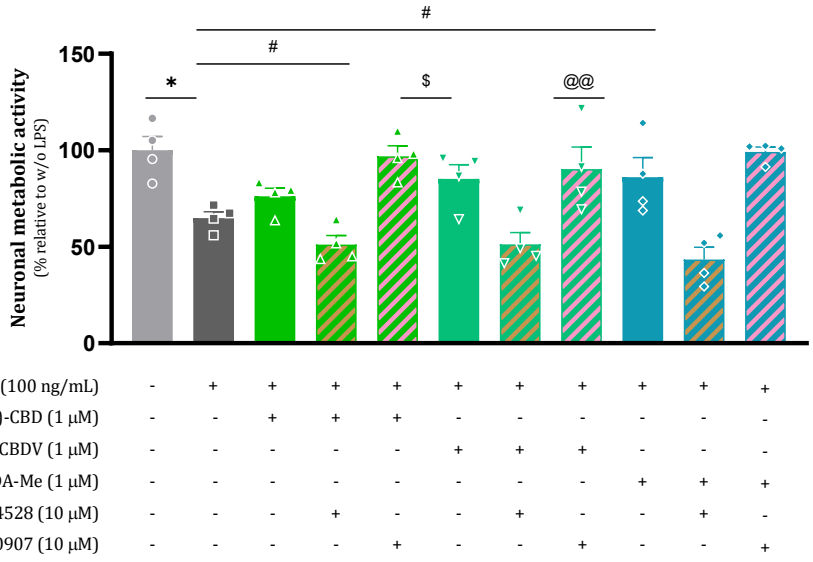


Figure 43. In vitro neuroprotective effects of (+)-CBD, (+)-CBDV and (+)-CBDA-Me, and involvement of CB2R and PPARγ. *Cell viability was evaluated using the MTT assay in M213-2O cells exposed to conditioned media derived from iMG cultures previously treated with one of the three compounds, with or without the CB2R antagonist SR144528 and/or the PPARγ inhibitor T0070907. Values were normalised to the mean of the group exposed to conditioned media from unstimulated iMG cells. Final values are expressed as mean ± SEM of four independent experiments, each performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test (* p < 0.05 vs. unstimulated conditioned microglial media-exposed group; # p < 0.05 vs. stimulated conditioned microglial media-exposed group; $ p < 0.05 vs. (+)-CBDV-treated conditioned microglial media-exposed group; @@ p < 0.01 vs. (+)-CBDA-Me-treated conditioned microglial media-exposed group).*

3.4. *In vivo* analysis of anti-inflammatory and neuroprotective effects

As a final step in this preclinical evaluation, we sought to investigate whether (+)-CBD and (+)-CBDV, identified as the most promising candidates based on their favourable pharmacological and pharmacokinetic profiles, could reduce glial reactivity and protect dopaminergic neurons in the SN *in vivo*. For this purpose, we used a well-established model of neuroinflammation based on the unilateral intrastriatal injection of LPS, which has previously been employed to study the anti-inflammatory and neuroprotective effects of CB2R agonists (García et al., 2011; Gómez-Gálvez et al., 2016).

3.4.1. (+)-Cannabidiol and (+)-cannabidivarin failed to significantly attenuate GFAP and CD68 immunoreactivity in the substantia nigra of lesioned mice

The stereotaxic administration of LPS into the striatum induced a marked increase in the expression of the microglial/infiltrating macrophage marker CD68 within the SN (**Figure 44A**), while the astroglial marker GFAP exhibited a more moderate rise in immunoreactivity (**Figure 44B**). The *i.p.* administration of either (+)-CBD or (+)-CBDV at a dose of 3 mg/kg failed to significantly attenuate the LPS-induced upregulation of these glial markers. Nevertheless, (+)-CBD demonstrated a slightly more favourable profile, with a modest trend towards reduced immunoreactivity for both CD68 and GFAP, whereas no clear effect was observed with (+)-CBDV (**Figures 44A** and **44B**).

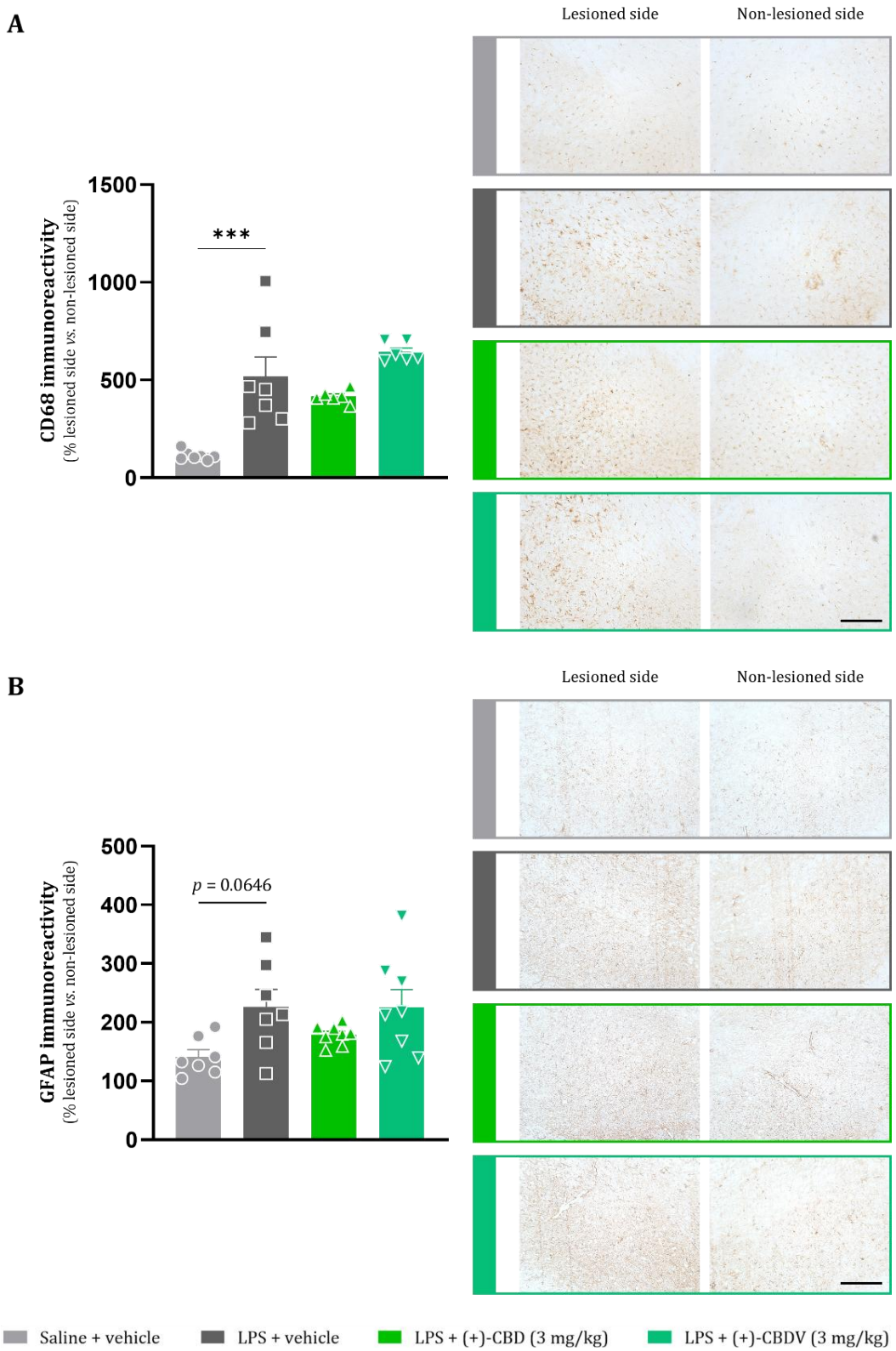


Figure 44. Effect of (+)-CBD or (+)-CBDV treatment on glial reactivity in the SNpc of LPS-lesioned mice*. Immunohistochemical analyses were conducted to assess the expression of (A) cluster of differentiation 68 (CD68) and (B) glial fibrillary acidic protein (GFAP). Immunoreactivity was quantified from 3-5 images per animal and expressed as the percentage of the lesioned side relative to the non-lesioned side. Final values are presented as mean ± SEM of 6-8 animals per group. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (*** p < 0.001 vs. Sham-operated vehicle group). Representative immunostaining images for each experimental group are shown to the right of the corresponding graph (scale bar = 200 µm).*

3.4.2. (+)-Cannabidiol, but not (+)-cannabidivarin, protected dopaminergic neurons in the substantia nigra against striatal inflammatory insult

The stereotaxic injection of LPS into the striatum also impacted dopaminergic neurons in the SN, as reflected by a reduction in TH immunoreactivity. Nevertheless, the extent of neuronal loss was less pronounced than that typically observed in neurotoxin-based models (**Figure 45**). Regarding the ability of the two compounds to confer neuroprotection against the striatal inflammatory insult, (+)-CBDV did not produce a statistically significant preservation of TH immunostaining compared to the vehicle-treated group (**Figure 45**). Conversely, (+)-CBD elicited a modest yet statistically significant increase in TH immunoreactivity, indicating a more discernible neuroprotective effect of this (+)-enantiomer.

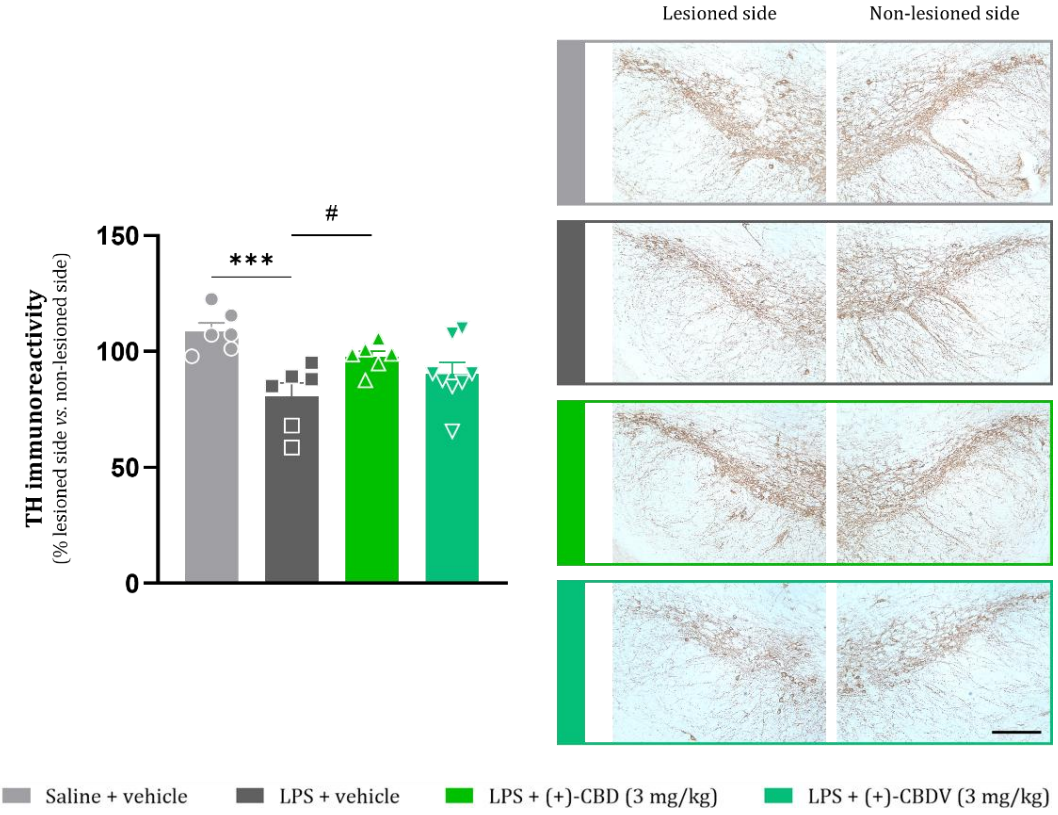


Figure 45. Effect of (+)-CBD and (+)-CBDV treatment on dopaminergic neuronal integrity in the SNpc of LPS-lesioned mice. *Immunohistochemical analyses were performed to evaluate the dopaminergic neuronal marker tyrosine hydroxylase (TH). Immunoreactivity was quantified from 3-5 images per animal and expressed as the percentage of the lesioned side relative to the non-lesioned side. Final values are presented as mean ± SEM of 6-8 animals per group. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (*** p < 0.001 vs. Sham-operated vehicle group; # p < 0.05 vs. LPS-lesioned vehicle group). Representative immunostaining images for each experimental group are shown to the right of the corresponding graph (scale bar = 200 μm).*

This effect aligns with the previously observed, slightly greater *in vivo* anti-inflammatory activity of (+)-CBD compared to (+)-CBDV (**Figures 44A** and **44B**). More importantly, it corresponds with the more pronounced improvement in motor deficits observed in the CRT following (+)-CBD treatment, in contrast to the limited effect elicited by (+)-CBDV (**Figure 46A**). Notably, no significant differences were detected between groups in the number of paw contacts during the behavioural test (**Figure 46B**), indicating that treatment with the (+)-enantiomers did not affect the general motility of the animals.

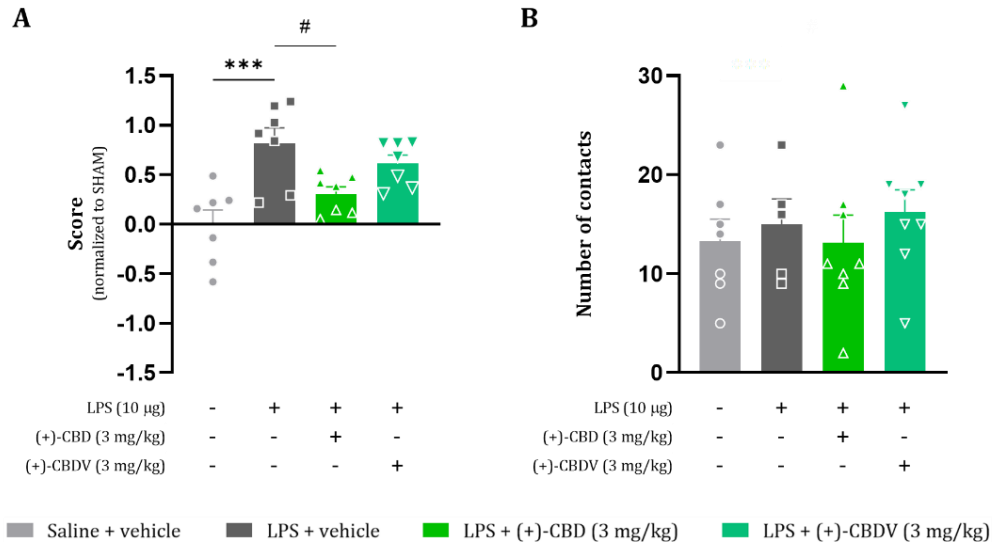


Figure 46. Effect of (+)-CBD or (+)-CBDV on the motor performance of LPS-lesioned mice in the cylinder rearing test (CRT). *(A) The motor score was calculated by subtracting the number of contacts made with the ipsilateral paw from the number of contacts made with the contralateral paw and dividing the result by (B) the total number of contacts. Values are expressed as mean ± SEM of 7 animals per group. Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparison test (*** $p < 0.001$ vs. Sham-operated vehicle-treated group; # $p < 0.05$ vs. LPS-lesioned vehicle-treated group).*

DISCUSSION

It is undeniable that throughout the 20th century and into the 21st, diseases such as cancer and bacterial or viral infections have received a substantial share of clinical research investment, given their significant impact on current global health. However, neurodegenerative diseases are swiftly gaining prominence in public health priorities. The prevalence of disorders linked to the degeneration of specific CNS areas is projected to rise substantially in the coming decades. This increase is largely driven by the exponential growth of the global population and the continuous rise in life expectancy, which will lead to a marked expansion of the population aged 60 and over. This trend is expected to impose a significant healthcare and economic burden in this century, particularly in developed regions such as Europe, where aging demographics further exacerbate the issue. The primary challenge faced by our societies in relation to neurodegenerative diseases lies in the lack of effective therapeutic strategies, owing to the heterogeneous nature of these pathologies, each characterized by distinct symptoms and pathogenic mechanisms; the deficiency in early diagnosis, which limits the potential therapeutic window; and the difficulty in pinpointing a single clear causal factor, hindering existing research. Despite the absence of disease-modifying therapies, some symptom-alleviating treatments have been developed in certain conditions, such as in PD.

PD is recognised as the first basal ganglia degenerative disease worldwide, with a prevalence that rises from 0.3% in the general population to 1% in individuals over 60 years, reaching 3% in those over 80 years (Balestrino & Schapira, 2020). Unlike other diseases, PD has effective therapy for managing motor symptoms since the late 1960s, based on the supplementation of DA through the administration of L-DOPA (Cotzias et al., 1969). However, complications associated with this treatment were soon identified, including on-off fluctuations and dyskinetic movements, which significantly limit its effectiveness within 5-10 years (Obeso et al., 2017). Although new strategies have emerged to provide symptomatic relief, including non-invasive surgical procedures, there remains an urgent need to explore new therapeutic strategies, with a particular emphasis on approaches that halt or slow the progression of the disease. Numerous clinical trials are currently underway, evaluating a range of therapeutic agents, including anti-inflammatory compounds, antioxidants, neuroprotective factors, and many others (McFarthing et al., 2024). However, no candidate has yet successfully completed all phases of clinical trials. This may be attributed, firstly, to the fact that diagnosis often occurs late, when neurodegeneration has already progressed significantly, preventing early treatment and reducing its potential impact. Furthermore, the multifactorial nature of this disease intensifies the problem, as it requires addressing multiple pathological processes that synergistically contribute to its progression. Therefore, breaking this vicious cycle would require therapeutic agents with a pleiotropic profile or, at least, a combination of various agents with complementary beneficial effects to ensure a broader spectrum of action. In this regard, the ECS and the cannabinoids represent a competent choice as therapeutic strategy for PD. In fact, a growing body of preclinical evidence demonstrates that the administration of certain cannabinoid compounds or the targeting of elements within the ECS exerts both symptomatic and neuroprotective effects in PD (Khaspekov & Illarioshkin, 2024). Nevertheless, no agents have successfully advanced beyond the preclinical phase in the context of PD. Therefore,

exploring novel compounds, targets and mechanisms of action of existing targets could be highly valuable. Thus, the aim of the present doctoral thesis project has been to advance the development of a cannabinoid-based pharmacological therapy for PD through preclinical evaluation of novel strategies in various PD models. This was done by exploring three distinct strategies including: (i) the potential role of CB1R in regulating α-syn dysregulation, (ii) the emerging role of GPR55 as a novel pharmacological target, and (iii) the pharmacological profile shift and differences in the mechanisms of action between CBD enantiomers and their derivatives.

Traditionally, CB1R has been studied in the context of PD either for its potential to alleviate motor symptoms associated with the condition or for its possible role in worsening the characteristic akinesia observed in patients. Specifically, most of the studies in search of a cannabinoid-based therapy for PD have demonstrated that CB1R antagonism or deletion exerts beneficial effects in managing parkinsonian hypokinetic symptoms (Di Marzo et al., 2000; Fernandez-Espejo et al., 2005; Van der Stelt et al., 2005; González et al., 2006) and mitigating complications arising from L-DOPA treatment (Angulo et al., 1986; Van der Stelt et al., 2005; Gutiérrez-Valdez et al., 2013). In contrast, although the use of CB1R agonists has also been associated with symptomatic relief (Sañudo-Peña et al., 1998; Morgese et al., 2007), in terms of neuroprotection, evidence points to the potentiality of CB1R activation in protecting dopaminergic neurons. The genetic deletion of this receptor increases vulnerability to 6-OHDA lesion in murine models (Angulo et al., 1986; Pérez-Rial et al., 2011), and its agonists have been reported to increase the survival of dopaminergic neurons in MPTP-lesioned mice (Chung et al., 2011). Diverse mechanisms have been described to be responsible for these neuroprotective actions of the CB1R, such as the reduction of glutamate-induced excitotoxicity by inducing a hyperpolarized state (Pintor et al., 2006), or the activation of survival pathways to maintain neuronal homeostasis (Blázquez et al., 2015). However, CB1R has also been linked to the ability to regulate protein homeostasis, particularly through the induction of autophagic flux (Casarejos et al., 2013; Vrechi et al., 2021). Building on this evidence and considering previous findings from our group, we sought to investigate the potential benefits of selective CB1R activation in the AAV9-α-syn^{A53T} murine model, which enables the localized overexpression of the mutated protein in the SN, leading to a dysregulation of protein homeostasis.

This pharmacological intervention, consisting of the *i.p.* administration of the CB1R-selective agonist ACEA (Hillard et al., 1999) in lesioned mice, has been assessed in other models of neurodegeneration. In these previous studies, improvements in cognitive and motor behaviour were reported, along with evidence of neuroprotective activity (Aso et al., 2012; Caltana et al., 2015; Gonzalo-Consuegra et al., 2024). Regarding PD, the beneficial motor effects of ACEA observed in preclinical studies have primarily been associated with the reduction of dyskinesias (Leija-Salazar et al., 2020). However, its potential motor improvements mediated by neuroprotection in murine PD models remain largely unexplored, despite being demonstrated for other CB1R-activating agents (Chung et al., 2011). In our model, we found that chronic exogenous stimulation of CB1R by ACEA was insufficient to fully reverse the motor impairments induced by

local overexpression of α-syn^{A53T} in the SN. Hypokinetic effects resulting from the activation of striatal CB1R (Gerdeman & Lovinger, 2001; Huang et al., 2001; Carriba et al., 2007) do not account for the lack of motor improvement, as no reduction was observed in the number of wall contacts or swings performed by the treated animals. This is consistent with the fact that the acute effects of CB1R activation immediately after compound administration were not assessed, since behavioural tests were conducted approximately 24 hours after the last dose, minimizing any measurement interferences (Strougo et al., 2008).

The absence of motor improvement does, however, correlate with the limited neuroprotection provided by the pharmacological treatment to dopaminergic neurons in the SN, further reinforcing the relationship between the extent of dopaminergic neuron loss and the severity of motor impairments (Grealish et al., 2010). Neuroprotective actions of ACEA have been identified *in vitro* against MPTP and rotenone-induced neurotoxicity (Iuvone et al., 2007; Cankara et al., 2021). Notably, lesions induced by these toxins are characterized by mitochondrial dysfunction (see **INTRODUCTION**). A pool of CB1Rs has been identified within these organelles, where they play a specialized role in regulating mitochondrial function (Bénard et al., 2012). Therefore, the ACEA-derived neuroprotection observed in these studies could be attributed, at least in part, to its effects on the subcellular compartment, promoting the restoration of mitochondrial homeostasis. Indeed, ACEA has been reported to upregulate the expression of mitochondrial CB1R in hippocampal neurons (Ma et al., 2015), and to enhance mitophagy in a neural context following haemorrhagic lesion (Liu et al., 2022). Nevertheless, it is important to note that this potential to improve mitochondrial function contrasts with previously described effects associated with this pool of receptors, namely the inhibition of cellular respiration (Bénard et al., 2012). This apparent contradiction may reflect the functional complexity of this receptor, which might be shaped by context-dependent factors (e.g., activation duration, dosage, cell type).

Regardless of the lack of effectiveness in preserving the lesioned neuronal population, ACEA treatment led to a further increase in the levels of the lysosomal marker LAMP-1, which may suggest that this cannabinoid promotes the clearance of the accumulated protein through degradative pathways, like autophagy. Activation of CB1R has already demonstrated the induction of autophagy in other pathological contexts, such as cancer (Velasco et al., 2016). Indeed, modulating autophagy may be a promising therapeutic strategy for neurodegenerative diseases, including PD, due to its role in the clearance of protein aggregates (Moors et al., 2017). Other *in vitro* studies have reported that CB1R activation increases the levels of autophagy markers, such as LC3-I/II, while reducing the accumulation of overexpressed proteins (Erustes et al., 2024; Vrechi et al., 2025). However, it is important to recognize that cellular systems overexpressing a protein may differ in several ways from more physiological contexts. In our model, the increased staining of LAMP-1 does not correlate with a reduction in α-syn levels; in fact, an upward trend is observed. Thus, the ACEA-induced elevation of LAMP-1 in treated animals may indicate aberrant lysosomal accumulation, rather than efficient autophagy, which may explain the absence of protection towards dopaminergic neurons.

Although some studies support the expression of CB1R in dopaminergic neurons (Davis et al., 2018; Baddenhausen et al., 2024), controversy still exists regarding the consistency of these findings. Therefore, it is important to consider other CB1R-expressing cell types that may contribute to the observed effects, such as astrocytes. These glial cells exhibit the second-highest expression of CB1R in brain tissue (www.brainrnaseq.com), providing additional sites where ACEA can exert its effects. In this line, we observed an exacerbation in the immunoreactivity of the astroglial marker GFAP after treatment with ACEA. Previous studies have shown that ACEA reduces astroglial response *in vivo* (Aso et al., 2012; Caltana et al., 2015), and promotes an anti-inflammatory astrocytic phenotype *in vitro* (Haspula & Clark, 2021). Although we observed an increase in astrocytic reactivity following ACEA treatment, suggesting a specific effect on this cell type, the functional implications of this modulation remain unexplored. Therefore, we cannot yet determine whether this astrocytic response exerts beneficial or detrimental effects, and further studies are required to clarify the significance of this alteration. Interestingly, it has been demonstrated that LAMP1-positive astrocytes play a role in limiting inflammation in the CNS (Sanmarco et al., 2021), and therefore, the increase in LAMP1 immunostaining could be associated with the elevation in GFAP, suggesting a significant role for astrocytes in the actions of ACEA. Moreover, it is important to note that, in a context of protein overload, astrocytes can take up proteins for degradation, thereby contributing to protein clearance, but may also contribute to the propagation of proteinopathy when a certain threshold is exceeded (Altay et al., 2022). This could also help explain the observed increase in α-syn immunoreactivity, as α-syn may be accumulating within these cells, similarly to what is observed in human pathology (Lindström et al., 2017). Additionally, the impact of ACEA on glial reactivity does not extend to CD68-positive cells. Cells expressing this marker, including microglia and infiltrating monocytes/macrophages, exhibit significantly lower CB1R expression compared to other neural cell types (www.brainrnaseq.com). Consequently, ACEA is expected to have a minimal impact on these cell populations, as observed in our results.

Beyond the SN, we also examined the striatum, the other major component of the basal ganglia affected in PD. This region is particularly relevant considering the presence of CB1R on striatal projection neurons, which are key postsynaptic targets of dopaminergic innervation (Herkenham et al., 1991b). Thus, we decided to explore the expression in striatum of various markers associated with dopaminergic transmission, as well as the CB1R. Neither the expression of D1 and D2 receptors nor that of the signalling molecule DARPP-32 was affected by the nigral lesion induced with AAV9-α-syn^{A53T} or by CB1R activation. It has been hypothesized that this lack of changes may reflect compensatory mechanisms aimed at preserving dopaminergic signalling (Chritin et al., 1996). However, alterations were observed after 6-OHDA lesion in phospho-DARPP-32 levels, as well as a reduction in total DARPP-32 protein (Yuste et al., 2012), which is consistent with the slight decrease we detected in *Ppp1r1b* mRNA levels. Activation of CB1R has also been associated with increased phosphorylation of DARPP-32 (Borgkvist et al., 2008), a mechanism that we have not quantified. Notably, we also did not detect changes in the expression of CB1R after AAV9-α-syn^{A53T} lesion or ACEA treatment, as was expected regarding previous

literature (Leija-Salazar et al., 2020), even though varying results may arise among different models. Recapitulating our findings, we found no evidence that either the lesion or ACEA treatment altered dopaminergic markers. However, additional experiments beyond gene expression analyses are necessary to draw definitive conclusions.

Given the involvement of CB1R in autophagy and the changes observed in LAMP-1 levels, we also investigated the expression of additional markers related to autophagy and the UPR to further assess the effects of ACEA in this context. Although in our model the primary proteinopathy-related insult is induced in the SN, we chose to assess markers related to protein homeostasis regulatory systems in the striatum, as Lewy pathology has also been identified in this brain region (Duda et al., 2002), along with substantial gene dysregulation observed in the AAV9-α-syn^{A53T} model (Burgaz et al., 2024). Autophagy-related markers (Atg7, p62 and Beclin-1) showed no changes in expression following the AAV9-α-syn^{A53T}-induced lesion or ACEA administration. The role of autophagy in PD pathogenesis remains controversial, as both increases and decreases in autophagic markers have been reported in preclinical models (Alvarez-Erviti et al., 2010; Marin & Aguilar, 2011; Xiong et al., 2013). In our study, the absence of changes in autophagic markers may be attributed to the likely lack of α-syn aggregates in the model used. Indeed, reproducing the aggregation of this protein in animal models has remained a significant challenge over the years (Giráldez-Pérez et al., 2014). Beyond autophagy, the UPR and ER stress have also been implicated in PD pathogenesis (Costa et al., 2020), and linked to α-syn pathology (Baek et al., 2016). When examining three markers involved in these mechanisms (BiP, CHOP, and GRP94), we observed a generalised reduction in their expression. Even though much of the evidence points to an increase in pathways associated with the UPR in the context of PD, reductions have also been reported in association with ageing or sporadic PD cases (Costa et al., 2020), which has been linked to a contribution to the vulnerability of dopaminergic neurons as a predisposing factor. Among the observed changes, the alteration in BiP was the most pronounced. This ER chaperone plays a crucial role in protein homeostasis, including in the context of PD, where its expression has been reported to exhibit inconsistent changes (Enogieru et al., 2019). However, it does appear that downregulation of BiP aggravates α-syn toxicity and susceptibility to neurotoxins (Salganik et al., 2015; Jiang et al., 2016), while its upregulation suppresses α-syn aggregation and toxicity (Gorbatyuk et al., 2012; Jiang et al., 2014). Therefore, the downregulation of *Hspa5* mRNA levels observed in our study may represent a detrimental alteration that further exacerbates the pathology, with ACEA failing to effectively counteract this effect, although a trend towards reversal was detected.

Moreover, BiP has gained particular interest in the field of cannabinoids, since it has recently been identified as an interactor of CB1R, with the capability to modulate the intracellular signalling triggered by this cannabinoid receptor (Costas-Insua et al., 2021). Despite the limited effects observed with ACEA, we sought to explore whether BiP deficiency in the same proteinopathy-related model could contribute to phenotypic worsening, considering the previously discussed evidence. However, our findings did not support this hypothesis. Neither

behaviourally nor histologically did partial genetic deficiency of BiP alter the effects induced by AAV9-α-syn^{A53T} in any way. Identical results were obtained when BiP-deficient mice were lesioned with 6-OHDA, with no increased vulnerability to neurotoxin-induced damage (Gómez-Almería et al., 2021). The partial nature of BiP deficiency, given that total deletion of the gene results in embryonic lethality, could account for the lack of significant findings, as it may allow the activation of compensatory mechanisms, preventing an effective deficiency. Nevertheless, the same study showed that BiP heterozygosity exacerbated pathological deterioration in experimental ALS. Notably, the ALS model employed was based on genetic modification, allowing for a more progressive phenotype compared to those generated from a neurotoxin-induced lesion, thereby providing greater opportunities to detect alterations between WT and BiP-deficient animals. Moving forward, investigating the role of this chaperone protein in a less acute PD model, where motor impairments, α-syn pathology and neuronal loss develop more progressively, would be of interest. Interestingly, in recent years, promising models related to this aspect have been developed and characterised (Carballo-Carbajal et al., 2019).

Returning to our results, despite the limited effects of BiP deficiency, we observed a slight exacerbation in Iba1 immunoreactivity in the lesioned SN of BiP-deficient mice, which was primarily reflected as an enhancement in statistical significance when comparing the lesion effect between WT and heterozygous animals. This heightened microglial immunoreactivity was also seen in the ALS model, accompanied by morphological changes in these glial cells that were consistent with a neurotoxic phenotype (Gómez-Almería et al., 2021). In line with this, the UPR has been identified as a regulator of neuroinflammation (Stone & Lin, 2015; Fernández et al., 2021). Microglia from patients suffering from multiple sclerosis, a neurodegenerative disorder with a significant inflammatory component, show increased expression of BiP (McMahon et al., 2012). *In vitro* studies have also shown an upregulation of this chaperone in the glial C6 cell line after ER stress-inducing stimuli, suggesting a protective role (Suyama et al., 2011). Notably, while ER stress triggers apoptotic signalling in neurons, it promotes a pro-inflammatory phenotype in glial cells (Hosoi et al., 2013; Sims et al., 2022; Sokołowska et al., 2024). ER stress-induced inflammation is driven by PERK-dependent signalling (Meares et al., 2014), which is suppressed by the BiP-PERK interaction (Sprenkle et al., 2017). Therefore, BiP deficiency may lead to a dysregulation of this signalling cascade, such as sustained PERK activation, resulting in increased expression of inflammatory molecules that, in turn, promote glial reactivity, as partially observed in our model. Moreover, these results are consistent with recent findings from intranasal administration of exogenous BiP, which produced anti-inflammatory effects by being internalised by microglia, reducing their number, preventing morphological changes, and inhibiting pro-inflammatory cytokine production in a lactacystin-induced model of PD (Pazi et al., 2024).

Overall, we set out to evaluate the relevance of CB1R and its interactor BiP as pharmacological targets in the AAV9-α-syn^{A53T} model; however, our findings did not align with our initial hypothesis. Although the role of CB1R as a potential pro-autophagic and neuroprotective target in PD may need to be reconsidered, other factors, particularly the suitability of the experimental

model, must also be considered. Employing more progressive models that better capture the gradual evolution of the disease phenotype could allow for a more comprehensive assessment of the therapeutic potential of this cannabinoid receptor beyond its symptomatic effects. Nevertheless, the limited therapeutic implications observed for CB1R and BiP in our model underscore the complexity of cannabinoid-related targets in PD and reinforces the need to expand the search beyond the classical CB1R and CB2R, which, in fact, have thus far failed to yield an effective therapeutic strategy. In this regard, we turned our attention to another promising candidate: GPR55, a recently-deorphanised orphan GPCR with growing relevance in neurodegenerative and inflammatory processes. This receptor has been found to exhibit affinity for a wide range of lipid-derived molecules, most notably LPI, which is considered its endogenous ligand (Oka et al., 2007), as well as several cannabinoids and cannabinoid-like compounds (Ryberg et al., 2007; Kapur et al., 2009; Sharir et al., 2012). Some of these compounds, acting through GPR55, have demonstrated neuroprotective and anti-inflammatory potential in different experimental models (Kallendrusch et al., 2013; Saliba et al., 2018; Burgaz et al., 2021c). However, one of the major challenges in elucidating the endogenous functions of this receptor, and in assessing its potential therapeutic relevance, lies in the lack of potent, well-validated, and especially selective GPR55 agonists and antagonists. This limitation has hampered pharmacological dissection of GPR55-mediated pathways. Given these constraints, alternative approaches, such as genetic models, become essential. The first generation of GPR55-deficient mice was produced by deleting amino acids 39 to 281 of the receptor (Johns et al., 2007; Staton et al., 2008). More recent models involve deletion of exon 2, which encompasses the entire coding region of the *Gpr55* gene (Sisay et al., 2013), thereby abolishing protein expression completely. These KO models have provided insight into the role of GPR55 in a range of physiological and pathological contexts, including motor activity (Wu et al., 2013; Bjursell et al., 2016), energy metabolism and thermal nociception (Bjursell et al., 2016), or cardiac function (Walsh et al., 2014; Schopohl et al., 2025). Interestingly, despite its involvement in diverse biological processes, GPR55-deficient mice do not exhibit an overt pathological phenotype or significant differences in gross morphology, physical development, or survival rates in adulthood (Staton et al., 2008). Furthermore, a detailed characterization of CNS function by Wu and colleagues revealed that the absence of GPR55 does not significantly impair basic behaviours, including muscle strength, general motor coordination, sensorimotor integration, learning and memory, or anxiety/depression-like behaviour (Wu et al., 2013). However, impairments were observed in more complex motor tasks such as the rotarod test, indicating a possible role of GPR55 in fine motor control or motor learning. Our current results align with these findings. We have found that GPR55 KO animals displayed increased latency to fall off the rotarod test, while no significant differences were observed in other locomotor parameters measured in the actimeter test. In the same characterization conducted by Wu and colleagues, no significant alterations were observed in the development of neuronal circuits and brain structures either, with identical patterns of intense fibrous TH immunoreactivity throughout the striatum and SN (Wu et al., 2013). Likewise, our immunohistochemical analysis revealed no gross alterations in the expression of TH, nor in

the astrocytic marker GFAP, suggesting that dopaminergic integrity and astrocytic homeostasis are preserved in the absence of the receptor. However, a significant reduction in microglial Iba1 immunoreactivity was detected in GPR55-deficient mice, indicating a possible role for this receptor in regulating basal microglial activity. This observation is particularly relevant, as GPR55 expression in microglial cells has been documented for over a decade (Pietr et al., 2009). Moreover, pharmacological inhibition of GPR55 has been shown to exert anti-inflammatory effects in reactive microglia (Saliba et al., 2018), likely mediated via NF-κB signalling pathway inhibition (Saliba et al., 2021). The reduction of Iba1 staining in our KO animals may therefore reflect a tonic pro-inflammatory action of GPR55, whose removal/inhibition could shift microglial towards a more quiescent or less reactive phenotype.

Building upon these initial results, we next sought to investigate the role of GPR55 in two distinct PD models, each characterized by a different primary pathological driver: α-syn^{A53T} overload by its local overexpression using AAV9 vectors, and mitochondrial dysfunction and oxidative stress induced by the neurotoxin 6-OHDA. Importantly, these models also differ in their associated inflammatory profiles. In the α-syn-based proteinopathy model, we observed a pronounced upregulation of both Iba1 and GFAP immunoreactivity accompanied by expression of pro-inflammatory cytokines, indicative of robust microglial and astrocytic activation. This heightened inflammatory response is consistent with the strong antigenic potential of misfolded α-syn species, known to trigger acquired immune mechanisms within the CNS (Sulzer et al., 2017). In contrast, the inflammatory response in the 6-OHDA model appears considerably more subtle, as evidenced by only modest changes in Iba1 and GFAP immunoreactivity. This likely reflects the secondary nature of the glial activation in this model, which arises as a downstream consequence of dopaminergic neuronal degeneration rather than serving as one of the primary pathological drivers. Although the mice used in each model differ in age by 2–4 months, it is unlikely that this age gap alone explains the reduced inflammation observed in the older 6-OHDA-lesioned animals, as aging is generally associated with an enhanced and prolonged neuroinflammatory response (Godbout et al., 2005). Moreover, age-associated changes are typically investigated using age differences of over one year in mice (Ritzel et al., 2019); therefore, a 2–4 month difference is unlikely to represent a biologically meaningful gap. Thus, using these two models, we first investigated how the expression of GPR55 is modulated by these distinct types of lesions, particularly in the striatum, which exhibits the highest expression of this receptor (Sawzdargo et al., 1999; Wu et al., 2013). Notably, we identified significant differences between both models in terms of the overall impact of the lesion: the proteinopathic model showed an increase in striatal GPR55 expression, accompanied by a greater inflammatory response, while the mitochondrial model, with less inflammation, exhibited minimal effects. A previous study using a distinct parkinsonian model, based on mitochondrial dysfunction induced by MPTP and characterized by a low inflammatory response, reported a reduction in striatal GPR55 expression (Celorrio et al., 2017). These findings suggest that GPR55 expression may be differentially modulated depending on the specific pathophysiological mechanisms engaged by each model. Notably, our direct measurements of GPR55 expression in PD patient samples, including the

midbrain and caudate-putamen nuclei, regions where the presence of this receptor has been confirmed (Sawzdargo et al., 1999; Henstridge et al., 2009), did not yield conclusive results, as no significant differences were observed between healthy controls and PD patients in any of the regions analysed, likely due to high inter-individual variability. Still, opposing trends may be inferred between both regions, with an upward tendency in the midbrain and a downward one in the striatum. This subtle pattern, at least in the case of striatum, is supported by a study employing nuclear-enriched transcript sort sequencing in *post-mortem* human tissue. That study reported reduced GPR55 transcription in donors with PD compared to non-neurodegenerative controls in both direct and indirect striatal MSNs (Hewer et al., 2023), suggesting a deficit in GPR55-mediated signalling in the disease. Additionally, studies in non-human primates have reported increased expression of GPR55–CB1R and GPR55–CB2R heteromers in the input nuclei of the basal ganglia of MPTP-treated animals (Martínez-Pinilla et al., 2020), which is consistent with the upward trend observed in AAV9-α-syn^{A53T} model. However, this does not necessarily indicate an overall increase in GPR55 expression, but may instead result from alterations in cannabinoid receptor levels, which are more consistently reported. No data regarding the SN were found in the literature, but the observed increase in GPR55 expression could be associated with the pronounced microglial response.

Although our hypothesis points to glial cells as the primary cellular substrate involved in these changes, we were not able to confirm the presence of the protein GPR55 in these cell types, as available analytical tools did not allow us to obtain reliable results. However, in the MPTP model and using *in situ* hybridization, GPR55 was reported to be expressed by neurons, but not by microglia and astrocytes (Celorrio et al., 2017), while other authors, using the same technique, have reported the expression of GPR55 mainly in Iba1-positive cells (Shen et al., 2022). Therefore, this type of study should be approached with caution, as it may yield controversial results.

Pending a more detailed analysis (e.g., single-cell techniques) to identify the specific cell types expressing GPR55 in the parkinsonian models we employed, we performed the same parkinsonian lesions in GPR55-deficient mice to evaluate the impact of the absence of this receptor on pathological progression. Overall, we observed a clear effect of GPR55 deficiency on motor behaviour following the lesion, as assessed through several behavioural tests designed to detect motor impairments resulting from unilateral brain damage. Notably, GPR55-deficient lesioned animals generally exhibited reduced lateralized motor preference compared to their lesioned WT counterparts. This effect is unlikely to be attributed to alterations in general motor activity, as the behavioural assays used are based on intra-individual comparisons between the two body sides, both equally influenced by the genetic manipulation. Although GPR55 deficiency has been previously associated with impaired motor performance, under a pathological insult the absence of GPR55 appears to mitigate lesion-induced motor deficits, suggesting a potentially protective role. This putative protection elicited by the lack of GPR55 correlates with the outcomes observed when analysing the intensity of TH immunoreactivity in the SN. In the AAV9-α-syn^{A53T} model, the lesion induced a marked reduction in the lesioned WT group, whereas this reduction was notably

attenuated in lesioned KO animals, indicating lower neuronal deterioration associated with the absence of the receptor. Interestingly, the protection of dopaminergic neurons conferred by GPR55 deficiency was less evident in the 6-OHDA mode and more pronounced in females than in males. Since GPR55 has also been reported to be expressed in dopaminergic neurons of the SN (Celorrio et al., 2017), although with some controversy (He et al., 2024), it is possible that within this degenerating cell population, GPR55 plays a selective role in activating intracellular pathways that promote pro-apoptotic signalling. Although it has been reported that GPR55 mediates cell death by overstimulating neuronal nitric oxide synthase and inducing oxidative stress through $G_{\alpha 13}$ signalling, this has been described in cancer cells (Akimov et al., 2021), and it remains unclear whether this is also the case in neurons undergoing degeneration. However, the use of GPR55 antagonists has suggested that the inhibition of this receptor may lead to neuroprotective effects associated with anti-inflammatory activity. In LPS-stimulated BV2 microglial cells, GPR55 antagonists significantly suppressed the expression and release of pro-inflammatory cytokines and chemokines (Sun et al., 2024). Comparable effects were observed in primary rat microglia, where GPR55 antagonists inhibited PGE_2 release and reduced the expression of mPGES-1 and COX-2 (Saliba et al., 2018; 2021). These anti-inflammatory actions were partially mediated through modulation of signalling pathways such as MAPK, PKC, and NF-κB (Saliba et al., 2021; Sun et al., 2024). Furthermore, GPR55 expression has also been detected in monocytes and neutrophils, two leukocyte subtypes that appear to play a relevant role in PD (Grozdanov et al., 2014; Muñoz-Delgado et al., 2021). In these immune cells, GPR55 activation enhances their pro-inflammatory responses (Chiurchiù et al., 2015) and promotes neutrophil migration (Balenga et al., 2011).

Our results confirmed the involvement of this orphan GPCR in neuroinflammatory responses, since in the AAV9-α-syn^{A53T} model, we observed a subtle decrease in the GFAP immunostaining in the SN of GPR55 KO animals, but a significant reduction in the density of 'reactive' C3-positive astrocytes in those animals, albeit only in females. There are limited data on the expression of GPR55 in astrocytes (Kallendrusch et al., 2013; García-Gutiérrez et al., 2018), and its precise function in this neural cell type remains unclear. One possibility is that the observed effects may result from indirect mechanisms involving altered microglial activity. Indeed, although no significant changes in Iba1 immunoreactivity were detected in the SN, we have found that the GPR55 deletion impacted the morphology of microglial cells, increasing their amoeboid-like conformation after the overexpression of mutated α-syn^{A53T}. Typically, this kind of morphology has been associated with activated 'M1' microglial phenotype. However, this dichotomic perspective is currently under revision, and it is not possible to assert that specific morphological changes correspond to a particular activation state or defined functionality in microglia (Paolicelli et al., 2022). Nevertheless, there is some consensus that a more ameboid morphology may promote a pro-phagocytic state (Levtova et al., 2017), which could facilitate the removal of protein aggregates without necessarily promoting inflammation (Hurley et al., 1999; Magnus et al., 2001). Notably, previous *in vitro* studies have reported that GPR55 activation in BV-2 cells and primary microglia suppresses LPS-induced phagocytosis. *In vivo*, morphological alterations in microglial

cells have been observed following pharmacological manipulation of GPR55 in the striatum of MPTP-lesioned mice, including a reduction in microglial branching (Celorrio et al., 2017). Therefore, GPR55 deficiency might modulate the microglial phenotype beyond changes detectable by Iba1 staining alone. Further studies are warranted to investigate this hypothesis. Our cytokine expression analysis in the striatum of AAV9-α-syn^{A53T}-lesioned mice further supports the notion that GPR55 deficiency affects the manner glial cells and/or infiltrated immune cells are responding to the insult, given that in GPR55-deficient animals, specifically in females, there is a trend toward lower expression levels of IL-1β and IFN-γ. Notably, the effects on glial cells and cytokine profiles were less impacted by GPR55 deficiency in the 6-OHDA-lesioned mice, which is consistent with the lower level of inflammation observed in this model. All these findings suggest that GPR55 may represent a promising immunomodulatory target in pathologies such as PD, where neuroinflammation plays a key role.

Another important finding was the consistent sex differences observed in this genetic model following the induction of a PD-like pathology, particularly with AAV9-α-syn^{A53T}. Research on the impact of sex in GPR55-deficient animals remains limited, with only a few studies suggesting a sexual dimorphism in the function of the receptor, and these primarily in non-neural physiological contexts (Schopohl et al., 2025). This is largely due to the predominant use of male mice in preclinical studies, especially those related to neurodegeneration, often aimed at avoiding the potential confounding effects of the estrous cycle on neuroprotection and related outcomes. However, our study has revealed a clear sex-dependent role of GPR55 in PD. Striatal *Gpr55* mRNA levels did not explain the origin of the observed sex differences, even though single-cell approaches might help to determine whether sex-dependent variations could be restricted to a specific cell type(s). Therefore, additional factors such as hormonal influences or interactions with signalling pathways may modulate the neuroprotective effects of the genetic deletion, as suggested by the sex-specific differences observed in the rotarod test performance. Gonadal hormones, particularly estradiol, have been proposed to display functional and organizational effects on the ECS (Reich et al., 2009; Rubino & Parolaro, 2011; Craft et al., 2013; Levine et al., 2021). Although not yet explored, sex differences in cannabinoid pharmacology could also impact on GPR55, regarding the close link between this intercellular system and the orphan receptor, which can be targeted by eCBs (Sharir et al., 2012) and form heteromers with both CB1R (Martínez-Pinilla et al., 2014) and CB2R (Balenga et al., 2014). Thus, it is plausible to suggest that GPR55 deletion might lead to sex-specific outcomes, given its integration into signalling networks that exhibit sexual dimorphism, such as the ECS. Additionally, estrogens are well known to exert protective effects against chronic neuroinflammation (Morale et al., 2006), acting not only on resident glial populations (Crespo-Castrillo et al., 2020), but also on peripheral monocyte-derived cells (Villa et al., 2016). In these cells, estrogen signalling might be modulated by the absence of GPR55, potentially enhancing an anti-inflammatory profile, an effect that would likely be less pronounced in male animals, given their considerably lower estrogen levels.

Overall, our findings suggest that GPR55 may play a detrimental role in PD in a sex-dependent manner, as its genetic deletion reduces the susceptibility of female mice to certain parkinsonian lesions. This points to the potential of GPR55 antagonists as a promising pharmacological strategy for PD, specifically by modulating excessive neuroinflammatory responses. However, this approach should be taken with caution, as GPR55, like other GPCRs, exhibits complex pharmacology, with both agonists and antagonists producing comparable effects (Celorrio et al., 2017; Fatemi et al., 2021). Therefore, it is critical to account for both the cellular context and the selective functionality of emerging ligands, as well as their potential sex-specific effects, to ensure targeted and effective therapeutic interventions.

Lastly, as the final objective of this thesis, we aimed to further investigate another additional strategy, that involves the modification of a widely studied pharmacological cannabinoid-based intervention: the CBD. A significant portion of pharmacological research on cannabinoids has focused on exploring the potential therapeutic benefits of phytocannabinoids found in the plant *Cannabis sativa*. While Δ^9-THC is the main plant-derived cannabinoid capable of interacting with both CB1R and CB2R receptors (Pertwee, 2008), which contributes to both its therapeutic potential and its unwanted adverse effects, the non-psychoactive CBD has gained increasing attention in the last decade due to its more favourable pharmacological profile from a clinical perspective (Martin-Santos et al., 2012). Indeed, extensive preclinical evidence (Lastres-Becker et al., 2005; Santos et al., 2015; Gugliandolo et al., 2020; Giuliano et al., 2021) and several clinical studies (Zuardi et al., 2009; Chagas et al., 2014a; 2014b) support the potential of CBD in the context of PD. However, despite the extensive research, the underlying mechanisms behind the reported beneficial effects remain elusive or not fully understood, particularly due to the low affinity of this phytocannabinoid for both CB1R and CB2R (Fernández-Ruiz et al., 2013). Alternative mechanisms and pathways have been proposed for its beneficial properties. Among these, CBD has been found to exert allosteric modulation of canonical cannabinoid receptors (Laprairie et al., 2015; Martínez-Pinilla et al., 2017), along with actions mediated by other molecular targets, including GPR55 (Pertwee, 2007), TRPV1 (Etemad et al., 2022), PPAR-γ (O'Sullivan, 2016), serotonin receptors (DeVuono et al., 2022; Billard et al., 2025) and the adenosine A2 receptor (Sánchez-Fernández et al., 2024). Additionally, CBD exhibits receptor-independent mechanisms, particularly its antioxidant properties, which stem from the free radical-scavenging capacity of its chemical structure. These studies have been usually conducted using the (–)-*trans* enantiomer of CBD, which is the predominant form found in the plant. However, due to its structural complexity, CBD can adopt other enantiomeric conformations. Such variations in the spatial arrangement of its chemical groups may significantly influence its interaction with different receptors, including cannabinoid receptors, thereby altering its binding profile and modulating its activity upon interaction. Indeed, previous studies have briefly explored this possibility, reporting differences in affinity and potency towards cannabinoid receptors among enantiomers of the same molecule (Mechoulam, 2000; Bisogno et al., 2001; Fride et al., 2004; Hanus et al., 2005; Morales et al., 2017). Thus, building on this knowledge and after confirming how the stereochemical shift between (–)-*trans* and (+)-*trans*-CBD influences their interaction

with CB1R and CB2R, we aimed to assess the functional activity of the (+)-*trans* enantiomer of this phytocannabinoid on the cannabinoid receptors, particularly its potential to elicit beneficial effects primarily mediated through CB2R, with a focus on anti-inflammatory activity. Beyond this phytocannabinoid, we extended the analysis to a natural derivative (CBDV) and three synthetically designed analogues (CBDA-Me, CBDA-Gly, CBDA-Hyp). The addition of these esterifications to (–)-CBD has been found to increase the affinity for both cannabinoid receptors (Götz et al., 2019). Therefore, we were interested in assessing these modifications in (+)-CBD as well. For these five (+)-compounds, key pharmacokinetic parameters, including absorption, solubility, permeability, and toxicity, were evaluated through *in silico* predictions to support their potential clinical translation.

Our data, already published (Rodríguez-Carreiro et al., 2025), fully corroborated the predictions, with (+)-enantiomers consistently displaying higher affinities than (–)-enantiomers for both receptors, particularly for the CB2R, with the exception of CBDA-Me. Quantification of receptor affinities, measured as K_i values, for the (+)-enantiomers at CB1R revealed the following order: CBDA-Hyp > CBDV = CBDA-Me = CBDA-Gly > CBD; with only CBDA-Hyp exhibiting a K_i in the low nanomolar range. For the CB2R, the affinity order was as follows: CBDA-Hyp > CBDA-Gly > CBDA-Me = CBDV = CBD; with all compounds exhibiting a K_i lower than 50 nM. These data are consistent with those reported by Golliher and colleagues, who found that (+)-CBD showed lower K_i values for both CB1R and CB2R compared to (–)-CBD (Golliher et al., 2021). Similarly, Bosquez-Berger and collaborators reported higher affinity for CB1R of (+)-CBD compared to its naturally occurring enantiomer, as well as greater potency in inhibiting depolarization-induced suppression of excitation. However, they also observed that the (+)-enantiomer was less potent than the (–)-counterpart in other CB1R-mediated effects, such as the suppression of cAMP accumulation (Bosquez-Berger et al., 2022). The trend of higher affinity in the (+)-enantiomeric series has also been reported for certain CBD derivatives. For instance, the (+)-enantiomer of 8,9-dihydro-7-hydroxy-CBD exhibited K_i values in the nanomolar range, whereas the (–)-enantiomer showed significantly lower affinity (Kozela et al., 2016). Furthermore, an earlier study comparing multiple CBD derivatives in both enantiomeric configurations found that the (+)-stereoisomers exhibited greater activity at CB1R within the low nanomolar range, while differences in CB2R binding were less pronounced (Hanus et al., 2005). This contrasts with our findings, where greater differences were observed for CB2R. However, since the CBD derivatives analysed in that study differ from those tested in our work, this discrepancy may be at least partially explained by differences in compound selection.

Collectively, our findings, in conjunction with previous results, confirm that the stereoisomeric change of CBD leads to a significant alteration in its binding profile at cannabinoid receptors, transitioning from a lack of interaction with (–)-CBD to a notable affinity with (+)-CBD. Although not yet performed, molecular docking studies are planned and will help elucidate how the change in stereoisomerism of this phytocannabinoid facilitates its binding to cannabinoid receptors, predicting the spatial orientation relative to the receptor. Nevertheless, we decided to continue

with the pharmacological evaluation, focusing on investigating the intrinsic activity of those compounds that exhibited affinities in the low nanomolar range. Thus, we found that (+)-CBD, (+)-CBDV, and (+)-CBDA-Me appear to act as agonists at the CB2R, while (+)-CBDA-Gly and (+)-CBDA-Hyp exhibited antagonist/inverse agonist-like activity at the same receptor. Additionally, an agonist profile was observed for the latter molecule at the CB1R. Regarding functional assays, it is important to note that data can often be controversial when comparing different methods of studying intrinsic activity. For instance, while in an agonist-stimulated GTPγS binding assay, we reported that (+)-CBDA-Hyp appeared to act as a CB2R antagonist and a CB1R agonist, opposite behaviour was observed in a cell-based assay to measure cAMP-mediated signalling (González-Mariscal et al., 2021). Therefore, further experiments will be necessary to resolve this controversy. However, based on the results we obtained, the fact that some of the (+)-enantiomers investigated in our study (CBD, CBDV, and CBDA-Me) act as agonists of CB2R supports the idea that (+)-CBD isomeric derivatives generally hold promising potential for the treatment of inflammatory disorders (Fride et al., 2004; Rao et al., 2025). Building on this indication, we aimed to study *in vitro* the potential anti-inflammatory and neuroprotective profiles of these three compounds, using appropriate cell-based assays (Gómez-Cañas et al., 2016). Our data strongly suggest that (+)-CBD, (+)-CBDV, and (+)-CBDA-Me can attenuate the effects of LPS exposure in cultured iMG microglial-like cells, reducing the expression of proinflammatory cytokines and enzymes, thereby likely mitigating the proinflammatory environment induced by this toxin. When assessing the impact of microglial conditioned media on neuronal metabolic activity, and indirectly on neuronal viability, we confirmed that the three CB2R (+)-agonists, at the optimal concentrations, reversed the neurotoxic effects of LPS-activated, microglia-derived conditioned media. Notably, this anti-inflammatory and neuroprotective activity seemed to involve the CB2R, but not the PPAR-γ, consistent with the binding profile observed for these compounds at the cannabinoid receptor compared to their (–)-enantiomers. We focused on these two receptors as they are the primary ones involved in the inflammatory actions of cannabinoids, although (+)-CBD has also been shown to be active on other receptors, such as CB1R, sphingosine-1-phosphate receptors (Bosquez-Berger et al., 2022) or TRPV1 (Bisogno et al., 2001).

As a final step and before proceeding with an *in vivo* characterisation of these compounds, we conducted an *in silico* analysis to predict the ADMET properties of the five compounds in the (+)-enantiomeric form, with (–)-CBD as a reference molecule. The rationale for this analysis is based on previous studies, where pharmacodynamic differences observed between both enantiomeric configurations might be linked to pharmacokinetic differences, particularly in the ability of (+)-enantiomers to cross the BBB. Indeed, it has been proposed that (+)-CBD and its derivatives could be useful to selectively target cannabinoid receptors at the periphery for treating gastrointestinal disorders or peripheral pain and inflammation (Fride et al., 2004). Thus, the predictive ADMET analysis aimed to further explore this hypothesis. The generated data identified (+)-CBD and (+)-CBDV as the most pharmacodynamically favourable compounds, exhibiting optimal profiles in terms of toxicity, absorption, solubility, and the ability to cross tissue barriers, including the BBB. In contrast, the other (+)-enantiomers showed less favourable predicted values, often falling

outside the expected range. These predictions challenge the idea of (+)-CBD and some derivatives as peripherally restricted agonists. Indeed, we decided to perform a final *in vivo* evaluation of (+)-CBD and (+)-CBDV in mice lesioned with LPS in the striatum. Interestingly, *i.p.* administration of these compounds produced distinct outcomes: while (+)-CBDV failed to improve any of the studied parameters affected by the pro-inflammatory insult, (+)-CBD exerted effects, attenuating the loss of TH immunoreactivity in the SN and improving motor performance in the CRT. The differences in the *in vivo* effects between the two molecules may arise from pharmacodynamic or pharmacokinetic factors that were not evident in the *in vitro* or *in silico* analyses, but which could manifest under more physiologically relevant conditions. Moreover, in contrast to previous studies that reported a lack of central activity of (+)-CBD (Fride et al., 2004), our findings provide evidence to the contrary, as demonstrated by its neuroprotective and motor effects. This controversy may be primarily explained by the fact that the study by Fride *et al*. assessed the lack of central activity based on parameters closely linked to CB1R activation. Conversely, our results demonstrate that this compound exhibits marked selectivity for CB2R, which could underlie the neuroprotective effects we observed. Notably, this CB2R-mediated activity does not appear to translate into modulation of glial reactivity, particularly in microglia or infiltrating monocytes, which are known to express this receptor at significant levels (Greco et al., 2021; Grabon et al., 2024). Although the doses employed in our study were lower than those used in other *in vivo* experiments with structurally related molecules (Fride et al., 2004; González-Mariscal et al., 2021), the observed effects on dopaminergic neurons suggest that our dosing regime falls within an effective therapeutic range. Therefore, the lack of glial effects may be more attributable to methodological aspects, as glial activation was assessed solely by immunostaining. It would thus be of interest to investigate whether (+)-CBD modulates additional parameters such as microglial morphology and cytokine profiles, analyses we plan to undertake in future studies. Additionally, another perspective we aim to address in follow-up studies is the potential for bidirectional *in vivo* biotransformation between enantiomers, which may influence their activity at cannabinoid receptors and help elucidate a key aspect of the mechanism of action of this non-classical phytocannabinoid.

In summary, this research builds upon previous investigations into the therapeutic potential of CBD in PD (Lastres-Becker et al., 2005; García-Arencibia et al., 2007; Gómez-Gálvez et al., 2016; Alves et al., 2024). Traditionally, the reported beneficial effects of CBD have been assigned to cannabinoid receptor-independent mechanisms, owing to the lack of affinity of the phytocannabinoid for the CB1R and CB2R (Fernández-Ruiz et al., 2013). However, it has long been recognized that changes in CBD stereoisomerism influence its binding profile to these receptors, potentially enhancing its pharmacological properties. Our study further advances this understanding by confirming that (+)-enantiomers exhibit higher affinities for CB1R and especially for CB2R, while also demonstrating their ability to interact with CB2R *in vitro* to induce anti-inflammatory and neuroprotective effects. These effects were also evaluated *in vivo* but only for (+)-CBD and (+)-CBDV, the compounds with the most favourable ADMET properties based on *in silico* predictions. In the murine PD model used, (+)-CBD demonstrated a clear neuroprotective

effect, as evidenced by increased TH immunoreactivity and improved performance in the CRT, although its impact on glial reactivity was less pronounced. While further studies are needed to advance the therapeutic potential of these (+)-enantiomers, our results suggest that (+)-CBD and its analogues may offer protection against neurodegeneration, likely through both CB2R-mediated and receptor-independent mechanisms.

Discussion summary

The findings presented in this thesis advance our understanding of the therapeutic potential of cannabinoids and cannabinoid-related targets in the context of PD. By exploring a range of novel pharmacological strategies, including modulation of the classical CB1R and its intracellular interactor BiP, investigation of the orphan receptor GPR55, and the characterization of structurally modified (+)-CBD enantiomers, we highlight both the complexity and the therapeutic versatility of the ECS. Although CB1R and BiP interventions showed limited efficacy in a proteinopathy-based model of PD, the results obtained from GPR55-deficient mice revealed a sex-dependent neuroprotective and immunomodulatory phenotype, underscoring the relevance of this receptor as a promising therapeutic target, which warrants further investigation. Furthermore, the pharmacological profiling of (+)-CBD and its derivatives demonstrated improved CB2R affinity and *in vitr*o anti-inflammatory and neuroprotective properties, which are also observed *in vivo*, although to a much lesser extent, yet still supported by favourable ADMET predictions. Collectively, these results underscore the value of targeting non-classical components of the ECS or using atypical compounds, while also accounting for variables such as sex differences and receptor stereoselectivity, as a foundation for developing more effective cannabinoid-based therapeutic strategies for PD and related neurodegenerative disorders.

CONCLUSIONS

CHAPTER 1. Cannabinoid type-1 receptor in α-synuclein dysregulation

1. In AAV9-α-syn^{A53T}-lesioned mice, pharmacological activation of CB1R did not confer clear neuroprotection to dopaminergic neurons in the SN nor improve symptoms. However, CB1R modulation influenced degradative pathways and astroglial responses, without detectable changes in microglial or infiltrating macrophage activity, or in the striatal gene expression profile.
2. Partial deficiency of the CB1R-interacting chaperone BiP in AAV9-α-syn^{A53T}-lesioned mice did not significantly affect nigral dopaminergic degeneration, motor function, or glial involvement, although a mild increase in microglial reactivity was observed.

CHAPTER 2. G protein-coupled receptor 55 and Parkinson's disease

3. GPR55 expression remains unchanged in affected brain regions of PD patients and in the striatum of mouse models subjected to intrastriatal 6-OHDA or intranigral of AAV9-α-syn^{A53T} inoculation, in both males and females; however, females displayed a more evident trend towards increased expression.
4. GPR55 appears to play a detrimental role in PD, as its genetic deletion protects dopaminergic neurons in the SN and improves motor performance in two distinct parkinsonian models. This neuroprotective effect was more pronounced in response to AAV9-α-syn^{A53T} and, to a lesser extent, 6-OHDA.
5. The neuroprotective afforded by GPR55 deletion, particularly in the AAV9-α-syn^{A53T} model, may be partly mediated by alterations in glial responses. This includes an increased cell body area-to-branch length ratio in Iba1-positive microglia and reduced astroglial reactivity in the SN, evidenced by decreased colocalisation of C3 and GFAP.
6. Sex-related differences indicate that the neuroprotective and glial modulatory effects of GPR55 deletion are more prominent in female than male mice, suggesting sexual dimorphism in the role of this receptor in PD.

CHAPTER 3. (+)-*trans*-enantiomer of cannabidiol and derivatives against neuroinflammation

7. The (+)-*trans* enantiomeric series of CBD, CBDV, CBDA-Me, CBDA-Gly, and CBDA-Hyp demonstrated enhanced affinity for both cannabinoid receptors compared to their (–)-*trans* counterparts, with notable selectivity for CB2R.
8. While (+)-CBD, (+)-CBDV, and (+)-CBDA-Me act as CB2R agonists, (+)-CBDA-Gly and (+)-CBDA-Hyp function as CB2R antagonists. Additionally, (+)-CBDA-Hyp shows agonistic activity at CB1R.
9. (+)-CBD, (+)-CBDV, and (+)-CBDA-Me exhibited *in vitro* anti-inflammatory effects, leading to neuroprotection in a CB2R-dependent manner.
10. Among compounds with the most favourable *in silico* predicted pharmacokinetics, (+)-CBD and (+)-CBDV showed differing efficacy in the LPS-induced parkinsonian mouse model: (+)-CBDV lacked significant *in vivo* benefits, whereas (+)-CBD elicited marked neuroprotective and symptomatic improvements, despite limited impact on glial reactivity.

BIBLIOGRAPHY

A

Abood, M. E., Rizvi, G., Sallapudi, N., & McAllister, S. D. (2001). Activation of the CB1 cannabinoid receptor protects cultured mouse spinal neurons against excitotoxicity. *Neuroscience Letters*, *309*(3), 197-201. https://doi.org/10.1016/s0304-3940(01)02065-1

Abounit, S., Bousset, L., Loria, F., Zhu, S., de Chaumont, F., Pieri, L., Olivo-Marin, J.-C., Melki, R., & Zurzolo, C. (2016). Tunneling nanotubes spread fibrillar α-synuclein by intercellular trafficking of lysosomes. *The EMBO Journal*, *35*(19), 2120-2138. https://doi.org/10.15252/embj.201593411

Adams, R., Baker, B. R., & Wearn, R. B. (1940). Structure of Cannabinol. III. Synthesis of Cannabinol, 1-Hydroxy-3-n-amyl-6,6,9-trimethyl-6-dibenzopyran[1]. *Journal of the American Chemical Society*, *62*(8), 2204-2207. https://doi.org/10.1021/ja01865a083

Adams, R., Hunt, M., & Clark, J. H. (1940). Structure of Cannabidiol, a Product Isolated from the Marihuana Extract of Minnesota Wild Hemp. I. *Journal of the American Chemical Society*, *62*(1), 196-200. https://doi.org/10.1021/ja01858a058

Aguareles, J., Paraíso-Luna, J., Palomares, B., Bajo-Grañeras, R., Navarrete, C., Ruiz-Calvo, A., García-Rincón, D., García-Taboada, E., Guzmán, M., Muñoz, E., & Galve-Roperh, I. (2019). Oral administration of the cannabigerol derivative VCE-003.2 promotes subventricular zone neurogenesis and protects against mutant huntingtin-induced neurodegeneration. *Translational Neurodegeneration*, *8*, 9. https://doi.org/10.1186/s40035-019-0148-x

Ahlskog, J. E. (2011). Does vigorous exercise have a neuroprotective effect in Parkinson disease? *Neurology*, *77*(3), 288-294. https://doi.org/10.1212/WNL.0b013e318225ab66

Ajalin, R., Al-Abdulrasul, H., Tuisku, J. M., Hirvonen, J., Lahdenpohja, S., Rinne, J. O., & Brück, A. (2024). Impaired Gait, Postural Instability, and Rigidity in Relation to CB1 Receptor Availability in Parkinson's Disease. *Movement Disorders: Official Journal of the Movement Disorder Society*. https://doi.org/10.1002/mds.30042

Ajalin, R. M., Al-Abdulrasul, H., Tuisku, J. M., Hirvonen, J. E. S., Vahlberg, T., Lahdenpohja, S., Rinne, J. O., & Brück, A. E. (2022). Cannabinoid Receptor Type 1 in Parkinson's Disease: A Positron Emission Tomography Study with [18 F]FMPEP-d2. *Movement Disorders: Official Journal of the Movement Disorder Society*, *37*(8), 1673-1682. https://doi.org/10.1002/mds.29117

Akimov, M. G., Gamisonia, A. M., Dudina, P. V., Gretskaya, N. M., Gaydaryova, A. A., Kuznetsov, A. S., Zinchenko, G. N., & Bezuglov, V. V. (2021). GPR55 Receptor Activation by the N-Acyl Dopamine Family Lipids Induces Apoptosis in Cancer Cells via the Nitric Oxide Synthase (nNOS) Over-Stimulation. *International Journal of Molecular Sciences*, *22*(2), 622. https://doi.org/10.3390/ijms22020622

Aladeen, T. S., Mattle, A. G., Zelen, K., Mesha, M., Rainka, M. M., Geist, T., Myers, B., & Mechtler, L. (2023). Medical Cannabis in the Treatment of Parkinson's Disease. *Clinical Neuropharmacology*, *46*(3), 98-104. https://doi.org/10.1097/WNF.0000000000000550

Alexander, G. E., Crutcher, M. D., & DeLong, M. R. (1990). Basal ganglia-thalamocortical circuits: Parallel substrates for motor, oculomotor, «prefrontal» and «limbic» functions. *Progress in Brain Research*, *85*, 119-146.

Altay, M. F., Liu, A. K. L., Holton, J. L., Parkkinen, L., & Lashuel, H. A. (2022). Prominent astrocytic alpha-synuclein pathology with unique post-translational modification signatures unveiled across Lewy body disorders. *Acta Neuropathologica Communications*, *10*(1), 163. https://doi.org/10.1186/s40478-022-01468-8

Alvarez-Erviti, L., Rodriguez-Oroz, M. C., Cooper, J. M., Caballero, C., Ferrer, I., Obeso, J. A., & Schapira, A. H. V. (2010). Chaperone-mediated autophagy markers in Parkinson disease brains. *Archives of Neurology*, *67*(12), 1464-1472. https://doi.org/10.1001/archneurol.2010.198

Alvarez-Fischer, D., Henze, C., Strenzke, C., Westrich, J., Ferger, B., Höglinger, G. U., Oertel, W. H., & Hartmann, A. (2008a). Characterization of the striatal 6-OHDA model of Parkinson's disease in wild type and alpha-synuclein-deleted mice. *Experimental Neurology*, *210*(1), 182-193. https://doi.org/10.1016/j.expneurol.2007.10.012

Alvarez-Fischer, D., Henze, C., Strenzke, C., Westrich, J., Ferger, B., Höglinger, G. U., Oertel, W. H., & Hartmann, A. (2008b). Characterization of the striatal 6-OHDA model of Parkinson's disease in wild type and alpha-synuclein-deleted mice. *Experimental Neurology*, *210*(1), 182-193. https://doi.org/10.1016/j.expneurol.2007.10.012

Alves, A. de D. F., Dias, F. C. R., Cadena, P. G., & Silva-Jr, V. A. (2024). Use of phytocanabinoids in animal models of Parkinson's disease: Systematic review. *Neurotoxicology*, *105*, 34-44. https://doi.org/10.1016/j.neuro.2024.08.002

Ambrosi, G., Cerri, S., & Blandini, F. (2014). A further update on the role of excitotoxicity in the pathogenesis of Parkinson's disease. *Journal of Neural Transmission (Vienna, Austria: 1996)*, *121*(8), 849-859. https://doi.org/10.1007/s00702-013-1149-z

Angelova, P. R., Ludtmann, M. H. R., Horrocks, M. H., Negoda, A., Cremades, N., Klenerman, D., Dobson, C. M., Wood, N. W., Pavlov, E. V., Gandhi, S., & Abramov, A. Y. (2016). Ca2+ is a key factor in α-synuclein-induced neurotoxicity. *Journal of Cell Science*, *129*(9), 1792-1801. https://doi.org/10.1242/jcs.180737

Angulo, J. A., Davis, L. G., Burkhart, B. A., & Christoph, G. R. (1986). Reduction of striatal dopaminergic neurotransmission elevates striatal proenkephalin mRNA. *European Journal of Pharmacology*, *130*(3), 341-343. https://doi.org/10.1016/0014-2999(86)90290-6

Araújo, B., Caridade-Silva, R., Soares-Guedes, C., Martins-Macedo, J., Gomes, E. D., Monteiro, S., & Teixeira, F. G. (2022). Neuroinflammation and Parkinson's Disease-From Neurodegeneration to Therapeutic Opportunities. *Cells*, *11*(18), 2908. https://doi.org/10.3390/cells11182908

Aso, E., Juvés, S., Maldonado, R., & Ferrer, I. (2013). CB2 cannabinoid receptor agonist ameliorates Alzheimer-like phenotype in AβPP/PS1 mice. *Journal of Alzheimer's Disease: JAD*, *35*(4), 847-858. https://doi.org/10.3233/JAD-130137

Aso, E., Palomer, E., Juvés, S., Maldonado, R., Muñoz, F. J., & Ferrer, I. (2012). CB1 agonist ACEA protects neurons and reduces the cognitive impairment of AβPP/PS1 mice. *Journal of Alzheimer's Disease: JAD*, *30*(2), 439-459. https://doi.org/10.3233/JAD-2012-111862

Atalay, S., Jarocka-Karpowicz, I., & Skrzydlewska, E. (2019). Antioxidative and Anti-Inflammatory Properties of Cannabidiol. *Antioxidants (Basel, Switzerland)*, *9*(1), 21. https://doi.org/10.3390/antiox9010021

Athari, S. Z., Farajdokht, F., Sadigh-Eteghad, S., Mohajeri, D., Nourazar, M. A., & Mohaddes, G. (2023). Hydroxychloroquine attenuated motor impairment and oxidative stress in a rat 6-hydroxydopamine model of Parkinson's disease. *The International Journal of Neuroscience*, *133*(11), 1252-1261. https://doi.org/10.1080/00207454.2022.2074848

Atwood, B. K., & Mackie, K. (2010). CB2: A cannabinoid receptor with an identity crisis. *British Journal of Pharmacology*, *160*(3), 467-479. https://doi.org/10.1111/j.1476-5381.2010.00729.x

Ayerra, L., Abellanas, M. A., Basurco, L., Tamayo, I., Conde, E., Tavira, A., Trigo, A., Vidaurre, C., Vilas, A., San Martin-Uriz, P., Luquin, E., Clavero, P., Mengual, E., Hervás-Stubbs, S., & Aymerich, M. S. (2024). Nigrostriatal degeneration determines dynamics of glial inflammatory and phagocytic activity. *Journal of Neuroinflammation*, *21*(1), 92. https://doi.org/10.1186/s12974-024-03091-x

B

Baddenhausen, S., Lutz, B., & Hofmann, C. (2024). Cannabinoid type-1 receptor signaling in dopaminergic Engrailed-1 expressing neurons modulates motivation and depressive-like behavior. *Frontiers in Molecular Neuroscience*, *17*, 1379889. https://doi.org/10.3389/fnmol.2024.1379889

Baek, J.-H., Whitfield, D., Howlett, D., Francis, P., Bereczki, E., Ballard, C., Hortobágyi, T., Attems, J., & Aarsland, D. (2016a). Unfolded protein response is activated in Lewy body dementias. *Neuropathology and Applied Neurobiology*, *42*(4), 352-365. https://doi.org/10.1111/nan.12260

Baek, J.-H., Whitfield, D., Howlett, D., Francis, P., Bereczki, E., Ballard, C., Hortobágyi, T., Attems, J., & Aarsland, D. (2016b). Unfolded protein response is activated in Lewy body dementias. *Neuropathology and Applied Neurobiology*, *42*(4), 352-365. https://doi.org/10.1111/nan.12260

Baker, D., Pryce, G., Davies, W. L., & Hiley, C. R. (2006). In silico patent searching reveals a new cannabinoid receptor. *Trends in Pharmacological Sciences*, *27*(1), 1-4. https://doi.org/10.1016/j.tips.2005.11.003

Balash, Y., Bar-Lev Schleider, L., Korczyn, A. D., Shabtai, H., Knaani, J., Rosenberg, A., Baruch, Y., Djaldetti, R., Giladi, N., & Gurevich, T. (2017). Medical Cannabis in Parkinson Disease: Real-Life Patients' Experience. *Clinical Neuropharmacology*, *40*(6), 268-272. https://doi.org/10.1097/WNF.0000000000000246

Balenga, N. A. B., Henstridge, C. M., Kargl, J., & Waldhoer, M. (2011). Pharmacology, signaling and physiological relevance of the G protein-coupled receptor 55. *Advances in Pharmacology (San Diego, Calif.)*, *62*, 251-277. https://doi.org/10.1016/B978-0-12-385952-5.00004-X

Balenga, N. A., Martínez-Pinilla, E., Kargl, J., Schröder, R., Peinhaupt, M., Platzer, W., Bálint, Z., Zamarbide, M., Dopeso-Reyes, I. G., Ricobaraza, A., Pérez-Ortiz, J. M., Kostenis, E., Waldhoer, M., Heinemann, A., & Franco, R. (2014). Heteromerization of GPR55 and cannabinoid CB2 receptors modulates signalling. *British Journal of Pharmacology*, *171*(23), 5387-5406. https://doi.org/10.1111/bph.12850

Balestrino, R., & Schapira, A. H. V. (2020). Parkinson disease. *European Journal of Neurology*, *27*(1), 27-42. https://doi.org/10.1111/ene.14108

Bao, L.-H., Zhang, Y.-N., Zhang, J.-N., Gu, L., Yang, H.-M., Huang, Y.-Y., Xia, N., & Zhang, H. (2018). Urate inhibits microglia activation to protect neurons in an LPS-induced model of Parkinson's disease. *Journal of Neuroinflammation*, *15*(1), 131. https://doi.org/10.1186/s12974-018-1175-8

Bellocchio, L., Cervino, C., Pasquali, R., & Pagotto, U. (2008). The endocannabinoid system and energy metabolism. *Journal of Neuroendocrinology*, *20*(6), 850-857. https://doi.org/10.1111/j.1365-2826.2008.01728.x

Bénard, G., Massa, F., Puente, N., Lourenço, J., Bellocchio, L., Soria-Gómez, E., Matias, I., Delamarre, A., Metna-Laurent, M., Cannich, A., Hebert-Chatelain, E., Mulle, C., Ortega-Gutiérrez, S., Martín-Fontecha, M., Klugmann, M., Guggenhuber, S., Lutz, B., Gertsch, J., Chaouloff, F., … Marsicano, G. (2012). Mitochondrial CB_1 receptors regulate neuronal energy metabolism. *Nature Neuroscience*, *15*(4), 558-564. https://doi.org/10.1038/nn.3053

Bender, A., Krishnan, K. J., Morris, C. M., Taylor, G. A., Reeve, A. K., Perry, R. H., Jaros, E., Hersheson, J. S., Betts, J., Klopstock, T., Taylor, R. W., & Turnbull, D. M. (2006). High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nature Genetics*, *38*(5), 515-517. https://doi.org/10.1038/ng1769

Benito, C., Núñez, E., Tolón, R. M., Carrier, E. J., Rábano, A., Hillard, C. J., & Romero, J. (2003). Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *23*(35), 11136-11141. https://doi.org/10.1523/JNEUROSCI.23-35-11136.2003

Benito, C., Romero, J. P., Tolón, R. M., Clemente, D., Docagne, F., Hillard, C. J., Guaza, C., & Romero, J. (2007). Cannabinoid CB1 and CB2 receptors and fatty acid amide hydrolase are specific markers of plaque cell subtypes in human multiple sclerosis. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *27*(9), 2396-2402. https://doi.org/10.1523/JNEUROSCI.4814-06.2007

Benito, C., Tolón, R. M., Pazos, M. R., Núñez, E., Castillo, A. I., & Romero, J. (2008). Cannabinoid CB2 receptors in human brain inflammation. *British Journal of Pharmacology*, *153*(2), 277-285. https://doi.org/10.1038/sj.bjp.0707505

Bentea, E., Van der Perren, A., Van Liefferinge, J., El Arfani, A., Albertini, G., Demuyser, T., Merckx, E., Michotte, Y., Smolders, I., Baekelandt, V., & Massie, A. (2015). Nigral proteasome inhibition in mice leads to motor and non-motor deficits and increased expression of Ser129 phosphorylated α-synuclein. *Frontiers in Behavioral Neuroscience*, *9*, 68. https://doi.org/10.3389/fnbeh.2015.00068

Bérard, M., Sheta, R., Malvaut, S., Rodriguez-Aller, R., Teixeira, M., Idi, W., Turmel, R., Alpaugh, M., Dubois, M., Dahmene, M., Salesse, C., Lamontagne-Proulx, J., St-Pierre, M.-K., Tavassoly, O., Luo, W., Del Cid-Pellitero, E., Qazi, R., Jeong, J.-W., Durcan, T. M., … Oueslati, A. (2022). A light-inducible protein clustering system for in vivo analysis of α-synuclein aggregation in Parkinson disease. *PLoS Biology*, *20*(3), e3001578. https://doi.org/10.1371/journal.pbio.3001578

Berwick, D. C., & Harvey, K. (2012). The importance of Wnt signalling for neurodegeneration in Parkinson's disease. *Biochemical Society Transactions*, *40*(5), 1123-1128. https://doi.org/10.1042/BST20120122

Billard, E., Torbey, A., Inserra, A., Grant, E., Bertazzo, A., De Gregorio, D., Comai, S., Chatenet, D., Gobbi, G., & Hébert, T. E. (2025). Pharmacological characterization of cannabidiol as a negative allosteric modulator of the 5-HT2A receptor. *Cellular Signalling*, *127*, 111588. https://doi.org/10.1016/j.cellsig.2025.111588

Billings, J. L., Gordon, S. L., Rawling, T., Doble, P. A., Bush, A. I., Adlard, P. A., Finkelstein, D. I., & Hare, D. J. (2019). L-3,4-dihydroxyphenylalanine (l-DOPA) modulates brain iron, dopaminergic neurodegeneration and motor dysfunction in iron overload and mutant alpha-synuclein mouse models of Parkinson's disease. *Journal of Neurochemistry*, *150*(1), 88-106. https://doi.org/10.1111/jnc.14676

Bilsland, L. G., Dick, J. R. T., Pryce, G., Petrosino, S., Di Marzo, V., Baker, D., & Greensmith, L. (2006). Increasing cannabinoid levels by pharmacological and genetic manipulation delay disease progression in SOD1 mice. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *20*(7), 1003-1005. https://doi.org/10.1096/fj.05-4743fje

Binda, K. H., Chacur, M., & Martins, D. O. (2023). Exercise Improves Orofacial Pain and Modifies Neuropeptide Expression in a Rat Model of Parkinson's Disease. *Neurotoxicity Research*, *41*(5), 459-470. https://doi.org/10.1007/s12640-023-00651-6

Bisogno, T., Hanus, L., De Petrocellis, L., Tchilibon, S., Ponde, D. E., Brandi, I., Moriello, A. S., Davis, J. B., Mechoulam, R., & Di Marzo, V. (2001). Molecular targets for cannabidiol and its synthetic analogues: Effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *British Journal of Pharmacology*, *134*(4), 845-852. https://doi.org/10.1038/sj.bjp.0704327

Bisogno, T., Ligresti, A., & Di Marzo, V. (2005). The endocannabinoid signalling system: Biochemical aspects. *Pharmacology, Biochemistry, and Behavior*, *81*(2), 224-238. https://doi.org/10.1016/j.pbb.2005.01.027

Bjursell, M., Ryberg, E., Wu, T., Greasley, P. J., Bohlooly-Y, M., & Hjorth, S. (2016). Deletion of Gpr55 Results in Subtle Effects on Energy Metabolism, Motor Activity and Thermal Pain Sensation. *PloS One*, *11*(12), e0167965. https://doi.org/10.1371/journal.pone.0167965

Blauwendraat, C., Nalls, M. A., & Singleton, A. B. (2020). The genetic architecture of Parkinson's disease. *The Lancet. Neurology*, *19*(2), 170-178. https://doi.org/10.1016/S1474-4422(19)30287-X

Blázquez, C., Chiarlone, A., Bellocchio, L., Resel, E., Pruunsild, P., García-Rincón, D., Sendtner, M., Timmusk, T., Lutz, B., Galve-Roperh, I., & Guzmán, M. (2015). The CB_1 cannabinoid receptor signals striatal neuroprotection via a PI3K/Akt/mTORC1/BDNF pathway. *Cell Death and Differentiation*, *22*(10), 1618-1629. https://doi.org/10.1038/cdd.2015.11

Bloem, B. R., Okun, M. S., & Klein, C. (2021). Parkinson's disease. *Lancet (London, England)*, *397*(10291), 2284-2303. https://doi.org/10.1016/S0140-6736(21)00218-X

Bolam, J. P., & Pissadaki, E. K. (2012). Living on the edge with too many mouths to feed: Why dopamine neurons die. *Movement Disorders: Official Journal of the Movement Disorder Society*, *27*(12), 1478-1483. https://doi.org/10.1002/mds.25135

Borgkvist, A., Marcellino, D., Fuxe, K., Greengard, P., & Fisone, G. (2008). Regulation of DARPP-32 phosphorylation by Delta9-tetrahydrocannabinol. *Neuropharmacology*, *54*(1), 31-35. https://doi.org/10.1016/j.neuropharm.2007.06.025

Borowska, M., Czarnywojtek, A., Sawicka-Gutaj, N., Woliński, K., Płazińska, M. T., Mikołajczak, P., & Ruchała, M. (2018). The effects of cannabinoids on the endocrine system. *Endokrynologia Polska*, *69*(6), 705-719. https://doi.org/10.5603/EP.a2018.0072

Bosquez-Berger, T., Wilson, S., Iliopoulos-Tsoutsouvas, C., Jiang, S., Wager-Miller, J., Nikas, S. P., Mackie, K. P., Makriyannis, A., & Straiker, A. (2022). Differential Enantiomer-Specific Signaling of Cannabidiol at CB1 Receptors. *Molecular Pharmacology*, *102*(6), 259-268. https://doi.org/10.1124/molpharm.121.000305

Bouaboula, M., Poinot-Chazel, C., Bourrié, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., & Casellas, P. (1995). Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *The Biochemical Journal*, *312 (Pt 2)*(Pt 2), 637-641. https://doi.org/10.1042/bj3120637

Braak, H., Del Tredici, K., Rüb, U., de Vos, R. A. I., Jansen Steur, E. N. H., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of Aging*, *24*(2), 197-211. https://doi.org/10.1016/s0197-4580(02)00065-9

Brailoiu, G. C., Deliu, E., Marcu, J., Hoffman, N. E., Console-Bram, L., Zhao, P., Madesh, M., Abood, M. E., & Brailoiu, E. (2014). Differential activation of intracellular versus plasmalemmal CB2 cannabinoid receptors. *Biochemistry*, *53*(30), 4990-4999. https://doi.org/10.1021/bi500632a

Breuer, A., Haj, C. G., Fogaça, M. V., Gomes, F. V., Silva, N. R., Pedrazzi, J. F., Del Bel, E. A., Hallak, J. C., Crippa, J. A., Zuardi, A. W., Mechoulam, R., & Guimarães, F. S. (2016). Fluorinated Cannabidiol Derivatives: Enhancement of Activity in Mice Models Predictive of Anxiolytic, Antidepressant and Antipsychotic Effects. *PloS One*, *11*(7), e0158779. https://doi.org/10.1371/journal.pone.0158779

Brooks, D. J., & Pavese, N. (2011). Imaging biomarkers in Parkinson's disease. *Progress in Neurobiology*, *95*(4), 614-628. https://doi.org/10.1016/j.pneurobio.2011.08.009

Bullock, T. A., Galpayage Dona, K. N. U., Hale, J. F., Morales, P., Jagerovic, N., Andrews, A. M., & Ramirez, S. H. (2023). Activation of CB2R by synthetic CB2R agonist, PM289, improves brain endothelial barrier properties, decreases inflammatory response and enhances endothelial repair. *NeuroImmune Pharmacology and Therapeutics*, *2*(4), 387-400. https://doi.org/10.1515/nipt-2023-0016

Burgaz, S., García, C., Gómez-Cañas, M., Muñoz, E., & Fernández-Ruiz, J. (2019). Development of An Oral Treatment with the PPAR-γ-Acting Cannabinoid VCE-003.2 Against the Inflammation-Driven Neuronal Deterioration in Experimental Parkinson's Disease. *Molecules (Basel, Switzerland)*, *24*(15), 2702. https://doi.org/10.3390/molecules24152702

Burgaz, S., García, C., Gómez-Cañas, M., Navarrete, C., García-Martín, A., Rolland, A., Del Río, C., Casarejos, M. J., Muñoz, E., Gonzalo-Consuegra, C., Muñoz, E., & Fernández-Ruiz, J. (2021). Neuroprotection with the cannabigerol quinone derivative VCE-003.2 and its analogs CBGA-Q and CBGA-Q-Salt in Parkinson's disease using 6-hydroxydopamine-lesioned mice. *Molecular and Cellular Neurosciences*, *110*, 103583. https://doi.org/10.1016/j.mcn.2020.103583

Burgaz, S., García, C., Gómez-Cañas, M., Rolland, A., Muñoz, E., & Fernández-Ruiz, J. (2021). Neuroprotection with the Cannabidiol Quinone Derivative VCE-004.8 (EHP-101) against 6-Hydroxydopamine in Cell and Murine Models of Parkinson's Disease. *Molecules (Basel, Switzerland)*, *26*(11), 3245. https://doi.org/10.3390/molecules26113245

Burgaz, S., García, C., Gonzalo-Consuegra, C., Gómez-Almería, M., Ruiz-Pino, F., Unciti, J. D., Gómez-Cañas, M., Alcalde, J., Morales, P., Jagerovic, N., Rodríguez-Cueto, C., de Lago, E., Muñoz, E., & Fernández-Ruiz, J. (2021). Preclinical Investigation in Neuroprotective Effects of the GPR55 Ligand VCE-006.1 in Experimental Models of Parkinson's Disease and Amyotrophic Lateral Sclerosis. *Molecules (Basel, Switzerland)*, *26*(24), 7643. https://doi.org/10.3390/molecules26247643

Burgaz, S., Navarro, E., Rodríguez-Carreiro, S., Navarrete, C., Garrido-Rodríguez, M., Lastres-Becker, I., Chocarro, J., Lanciego, J. L., Muñoz, E., & Fernández-Ruiz, J. (2024). Investigation in the cannabigerol derivative VCE-003.2 as a disease-modifying agent in a mouse model of experimental synucleinopathy. *Behavioral and Brain Functions: BBF*, *20*(1), 28. https://doi.org/10.1186/s12993-024-00256-9

C

Calatayud, C., Carola, G., Consiglio, A., & Raya, A. (2017). Modeling the genetic complexity of Parkinson's disease by targeted genome edition in iPS cells. *Current Opinion in Genetics & Development*, *46*, 123-131. https://doi.org/10.1016/j.gde.2017.06.002

Callén, L., Moreno, E., Barroso-Chinea, P., Moreno-Delgado, D., Cortés, A., Mallol, J., Casadó, V., Lanciego, J. L., Franco, R., Lluis, C., Canela, E. I., & McCormick, P. J. (2012). Cannabinoid receptors CB1 and CB2 form functional heteromers in brain. *The Journal of Biological Chemistry*, *287*(25), 20851-20865. https://doi.org/10.1074/jbc.M111.335273

Caltana, L., Saez, T. M., Aronne, M. P., & Brusco, A. (2015). Cannabinoid receptor type 1 agonist ACEA improves motor recovery and protects neurons in ischemic stroke in mice. *Journal of Neurochemistry*, *135*(3), 616-629. https://doi.org/10.1111/jnc.13288

Cankara, F. N., Çelik, Z. B., & Günaydın, C. (2021). Cannabinoid receptor-1 has an effect on CD200 under rotenone and alpha-synuclein induced stress. *Neuroscience Letters*, *755*, 135908. https://doi.org/10.1016/j.neulet.2021.135908

Cao, X., Liang, L., Hadcock, J. R., Iredale, P. A., Griffith, D. A., Menniti, F. S., Factor, S., Greenamyre, J. T., & Papa, S. M. (2007). Blockade of cannabinoid type 1 receptors augments the antiparkinsonian action of levodopa without affecting dyskinesias in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated rhesus monkeys. *The Journal of Pharmacology and Experimental Therapeutics*, *323*(1), 318-326. https://doi.org/10.1124/jpet.107.125666

Carballo-Carbajal, I., Laguna, A., Romero-Giménez, J., Cuadros, T., Bové, J., Martinez-Vicente, M., Parent, A., Gonzalez-Sepulveda, M., Peñuelas, N., Torra, A., Rodríguez-Galván, B., Ballabio, A., Hasegawa, T., Bortolozzi, A., Gelpi, E., & Vila, M. (2019). Brain tyrosinase overexpression implicates age-dependent neuromelanin production in Parkinson's disease pathogenesis. *Nature Communications*, *10*(1), 973. https://doi.org/10.1038/s41467-019-08858-y

Carbone, C., Costa, A., Provensi, G., Mannaioni, G., & Masi, A. (2017). The Hyperpolarization-Activated Current Determines Synaptic Excitability, Calcium Activity and Specific Viability of Substantia Nigra Dopaminergic Neurons. *Frontiers in Cellular Neuroscience*, *11*, 187. https://doi.org/10.3389/fncel.2017.00187

Carriba, P., Ortiz, O., Patkar, K., Justinova, Z., Stroik, J., Themann, A., Müller, C., Woods, A. S., Hope, B. T., Ciruela, F., Casadó, V., Canela, E. I., Lluis, C., Goldberg, S. R., Moratalla, R., Franco, R., & Ferré, S. (2007). Striatal adenosine A2A and cannabinoid CB1 receptors form functional heteromeric complexes that mediate the motor effects of cannabinoids. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *32*(11), 2249-2259. https://doi.org/10.1038/sj.npp.1301375

Carroll, C. B., Bain, P. G., Teare, L., Liu, X., Joint, C., Wroath, C., Parkin, S. G., Fox, P., Wright, D., Hobart, J., & Zajicek, J. P. (2004). Cannabis for dyskinesia in Parkinson disease: A randomized double-blind crossover study. *Neurology*, *63*(7), 1245-1250. https://doi.org/10.1212/01.wnl.0000140288.48796.8e

Carta, A. R., Pisanu, A., & Carboni, E. (2011). Do PPAR-Gamma Agonists Have a Future in Parkinson's Disease Therapy? *Parkinson's Disease*, *2011*, 689181. https://doi.org/10.4061/2011/689181

Casarejos, M. J., Perucho, J., Gomez, A., Muñoz, M. P., Fernandez-Estevez, M., Sagredo, O., Fernandez Ruiz, J., Guzman, M., de Yebenes, J. G., & Mena, M. A. (2013). Natural cannabinoids improve dopamine neurotransmission and tau and amyloid pathology in a mouse model of tauopathy. *Journal of Alzheimer's Disease: JAD*, *35*(3), 525-539. https://doi.org/10.3233/JAD-130050

Castillo, A., Tolón, M. R., Fernández-Ruiz, J., Romero, J., & Martinez-Orgado, J. (2010). The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB(2) and adenosine receptors. *Neurobiology of Disease*, *37*(2), 434-440. https://doi.org/10.1016/j.nbd.2009.10.023

Castillo, P. E., Younts, T. J., Chávez, A. E., & Hashimotodani, Y. (2012). Endocannabinoid signaling and synaptic function. *Neuron*, *76*(1), 70-81. https://doi.org/10.1016/j.neuron.2012.09.020

Castro-Sánchez, S., García-Yagüe, Á. J., López-Royo, T., Casarejos, M., Lanciego, J. L., & Lastres-Becker, I. (2018). Cx3cr1-deficiency exacerbates alpha-synuclein-A53T induced neuroinflammation and neurodegeneration in a mouse model of Parkinson's disease. *Glia*, *66*(8), 1752-1762. https://doi.org/10.1002/glia.23338

Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I., & Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science (New York, N.Y.)*, *288*(5464), 306-313. https://doi.org/10.1126/science.288.5464.306

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., & Julius, D. (1997). The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature*, *389*(6653), 816-824. https://doi.org/10.1038/39807

Cebrián, C., Zucca, F. A., Mauri, P., Steinbeck, J. A., Studer, L., Scherzer, C. R., Kanter, E., Budhu, S., Mandelbaum, J., Vonsattel, J. P., Zecca, L., Loike, J. D., & Sulzer, D. (2014). MHC-I expression renders catecholaminergic neurons susceptible to T-cell-mediated degeneration. *Nature Communications*, *5*, 3633. https://doi.org/10.1038/ncomms4633

Celorrio, M., Fernández-Suárez, D., Rojo-Bustamante, E., Echeverry-Alzate, V., Ramírez, M. J., Hillard, C. J., López-Moreno, J. A., Maldonado, R., Oyarzábal, J., Franco, R., & Aymerich, M. S. (2016). Fatty acid amide hydrolase inhibition for the symptomatic relief of Parkinson's disease. *Brain, Behavior, and Immunity*, *57*, 94-105. https://doi.org/10.1016/j.bbi.2016.06.010

Celorrio, M., Rojo-Bustamante, E., Fernández-Suárez, D., Sáez, E., Estella-Hermoso de Mendoza, A., Müller, C. E., Ramírez, M. J., Oyarzábal, J., Franco, R., & Aymerich, M. S. (2017). GPR55: A therapeutic target for Parkinson's disease? *Neuropharmacology*, *125*, 319-332. https://doi.org/10.1016/j.neuropharm.2017.08.017

Chagas, M. H. N., Eckeli, A. L., Zuardi, A. W., Pena-Pereira, M. A., Sobreira-Neto, M. A., Sobreira, E. T., Camilo, M. R., Bergamaschi, M. M., Schenck, C. H., Hallak, J. E. C., Tumas, V., & Crippa, J. a. S. (2014). Cannabidiol can improve complex sleep-related behaviours associated with rapid eye movement sleep behaviour disorder in Parkinson's disease patients: A case series. *Journal of Clinical Pharmacy and Therapeutics*, *39*(5), 564-566. https://doi.org/10.1111/jcpt.12179

Chagas, M. H. N., Zuardi, A. W., Tumas, V., Pena-Pereira, M. A., Sobreira, E. T., Bergamaschi, M. M., dos Santos, A. C., Teixeira, A. L., Hallak, J. E. C., & Crippa, J. A. S. (2014). Effects of cannabidiol in the treatment of patients with Parkinson's disease: An exploratory double-blind trial. *Journal of Psychopharmacology (Oxford, England)*, *28*(11), 1088-1098. https://doi.org/10.1177/0269881114550355

Chahl, L. A. (2024). TRPV1 Channels in the Central Nervous System as Drug Targets. *Pharmaceuticals (Basel, Switzerland)*, *17*(6), 756. https://doi.org/10.3390/ph17060756

Chakrabarti, S., & Bisaglia, M. (2023). Oxidative Stress and Neuroinflammation in Parkinson's Disease: The Role of Dopamine Oxidation Products. *Antioxidants (Basel, Switzerland)*, *12*(4), 955. https://doi.org/10.3390/antiox12040955

Chandran, J. S., Lin, X., Zapata, A., Höke, A., Shimoji, M., Moore, S. O., Galloway, M. P., Laird, F. M., Wong, P. C., Price, D. L., Bailey, K. R., Crawley, J. N., Shippenberg, T., & Cai, H. (2008). Progressive behavioral deficits in DJ-1-deficient mice are associated with normal nigrostriatal function. *Neurobiology of Disease*, *29*(3), 505-514. https://doi.org/10.1016/j.nbd.2007.11.011

Chang, E. E. S., Ho, P. W.-L., Liu, H.-F., Pang, S. Y.-Y., Leung, C.-T., Malki, Y., Choi, Z. Y.-K., Ramsden, D. B., & Ho, S.-L. (2022). LRRK2 mutant knock-in mouse models: Therapeutic relevance in Parkinson's disease. *Translational Neurodegeneration*, *11*(1), 10. https://doi.org/10.1186/s40035-022-00285-2

Chavarría, C., Rodríguez-Bottero, S., Quijano, C., Cassina, P., & Souza, J. M. (2018). Impact of monomeric, oligomeric and fibrillar alpha-synuclein on astrocyte reactivity and toxicity to neurons. *The Biochemical Journal*, *475*(19), 3153-3169. https://doi.org/10.1042/BCJ20180297

Chen, J., Hou, C., Chen, X., Wang, D., Yang, P., He, X., Zhou, J., & Li, H. (2016). Protective effect of cannabidiol on hydrogen peroxide-induced apoptosis, inflammation and oxidative stress in nucleus pulposus cells. *Molecular Medicine Reports*, *14*(3), 2321-2327. https://doi.org/10.3892/mmr.2016.5513

Chen, R., Gu, X., & Wang, X. (2022). α-Synuclein in Parkinson's disease and advances in detection. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *529*, 76-86. https://doi.org/10.1016/j.cca.2022.02.006

Cheng, Y., & Prusoff, W. H. (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochemical Pharmacology*, *22*(23), 3099-3108. https://doi.org/10.1016/0006-2952(73)90196-2

Cherif, H., Argaw, A., Cécyre, B., Bouchard, A., Gagnon, J., Javadi, P., Desgent, S., Mackie, K., & Bouchard, J.-F. (2015a). Role of GPR55 during Axon Growth and Target Innervation. *eNeuro*, *2*(5), ENEURO.0011-15.2015. https://doi.org/10.1523/ENEURO.0011-15.2015

Cherif, H., Argaw, A., Cécyre, B., Bouchard, A., Gagnon, J., Javadi, P., Desgent, S., Mackie, K., & Bouchard, J.-F. (2015b). Role of GPR55 during Axon Growth and Target Innervation. *eNeuro*, *2*(5), ENEURO.0011-15.2015. https://doi.org/10.1523/ENEURO.0011-15.2015

Chesselet, M.-F., Richter, F., Zhu, C., Magen, I., Watson, M. B., & Subramaniam, S. R. (2012). A progressive mouse model of Parkinson's disease: The Thy1-aSyn («Line 61») mice. *Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics*, *9*(2), 297-314. https://doi.org/10.1007/s13311-012-0104-2

Chiarlone, A., Bellocchio, L., Blázquez, C., Resel, E., Soria-Gómez, E., Cannich, A., Ferrero, J. J., Sagredo, O., Benito, C., Romero, J., Sánchez-Prieto, J., Lutz, B., Fernández-Ruiz, J., Galve-Roperh, I., & Guzmán, M. (2014). A restricted population of CB1 cannabinoid receptors with neuroprotective activity. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(22), 8257-8262. https://doi.org/10.1073/pnas.1400988111

Chiba, T., Ueno, S., Obara, Y., & Nakahata, N. (2011). A synthetic cannabinoid, CP55940, inhibits lipopolysaccharide-induced cytokine mRNA expression in a cannabinoid receptor-independent mechanism in rat cerebellar granule cells. *The Journal of Pharmacy and Pharmacology*, *63*(5), 636-647. https://doi.org/10.1111/j.2042-7158.2011.01250.x

Chiurchiù, V., Lanuti, M., De Bardi, M., Battistini, L., & Maccarrone, M. (2015). The differential characterization of GPR55 receptor in human peripheral blood reveals a distinctive expression in monocytes and NK cells and a proinflammatory role in these innate cells. *International Immunology*, *27*(3), 153-160. https://doi.org/10.1093/intimm/dxu097

Choi, D.-Y., Liu, M., Hunter, R. L., Cass, W. A., Pandya, J. D., Sullivan, P. G., Shin, E.-J., Kim, H.-C., Gash, D. M., & Bing, G. (2009). Striatal neuroinflammation promotes Parkinsonism in rats. *PloS One*, *4*(5), e5482. https://doi.org/10.1371/journal.pone.0005482

Choong, C.-J., Aguirre, C., Kakuda, K., Beck, G., Nakanishi, H., Kimura, Y., Shimma, S., Nabekura, K., Hideshima, M., Doi, J., Yamaguchi, K., Nakajima, K., Wadayama, T., Hayakawa, H., Baba, K., Ogawa, K., Takeuchi, T., Badawy, S. M. M., Murayama, S., … Ikenaka, K. (2023). Phosphatidylinositol-3,4,5-trisphosphate interacts with alpha-synuclein and initiates its aggregation and formation of Parkinson's disease-related fibril polymorphism. *Acta Neuropathologica*, *145*(5), 573-595. https://doi.org/10.1007/s00401-023-02555-3

Chrétien, F., Le Pavec, G., Vallat-Decouvelaere, A.-V., Delisle, M.-B., Uro-Coste, E., Ironside, J. W., Gambetti, P., Parchi, P., Créminon, C., Dormont, D., Mikol, J., Gray, F., & Gras, G. (2004). Expression of excitatory amino acid transporter-1 (EAAT-1) in brain macrophages and microglia of patients with prion diseases. *Journal of Neuropathology and Experimental Neurology*, *63*(10), 1058-1071. https://doi.org/10.1093/jnen/63.10.1058

Chritin, M., Blanchard, V., Raisman-Vozari, R., Feuerstein, C., Agid, Y., Javoy-Agid, F., & Savasta, M. (1996). DA uptake sites, D1 and D2 receptors, D2 and preproenkephalin mRNAs and Fos immunoreactivity in rat striatal subregions after partial dopaminergic degeneration. *The European Journal of Neuroscience*, *8*(12), 2511-2520. https://doi.org/10.1111/j.1460-9568.1996.tb01545.x

Chrysagis, N., Trompouki, G., Petropaulis, D., Koumantakis, G. A., Krekoukias, G., Theotokatos, G., Skordilis, E., & Sakellari, V. (2024). Effect of Boxing Exercises on the Functional Ability and Quality of Life of Individuals with Parkinson's Disease: A Systematic Review. *European Journal of Investigation in Health, Psychology and Education*, *14*(5), 1295-1310. https://doi.org/10.3390/ejihpe14050085

Chung, H. K., Ho, H.-A., Pérez-Acuña, D., & Lee, S.-J. (2019). Modeling α-Synuclein Propagation with Preformed Fibril Injections. *Journal of Movement Disorders*, *12*(3), 139-151. https://doi.org/10.14802/jmd.19046

Chung, Y. C., Bok, E., Huh, S. H., Park, J.-Y., Yoon, S.-H., Kim, S. R., Kim, Y.-S., Maeng, S., Park, S. H., & Jin, B. K. (2011). Cannabinoid receptor type 1 protects nigrostriatal dopaminergic neurons against MPTP neurotoxicity by inhibiting microglial activation. *Journal of Immunology (Baltimore, Md.: 1950)*, *187*(12), 6508-6517. https://doi.org/10.4049/jimmunol.1102435

Church, F. C. (2021). Treatment Options for Motor and Non-Motor Symptoms of Parkinson's Disease. *Biomolecules*, *11*(4), 612. https://doi.org/10.3390/biom11040612

Concannon, R. M., Okine, B. N., Finn, D. P., & Dowd, E. (2015). Differential upregulation of the cannabinoid CB_2 receptor in neurotoxic and inflammation-driven rat models of Parkinson's disease. *Experimental Neurology*, *269*, 133-141. https://doi.org/10.1016/j.expneurol.2015.04.007

Concannon, R. M., Okine, B. N., Finn, D. P., & Dowd, E. (2016). Upregulation of the cannabinoid CB2 receptor in environmental and viral inflammation-driven rat models of Parkinson's disease. *Experimental Neurology*, *283*(Pt A), 204-212. https://doi.org/10.1016/j.expneurol.2016.06.014

Cookson, M. R. (2015). LRRK2 Pathways Leading to Neurodegeneration. *Current Neurology and Neuroscience Reports*, *15*(7), 42. https://doi.org/10.1007/s11910-015-0564-y

Costa, C. A. da, Manaa, W. E., Duplan, E., & Checler, F. (2020). The Endoplasmic Reticulum Stress/Unfolded Protein Response and Their Contributions to Parkinson's Disease Physiopathology. *Cells*, *9*(11), 2495. https://doi.org/10.3390/cells9112495

Costa, L., Amaral, C., Teixeira, N., Correia-da-Silva, G., & Fonseca, B. M. (2016). Cannabinoid-induced autophagy: Protective or death role? *Prostaglandins & Other Lipid Mediators*, *122*, 54-63. https://doi.org/10.1016/j.prostaglandins.2015.12.006

Costas-Insua, C., Hermoso-López, A., Moreno, E., Montero-Fernández, C., Álvaro-Blázquez, A., Maroto, I. B., Sánchez-Ruiz, A., Diez-Alarcia, R., Blázquez, C., Morales, P., Canela, E. I., Casadó, V., Urigüen, L., Perea, G., Bellocchio, L., Rodríguez-Crespo, I., & Guzmán, M. (2024). The CB1 receptor interacts with cereblon and drives cereblon deficiency-associated memory shortfalls. *EMBO Molecular Medicine*, *16*(4), 755-783. https://doi.org/10.1038/s44321-024-00054-w

Costas-Insua, C., Moreno, E., Maroto, I. B., Ruiz-Calvo, A., Bajo-Grañeras, R., Martín-Gutiérrez, D., Diez-Alarcia, R., Vilaró, M. T., Cortés, R., García-Font, N., Martín, R., Espina, M., Botta, J., Ginés, S., McCormick, P. J., Sánchez-Prieto, J., Galve-Roperh, I., Mengod, G., Urigüen, L., … Guzmán, M. (2021). Identification of BiP as a CB1 Receptor-Interacting Protein That Fine-Tunes Cannabinoid Signaling in the Mouse Brain. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *41*(38), 7924-7941. https://doi.org/10.1523/JNEUROSCI.0821-21.2021

Cotzias, G. C., Papavasiliou, P. S., & Gellene, R. (1969). Modification of Parkinsonism—Chronic treatment with L-dopa. *The New England Journal of Medicine*, *280*(7), 337-345. https://doi.org/10.1056/NEJM196902132800701

Craft, R. M., Marusich, J. A., & Wiley, J. L. (2013). Sex differences in cannabinoid pharmacology: A reflection of differences in the endocannabinoid system? *Life Sciences*, *92*(8-9), 476-481. https://doi.org/10.1016/j.lfs.2012.06.009

Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., & Gilula, N. B. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature*, *384*(6604), 83-87. https://doi.org/10.1038/384083a0

Cravatt, B. F., & Lichtman, A. H. (2004). The endogenous cannabinoid system and its role in nociceptive behavior. *Journal of Neurobiology*, *61*(1), 149-160. https://doi.org/10.1002/neu.20080

Crespo-Castrillo, A., Arévalo, M. Á., García-Segura, L. M., & Yanguas-Casás, N. (2020). *Estrogenic Regulation of Glia and Neuroinflammation*. https://doi.org/10.13039/100012619

D

Damier, P., Hirsch, E. C., Agid, Y., & Graybiel, A. M. (1999). The substantia nigra of the human brain. II. Patterns of loss of dopamine-containing neurons in Parkinson's disease. *Brain: A Journal of Neurology*, *122 (Pt 8)*, 1437-1448. https://doi.org/10.1093/brain/122.8.1437

D'Autréaux, B., & Toledano, M. B. (2007). ROS as signalling molecules: Mechanisms that generate specificity in ROS homeostasis. *Nature Reviews. Molecular Cell Biology*, *8*(10), 813-824. https://doi.org/10.1038/nrm2256

Davin, A., Chabardès, S., Belaid, H., Fagret, D., Djaileb, L., Dauvilliers, Y., David, O., Torres-Martinez, N., & Piallat, B. (2022). Early onset of sleep/wake disturbances in a progressive macaque model of Parkinson's disease. *Scientific Reports*, *12*(1), 17499. https://doi.org/10.1038/s41598-022-22381-z

Davis, M. I., Crittenden, J. R., Feng, A. Y., Kupferschmidt, D. A., Naydenov, A., Stella, N., Graybiel, A. M., & Lovinger, D. M. (2018). The cannabinoid-1 receptor is abundantly expressed in striatal striosomes and striosome-dendron bouquets of the substantia nigra. *PloS One*, *13*(2), e0191436. https://doi.org/10.1371/journal.pone.0191436

Davis, M. I., Ronesi, J., & Lovinger, D. M. (2003). A predominant role for inhibition of the adenylate cyclase/protein kinase A pathway in ERK activation by cannabinoid receptor 1 in N1E-115 neuroblastoma cells. *The Journal of Biological Chemistry*, *278*(49), 48973-48980. https://doi.org/10.1074/jbc.M305697200

De Biase, L. M., Schuebel, K. E., Fusfeld, Z. H., Jair, K., Hawes, I. A., Cimbro, R., Zhang, H.-Y., Liu, Q.-R., Shen, H., Xi, Z.-X., Goldman, D., & Bonci, A. (2017). Local Cues Establish and Maintain Region-Specific Phenotypes of Basal Ganglia Microglia. *Neuron*, *95*(2), 341-356.e6. https://doi.org/10.1016/j.neuron.2017.06.020

de la Rosa, T., Calvo, V. S., Gonçalves, V. C., Ferreira, C. B., Cabral, L. M., Souza, F. da C., Scerni, D. A., Scorza, F. A., Moreira, T. S., & Takakura, A. C. (2022). Respiratory deficits in a female rat model of Parkinson's disease. *Experimental Physiology*, *107*(11), 1349-1359. https://doi.org/10.1113/EP090378

de Lau, L. M. L., & Breteler, M. M. B. (2006). Epidemiology of Parkinson's disease. *The Lancet. Neurology*, *5*(6), 525-535. https://doi.org/10.1016/S1474-4422(06)70471-9

de Mattos, B. A., Bonato, J. M., Splendor, M. C., Del Bel, E., Milani, H., & de Oliveira, R. M. W. (2024). Cannabidiol improves non-motor symptoms, attenuates neuroinflammation, and favours hippocampal newborn neuronal maturation in a rat model of Parkinsonism. *Acta Neuropsychiatrica*, *36*(5), 307-319. https://doi.org/10.1017/neu.2024.15

De Meij, J., Alfanek, Z., Morel, L., Decoeur, F., Leyrolle, Q., Picard, K., Carrier, M., Aubert, A., Séré, A., Lucas, C., Laforest, G., Helbling, J.-C., Tremblay, M.-E., Cota, D., Moisan, M.-P., Marsicano, G., Layé, S., & Nadjar, A. (2021). Microglial Cannabinoid Type 1 Receptor Regulates Brain Inflammation in a Sex-Specific Manner. *Cannabis and Cannabinoid Research*, *6*(6), 488-507. https://doi.org/10.1089/can.2020.0170

De Miranda, B. R., Goldman, S. M., Miller, G. W., Greenamyre, J. T., & Dorsey, E. R. (2022). Preventing Parkinson's Disease: An Environmental Agenda. *Journal of Parkinson's Disease*, *12*(1), 45-68. https://doi.org/10.3233/JPD-212922

De Petrocellis, L., Ligresti, A., Moriello, A. S., Allarà, M., Bisogno, T., Petrosino, S., Stott, C. G., & Di Marzo, V. (2011). Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. *British Journal of Pharmacology*, *163*(7), 1479-1494. https://doi.org/10.1111/j.1476-5381.2010.01166.x

De Petrocellis, L., Nabissi, M., Santoni, G., & Ligresti, A. (2017). Actions and Regulation of Ionotropic Cannabinoid Receptors. *Advances in Pharmacology (San Diego, Calif.)*, *80*, 249-289. https://doi.org/10.1016/bs.apha.2017.04.001

Deas, E., Cremades, N., Angelova, P. R., Ludtmann, M. H. R., Yao, Z., Chen, S., Horrocks, M. H., Banushi, B., Little, D., Devine, M. J., Gissen, P., Klenerman, D., Dobson, C. M., Wood, N. W., Gandhi, S., & Abramov, A. Y. (2016). Alpha-Synuclein Oligomers Interact with Metal Ions to Induce Oxidative Stress and Neuronal Death in Parkinson's Disease. *Antioxidants & Redox Signaling*, *24*(7), 376-391. https://doi.org/10.1089/ars.2015.6343

del Río, C., Navarrete, C., Collado, J. A., Bellido, M. L., Gómez-Cañas, M., Pazos, M. R., Fernández-Ruiz, J., Pollastro, F., Appendino, G., Calzado, M. A., Cantarero, I., & Muñoz, E. (2016). The cannabinoid quinol VCE-004.8 alleviates bleomycin-induced scleroderma and exerts potent antifibrotic effects through peroxisome proliferator-activated receptor-γ and CB2 pathways. *Scientific Reports*, *6*, 21703. https://doi.org/10.1038/srep21703

Deng, I., Corrigan, F., Zhai, G., Zhou, X.-F., & Bobrovskaya, L. (2020). Lipopolysaccharide animal models of Parkinson's disease: Recent progress and relevance to clinical disease. *Brain, Behavior, & Immunity - Health*, *4*, 100060. https://doi.org/10.1016/j.bbih.2020.100060

Devane, W. A., Dysarz, F. A., Johnson, M. R., Melvin, L. S., & Howlett, A. C. (1988). Determination and characterization of a cannabinoid receptor in rat brain. *Molecular Pharmacology*, *34*(5), 605-613.

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., & Mechoulam, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (New York, N.Y.)*, *258*(5090), 1946-1949. https://doi.org/10.1126/science.1470919

Devinsky, O., Cross, J. H., & Wright, S. (2017). Trial of Cannabidiol for Drug-Resistant Seizures in the Dravet Syndrome. *The New England Journal of Medicine*, *377*(7), 699-700. https://doi.org/10.1056/NEJMc1708349

DeVuono, M. V., La Caprara, O., Petrie, G. N., Limebeer, C. L., Rock, E. M., Hill, M. N., & Parker, L. A. (2022). Cannabidiol Interferes with Establishment of Δ9-Tetrahydrocannabinol-Induced Nausea Through a 5-HT1A Mechanism. *Cannabis and Cannabinoid Research*, *7*(1), 58-64. https://doi.org/10.1089/can.2020.0083

di Domenico, A., Carola, G., Calatayud, C., Pons-Espinal, M., Muñoz, J. P., Richaud-Patin, Y., Fernandez-Carasa, I., Gut, M., Faella, A., Parameswaran, J., Soriano, J., Ferrer, I., Tolosa, E., Zorzano, A., Cuervo, A. M., Raya, A., & Consiglio, A. (2019). Patient-Specific iPSC-Derived Astrocytes Contribute to Non-Cell-Autonomous Neurodegeneration in Parkinson's Disease. *Stem Cell Reports*, *12*(2), 213-229. https://doi.org/10.1016/j.stemcr.2018.12.011

Di Maio, R., Barrett, P. J., Hoffman, E. K., Barrett, C. W., Zharikov, A., Borah, A., Hu, X., McCoy, J., Chu, C. T., Burton, E. A., Hastings, T. G., & Greenamyre, J. T. (2016). α-Synuclein binds to TOM20 and inhibits mitochondrial protein import in Parkinson's disease. *Science Translational Medicine*, *8*(342), 342ra78. https://doi.org/10.1126/scitranslmed.aaf3634

Di Maio, R., Hoffman, E. K., Rocha, E. M., Keeney, M. T., Sanders, L. H., De Miranda, B. R., Zharikov, A., Van Laar, A., Stepan, A. F., Lanz, T. A., Kofler, J. K., Burton, E. A., Alessi, D. R., Hastings, T. G., & Greenamyre, J. T. (2018). LRRK2 activation in idiopathic Parkinson's disease. *Science Translational Medicine*, *10*(451), eaar5429. https://doi.org/10.1126/scitranslmed.aar5429

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C., & Piomelli, D. (1994). Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature*, *372*(6507), 686-691. https://doi.org/10.1038/372686a0

Di Marzo, V., Hill, M. P., Bisogno, T., Crossman, A. R., & Brotchie, J. M. (2000a). Enhanced levels of endogenous cannabinoids in the globus pallidus are associated with a reduction in movement in an animal model of Parkinson's disease. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *14*(10), 1432-1438. https://doi.org/10.1096/fj.14.10.1432

Di Marzo, V., Hill, M. P., Bisogno, T., Crossman, A. R., & Brotchie, J. M. (2000b). Enhanced levels of endogenous cannabinoids in the globus pallidus are associated with a reduction in movement in an animal model of Parkinson's disease. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *14*(10), 1432-1438. https://doi.org/10.1096/fj.14.10.1432

Dias, V., Junn, E., & Mouradian, M. M. (2013). The role of oxidative stress in Parkinson's disease. *Journal of Parkinson's Disease*, *3*(4), 461-491. https://doi.org/10.3233/JPD-130230

Díaz-Alonso, J., Paraíso-Luna, J., Navarrete, C., Del Río, C., Cantarero, I., Palomares, B., Aguareles, J., Fernández-Ruiz, J., Bellido, M. L., Pollastro, F., Appendino, G., Calzado, M. A., Galve-Roperh, I., & Muñoz, E. (2016). VCE-003.2, a novel cannabigerol derivative, enhances neuronal progenitor cell survival and alleviates symptomatology in murine models of Huntington's disease. *Scientific Reports*, *6*, 29789. https://doi.org/10.1038/srep29789

Dinh, T. P., Carpenter, D., Leslie, F. M., Freund, T. F., Katona, I., Sensi, S. L., Kathuria, S., & Piomelli, D. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(16), 10819-10824. https://doi.org/10.1073/pnas.152334899

Do, J., McKinney, C., Sharma, P., & Sidransky, E. (2019). Glucocerebrosidase and its relevance to Parkinson disease. *Molecular Neurodegeneration*, *14*(1), 36. https://doi.org/10.1186/s13024-019-0336-2

Dobricic, V., Schilling, M., Farkas, I., Gveric, D. O., Ohlei, O., Schulz, J., Middleton, L., Gentleman, S. M., Parkkinen, L., Bertram, L., & Lill, C. M. (2022). Common signatures of differential microRNA expression in Parkinson's and Alzheimer's disease brains. *Brain Communications*, *4*(6), fcac274. https://doi.org/10.1093/braincomms/fcac274

Domen, C. H., Sillau, S., Liu, Y., Adkins, M., Rajkovic, S., Bainbridge, J., Sempio, C., Klawitter, J., & Leehey, M. A. (2023). Cognitive Safety Data from a Randomized, Double-Blind, Parallel-Group, Placebo-Controlled Phase IIb Study of the Effects of a Cannabidiol and Δ9-Tetrahydrocannabinol Drug on Parkinson's Disease-Related Motor Symptoms. *Movement Disorders: Official Journal of the Movement Disorder Society*, *38*(7), 1341-1346. https://doi.org/10.1002/mds.29447

Dorsey, E. R., & Bloem, B. R. (2018). The Parkinson Pandemic-A Call to Action. *JAMA Neurology*, *75*(1), 9-10. https://doi.org/10.1001/jamaneurol.2017.3299

Dovonou, A., Bolduc, C., Soto Linan, V., Gora, C., Peralta III, M. R., & Lévesque, M. (2023). Animal models of Parkinson's disease: Bridging the gap between disease hallmarks and research questions. *Translational Neurodegeneration*, *12*, 36. https://doi.org/10.1186/s40035-023-00368-8

Duda, J. E., Giasson, B. I., Mabon, M. E., Lee, V. M.-Y., & Trojanowski, J. Q. (2002). Novel antibodies to synuclein show abundant striatal pathology in Lewy body diseases. *Annals of Neurology*, *52*(2), 205-210. https://doi.org/10.1002/ana.10279

Dusonchet, J., Kochubey, O., Stafa, K., Young, S. M., Zufferey, R., Moore, D. J., Schneider, B. L., & Aebischer, P. (2011). A rat model of progressive nigral neurodegeneration induced by the Parkinson's disease-associated G2019S mutation in LRRK2. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *31*(3), 907-912. https://doi.org/10.1523/JNEUROSCI.5092-10.2011

E

Ellert-Miklaszewska, A., Ciechomska, I. A., & Kaminska, B. (2021). Synthetic Cannabinoids Induce Autophagy and Mitochondrial Apoptotic Pathways in Human Glioblastoma Cells Independently of Deficiency in TP53 or PTEN Tumor Suppressors. *Cancers*, *13*(3), 419. https://doi.org/10.3390/cancers13030419

Enogieru, A. B., Omoruyi, S. I., Hiss, D. C., & Ekpo, O. E. (2019). GRP78/BIP/HSPA5 as a Therapeutic Target in Models of Parkinson's Disease: A Mini Review. *Advances in Pharmacological Sciences*, *2019*, 2706783. https://doi.org/10.1155/2019/2706783

Eraso-Pichot, A., Pouvreau, S., Olivera-Pinto, A., Gomez-Sotres, P., Skupio, U., & Marsicano, G. (2023). Endocannabinoid signaling in astrocytes. *Glia*, *71*(1), 44-59. https://doi.org/10.1002/glia.24246

Erny, D., Hrabě de Angelis, A. L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., Keren-Shaul, H., Mahlakoiv, T., Jakobshagen, K., Buch, T., Schwierzeck, V., Utermöhlen, O., Chun, E., Garrett, W. S., McCoy, K. D., Diefenbach, A., Staeheli,

P., Stecher, B., Amit, I., & Prinz, M. (2015). Host microbiota constantly control maturation and function of microglia in the CNS. *Nature Neuroscience*, *18*(7), 965-977. https://doi.org/10.1038/nn.4030

Erustes, A. G., Abílio, V. C., Bincoletto, C., Piacentini, M., Pereira, G. J. S., & Smaili, S. S. (2024). Cannabidiol induces autophagy via CB1 receptor and reduces α-synuclein cytosolic levels. *Brain Research*, *1850*, 149414. https://doi.org/10.1016/j.brainres.2024.149414

Erustes, A. G., D'Eletto, M., Guarache, G. C., Ureshino, R. P., Bincoletto, C., da Silva Pereira, G. J., Piacentini, M., & Smaili, S. S. (2021). Overexpression of α-synuclein inhibits mitochondrial Ca2+ trafficking between the endoplasmic reticulum and mitochondria through MAMs by altering the GRP75-IP3R interaction. *Journal of Neuroscience Research*, *99*(11), 2932-2947. https://doi.org/10.1002/jnr.24952

Escartin, C., Galea, E., Lakatos, A., O'Callaghan, J. P., Petzold, G. C., Serrano-Pozo, A., Steinhäuser, C., Volterra, A., Carmignoto, G., Agarwal, A., Allen, N. J., Araque, A., Barbeito, L., Barzilai, A., Bergles, D. E., Bonvento, G., Butt, A. M., Chen, W.-T., Cohen-Salmon, M., … Verkhratsky, A. (2021). Reactive astrocyte nomenclature, definitions, and future directions. *Nature Neuroscience*, *24*(3), 312-325. https://doi.org/10.1038/s41593-020-00783-4

Eslamboli, A., Romero-Ramos, M., Burger, C., Bjorklund, T., Muzyczka, N., Mandel, R. J., Baker, H., Ridley, R. M., & Kirik, D. (2007). Long-term consequences of human alpha-synuclein overexpression in the primate ventral midbrain. *Brain: A Journal of Neurology*, *130*(Pt 3), 799-815. https://doi.org/10.1093/brain/awl382

Espadas, I., Keifman, E., Palomo-Garo, C., Burgaz, S., García, C., Fernández-Ruiz, J., & Moratalla, R. (2020). Beneficial effects of the phytocannabinoid Δ9-THCV in L-DOPA-induced dyskinesia in Parkinson's disease. *Neurobiology of Disease*, *141*, 104892. https://doi.org/10.1016/j.nbd.2020.104892

Espejo-Porras, F., Fernández-Ruiz, J., & de Lago, E. (2018). Analysis of endocannabinoid receptors and enzymes in the post-mortem motor cortex and spinal cord of amyotrophic lateral sclerosis patients. *Amyotrophic Lateral Sclerosis & Frontotemporal Degeneration*, *19*(5-6), 377-386. https://doi.org/10.1080/21678421.2018.1425454

Espejo-Porras, F., Piscitelli, F., Verde, R., Ramos, J. A., Di Marzo, V., de Lago, E., & Fernández-Ruiz, J. (2015). Changes in the endocannabinoid signaling system in CNS structures of TDP-43 transgenic mice: Relevance for a neuroprotective therapy in TDP-43-related disorders. *Journal of Neuroimmune Pharmacology: The Official Journal of the Society on NeuroImmune Pharmacology*, *10*(2), 233-244. https://doi.org/10.1007/s11481-015-9602-4

Esteves, A. R., Swerdlow, R. H., & Cardoso, S. M. (2014). LRRK2, a puzzling protein: Insights into Parkinson's disease pathogenesis. *Experimental Neurology*, *261*, 206-216. https://doi.org/10.1016/j.expneurol.2014.05.025

Etemad, L., Karimi, G., Alavi, M. S., & Roohbakhsh, A. (2022). Pharmacological effects of cannabidiol by transient receptor potential channels. *Life Sciences*, *300*, 120582. https://doi.org/10.1016/j.lfs.2022.120582

F

Fatemi, I., Abdollahi, A., Shamsizadeh, A., Allahtavakoli, M., & Roohbakhsh, A. (2021). The effect of intra-striatal administration of GPR55 agonist (LPI) and antagonist (ML193) on sensorimotor and motor functions in a Parkinson's disease rat model. *Acta Neuropsychiatrica*, *33*(1), 15-21. https://doi.org/10.1017/neu.2020.30

Fearnley, J. M., & Lees, A. J. (1991). Ageing and Parkinson's disease: Substantia nigra regional selectivity. *Brain: A Journal of Neurology*, *114 (Pt 5)*, 2283-2301. https://doi.org/10.1093/brain/114.5.2283

Feigenbaum, J. J., Bergmann, F., Richmond, S. A., Mechoulam, R., Nadler, V., Kloog, Y., & Sokolovsky, M. (1989). Nonpsychotropic cannabinoid acts as a functional N-methyl-D-aspartate receptor blocker. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(23), 9584-9587. https://doi.org/10.1073/pnas.86.23.9584

Feng, L., Lo, H., You, H., Wu, W., Cheng, X., Xin, J., Ye, Z., Chen, X., & Pan, X. (2023). Loss of cannabinoid receptor 2 promotes α-Synuclein-induced microglial synaptic pruning in nucleus accumbens by modulating the pCREB-c-Fos signaling pathway and complement system. *Experimental Neurology*, *359*, 114230. https://doi.org/10.1016/j.expneurol.2022.114230

Fernández, D., Geisse, A., Bernales, J. I., Lira, A., & Osorio, F. (2021). The Unfolded Protein Response in Immune Cells as an Emerging Regulator of Neuroinflammation. *Frontiers in Aging Neuroscience*, *13*, 682633. https://doi.org/10.3389/fnagi.2021.682633

Fernandez-Espejo, E., Caraballo, I., de Fonseca, F. R., El Banoua, F., Ferrer, B., Flores, J. A., & Galan-Rodriguez, B. (2005). Cannabinoid CB1 antagonists possess antiparkinsonian efficacy only in rats with very severe nigral lesion in experimental parkinsonism. *Neurobiology of Disease*, *18*(3), 591-601. https://doi.org/10.1016/j.nbd.2004.10.015

Fernández-Espejo, E., Rodríguez de Fonseca, F., Suárez, J., Tolosa, E., Vilas, D., Aldecoa, I., Berenguer, J., & Damas-Hermoso, F. (2021). Native α-Synuclein, 3-Nitrotyrosine Proteins, and Patterns of Nitro-α-Synuclein-Immunoreactive Inclusions in Saliva and Submandibulary Gland in Parkinson's Disease. *Antioxidants (Basel, Switzerland)*, *10*(5), 715. https://doi.org/10.3390/antiox10050715

Fernández-Ruiz, J. (2009). The endocannabinoid system as a target for the treatment of motor dysfunction. *British Journal of Pharmacology*, *156*(7), 1029-1040. https://doi.org/10.1111/j.1476-5381.2008.00088.x

Fernández-Ruiz, J., Moro, M. A., & Martínez-Orgado, J. (2015). Cannabinoids in Neurodegenerative Disorders and Stroke/Brain Trauma: From Preclinical Models to Clinical Applications. *Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics*, *12*(4), 793-806. https://doi.org/10.1007/s13311-015-0381-7

Fernández-Ruiz, J., Romero, J., Velasco, G., Tolón, R. M., Ramos, J. A., & Guzmán, M. (2007). Cannabinoid CB2 receptor: A new target for controlling neural cell survival? *Trends in Pharmacological Sciences*, *28*(1), 39-45. https://doi.org/10.1016/j.tips.2006.11.001

Fernández-Ruiz, J., Sagredo, O., Pazos, M. R., García, C., Pertwee, R., Mechoulam, R., & Martínez-Orgado, J. (2013). Cannabidiol for neurodegenerative disorders: Important new clinical applications for this phytocannabinoid? *British Journal of Clinical Pharmacology*, *75*(2), 323-333. https://doi.org/10.1111/j.1365-2125.2012.04341.x

Fernández-Suárez, D., Celorrio, M., Riezu-Boj, J. I., Ugarte, A., Pacheco, R., González, H., Oyarzabal, J., Hillard, C. J., Franco, R., & Aymerich, M. S. (2014). Monoacylglycerol lipase inhibitor JZL184 is neuroprotective and alters glial cell phenotype in the chronic MPTP mouse model. *Neurobiology of Aging*, *35*(11), 2603-2616. https://doi.org/10.1016/j.neurobiolaging.2014.05.021

Fleming, S. M., Ekhator, O. R., & Ghisays, V. (2013). Assessment of sensorimotor function in mouse models of Parkinson's disease. *Journal of Visualized Experiments: JoVE*, *76*, 50303. https://doi.org/10.3791/50303

Fox, S. H., Katzenschlager, R., Lim, S.-Y., Ravina, B., Seppi, K., Coelho, M., Poewe, W., Rascol, O., Goetz, C. G., & Sampaio, C. (2011). The Movement Disorder Society Evidence-Based Medicine Review Update: Treatments for the motor symptoms of Parkinson's disease. *Movement Disorders: Official Journal of the Movement Disorder Society*, *26 Suppl 3*, S2-41. https://doi.org/10.1002/mds.23829

Fox, S. H., Lang, A. E., & Brotchie, J. M. (2006). Translation of nondopaminergic treatments for levodopa-induced dyskinesia from MPTP-lesioned nonhuman primates to phase IIa clinical studies: Keys to success and roads to failure. *Movement Disorders: Official Journal of the Movement Disorder Society*, *21*(10), 1578-1594. https://doi.org/10.1002/mds.20936

Frankel, J. P., Hughes, A., Lees, A. J., & Stern, G. M. (1990). Marijuana for parkinsonian tremor. *Journal of Neurology, Neurosurgery, and Psychiatry*, *53*(5), 436. https://doi.org/10.1136/jnnp.53.5.436

Freitas, M. E., Ruiz-Lopez, M., & Fox, S. H. (2016). Novel Levodopa Formulations for Parkinson's Disease. *CNS Drugs*, *30*(11), 1079-1095. https://doi.org/10.1007/s40263-016-0386-8

Fride, E., Feigin, C., Ponde, D. E., Breuer, A., Hanus, L., Arshavsky, N., & Mechoulam, R. (2004a). (+)-Cannabidiol analogues which bind cannabinoid receptors but exert peripheral activity only. *European Journal of Pharmacology*, *506*(2), 179-188. https://doi.org/10.1016/j.ejphar.2004.10.049

Fride, E., Feigin, C., Ponde, D. E., Breuer, A., Hanus, L., Arshavsky, N., & Mechoulam, R. (2004b). (+)-Cannabidiol analogues which bind cannabinoid receptors but exert peripheral activity only. *European Journal of Pharmacology*, *506*(2), 179-188. https://doi.org/10.1016/j.ejphar.2004.10.049

Furukawa, K., Shima, A., Kambe, D., Nishida, A., Wada, I., Sakamaki, H., Yoshimura, K., Terada, Y., Sakato, Y., Mitsuhashi, M., Sawamura, M., Nakanishi, E., Taruno, Y., Yamakado, H., Fushimi, Y., Okada, T., Nakamoto, Y., Takahashi, R., & Sawamoto, N. (2022). Motor Progression and Nigrostriatal Neurodegeneration in Parkinson Disease. *Annals of Neurology*, *92*(1), 110-121. https://doi.org/10.1002/ana.26373

G

Gagne, J. J., & Power, M. C. (2010). Anti-inflammatory drugs and risk of Parkinson disease: A meta-analysis. *Neurology*, *74*(12), 995-1002. https://doi.org/10.1212/WNL.0b013e3181d5a4a3

Gajghate, S., Li, H., & Rom, S. (2024). GPR55 Inactivation Diminishes Splenic Responses and Improves Neurological Outcomes in the Mouse Ischemia/Reperfusion Stroke Model. *Cells*, *13*(3), 280. https://doi.org/10.3390/cells13030280

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G., & Casellas, P. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte

subpopulations. *European Journal of Biochemistry*, *232*(1), 54-61. https://doi.org/10.1111/j.1432-1033.1995.tb20780.x

Galve-Roperh, I., Rueda, D., Gómez del Pulgar, T., Velasco, G., & Guzmán, M. (2002). Mechanism of extracellular signal-regulated kinase activation by the CB(1) cannabinoid receptor. *Molecular Pharmacology*, *62*(6), 1385-1392. https://doi.org/10.1124/mol.62.6.1385

Gao, H.-M., Zhang, F., Zhou, H., Kam, W., Wilson, B., & Hong, J.-S. (2011). Neuroinflammation and α-synuclein dysfunction potentiate each other, driving chronic progression of neurodegeneration in a mouse model of Parkinson's disease. *Environmental Health Perspectives*, *119*(6), 807-814. https://doi.org/10.1289/ehp.1003013

Gaoni, Y., & Mechoulam, R. (1964). Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. *Journal of the American Chemical Society*, *86*(8), 1646-1647. https://doi.org/10.1021/ja01062a046

García, C., Gómez-Cañas, M., Burgaz, S., Palomares, B., Gómez-Gálvez, Y., Palomo-Garo, C., Campo, S., Ferrer-Hernández, J., Pavicic, C., Navarrete, C., Luz Bellido, M., García-Arencibia, M., Ruth Pazos, M., Muñoz, E., & Fernández-Ruiz, J. (2018). Benefits of VCE-003.2, a cannabigerol quinone derivative, against inflammation-driven neuronal deterioration in experimental Parkinson's disease: Possible involvement of different binding sites at the PPARγ receptor. *Journal of Neuroinflammation*, *15*(1), 19. https://doi.org/10.1186/s12974-018-1060-5

García, C., Palomo-Garo, C., García-Arencibia, M., Ramos, J., Pertwee, R., & Fernández-Ruiz, J. (2011). Symptom-relieving and neuroprotective effects of the phytocannabinoid Δ^9-THCV in animal models of Parkinson's disease. *British Journal of Pharmacology*, *163*(7), 1495-1506. https://doi.org/10.1111/j.1476-5381.2011.01278.x

García, M. C., Cinquina, V., Palomo-Garo, C., Rábano, A., & Fernández-Ruiz, J. (2015). Identification of CB_2 receptors in human nigral neurons that degenerate in Parkinson's disease. *Neuroscience Letters*, *587*, 1-4. https://doi.org/10.1016/j.neulet.2014.12.003

García-Arencibia, M., García, C., & Fernández-Ruiz, J. (2009). Cannabinoids and Parkinson's disease. *CNS & Neurological Disorders Drug Targets*, *8*(6), 432-439. https://doi.org/10.2174/187152709789824642

García-Arencibia, M., García, C., Kurz, A., Rodríguez-Navarro, J. A., Gispert-Sáchez, S., Mena, M. A., Auburger, G., de Yébenes, J. G., & Fernández-Ruiz, J. (2009). Cannabinoid CB1 receptors are early downregulated followed by a further upregulation in the basal ganglia of mice with deletion of specific park genes. *Journal of Neural Transmission. Supplementum*, *73*, 269-275. https://doi.org/10.1007/978-3-211-92660-4_22

García-Arencibia, M., González, S., de Lago, E., Ramos, J. A., Mechoulam, R., & Fernández-Ruiz, J. (2007a). Evaluation of the neuroprotective effect of cannabinoids in a rat model of Parkinson's disease: Importance of antioxidant and cannabinoid receptor-independent properties. *Brain Research*, *1134*(1), 162-170. https://doi.org/10.1016/j.brainres.2006.11.063

García-Arencibia, M., González, S., de Lago, E., Ramos, J. A., Mechoulam, R., & Fernández-Ruiz, J. (2007b). Evaluation of the neuroprotective effect of cannabinoids in a rat model of Parkinson's disease: Importance of antioxidant and cannabinoid receptor-independent properties. *Brain Research*, *1134*(1), 162-170. https://doi.org/10.1016/j.brainres.2006.11.063

Garcia-Arencibia, M., Molina-Holgado, E., & Molina-Holgado, F. (2019). Effect of endocannabinoid signalling on cell fate: Life, death, differentiation and proliferation of brain cells. *British Journal of Pharmacology*, *176*(10), 1361-1369. https://doi.org/10.1111/bph.14369

García-Gutiérrez, M. S., Navarrete, F., Navarro, G., Reyes-Resina, I., Franco, R., Lanciego, J. L., Giner, S., & Manzanares, J. (2018). Alterations in Gene and Protein Expression of Cannabinoid CB2 and GPR55 Receptors in the Dorsolateral Prefrontal Cortex of Suicide Victims. *Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics*, *15*(3), 796-806. https://doi.org/10.1007/s13311-018-0610-y

Gasperi, V., Dainese, E., Oddi, S., Sabatucci, A., & Maccarrone, M. (2013). GPR55 and its interaction with membrane lipids: Comparison with other endocannabinoid-binding receptors. *Current Medicinal Chemistry*, *20*(1), 64-78.

Gegg, M. E., Burke, D., Heales, S. J. R., Cooper, J. M., Hardy, J., Wood, N. W., & Schapira, A. H. V. (2012). Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. *Annals of Neurology*, *72*(3), 455-463. https://doi.org/10.1002/ana.23614

Gérard, C. M., Mollereau, C., Vassart, G., & Parmentier, M. (1991). Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *The Biochemical Journal*, *279 (Pt 1)*(Pt 1), 129-134. https://doi.org/10.1042/bj2790129

Gerdeman, G., & Lovinger, D. M. (2001). CB1 cannabinoid receptor inhibits synaptic release of glutamate in rat dorsolateral striatum. *Journal of Neurophysiology*, *85*(1), 468-471. https://doi.org/10.1152/jn.2001.85.1.468

Giasson, B. I., Duda, J. E., Quinn, S. M., Zhang, B., Trojanowski, J. Q., & Lee, V. M.-Y. (2002). Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron*, *34*(4), 521-533. https://doi.org/10.1016/s0896-6273(02)00682-7

Giordano, M., Takashima, H., Herranz, A., Poltorak, M., Geller, H. M., Marone, M., & Freed, W. J. (1993). Immortalized GABAergic cell lines derived from rat striatum using a temperature-sensitive allele of the SV40 large T antigen. *Experimental Neurology*, *124*(2), 395-400. https://doi.org/10.1006/exnr.1993.1213

Giráldez-Pérez, R., Antolín-Vallespín, M., Muñoz, M., & Sánchez-Capelo, A. (2014). Models of α-synuclein aggregation in Parkinson's disease. *Acta Neuropathologica Communications*, *2*, 176. https://doi.org/10.1186/s40478-014-0176-9

Gispert, S., Ricciardi, F., Kurz, A., Azizov, M., Hoepken, H.-H., Becker, D., Voos, W., Leuner, K., Müller, W. E., Kudin, A. P., Kunz, W. S., Zimmermann, A., Roeper, J., Wenzel, D., Jendrach, M., García-Arencíbia, M., Fernández-Ruiz, J., Huber, L., Rohrer, H., … Auburger, G. (2009). Parkinson phenotype in aged PINK1-deficient mice is accompanied by progressive mitochondrial dysfunction in absence of neurodegeneration. *PloS One*, *4*(6), e5777. https://doi.org/10.1371/journal.pone.0005777

Giuliano, C., Francavilla, M., Ongari, G., Petese, A., Ghezzi, C., Rossini, N., Blandini, F., & Cerri, S. (2021). Neuroprotective and Symptomatic Effects of Cannabidiol in an Animal Model of Parkinson's Disease. *International Journal of Molecular Sciences*, *22*(16), 8920. https://doi.org/10.3390/ijms22168920

Glass, M., & Felder, C. C. (1997). Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: Evidence for a Gs linkage to the CB1 receptor. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *17*(14), 5327-5333. https://doi.org/10.1523/JNEUROSCI.17-14-05327.1997

Godbout, J. P., Chen, J., Abraham, J., Richwine, A. F., Berg, B. M., Kelley, K. W., & Johnson, R. W. (2005). Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *19*(10), 1329-1331. https://doi.org/10.1096/fj.05-3776fje

Goldberg, M. S., Fleming, S. M., Palacino, J. J., Cepeda, C., Lam, H. A., Bhatnagar, A., Meloni, E. G., Wu, N., Ackerson, L. C., Klapstein, G. J., Gajendiran, M., Roth, B. L., Chesselet, M.-F., Maidment, N. T., Levine, M. S., & Shen, J. (2003). Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. *The Journal of Biological Chemistry*, *278*(44), 43628-43635. https://doi.org/10.1074/jbc.M308947200

Golliher, A. E., Tenorio, A. J., Dimauro, N. O., Mairata, N. R., Holguin, F. O., & Maio, W. (2021). Using (+)-Carvone to access novel derivatives of (+)-ent-Cannabidiol: The first asymmetric syntheses of (+)-ent-CBDP and (+)-ent-CBDV. *Tetrahedron Letters*, *67*, 152891. https://doi.org/10.1016/j.tetlet.2021.152891

Gómez-Almería, M., Burgaz, S., Costas-Insua, C., Rodríguez-Cueto, C., Santos-García, I., Rodríguez-Crespo, I., García, C., Guzmán, M., de Lago, E., & Fernández-Ruiz, J. (2021). BiP Heterozigosity Aggravates Pathological Deterioration in Experimental Amyotrophic Lateral Sclerosis. *International Journal of Molecular Sciences*, *22*(22), 12533. https://doi.org/10.3390/ijms222212533

Gómez-Cañas, M., Morales, P., García-Toscano, L., Navarrete, C., Muñoz, E., Jagerovic, N., Fernández-Ruiz, J., García-Arencibia, M., & Pazos, M. R. (2016). Biological characterization of PM226, a chromenoisoxazole, as a selective CB 2 receptor agonist with neuroprotective profile. *Pharmacological Research*, *110*, 205-215. https://doi.org/10.1016/j.phrs.2016.03.021

Gómez-Gálvez, Y., Palomo-Garo, C., Fernández-Ruiz, J., & García, C. (2016). Potential of the cannabinoid CB(2) receptor as a pharmacological target against inflammation in Parkinson's disease. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *64*, 200-208. https://doi.org/10.1016/j.pnpbp.2015.03.017

Gomez-Isla, T., Irizarry, M. C., Mariash, A., Cheung, B., Soto, O., Schrump, S., Sondel, J., Kotilinek, L., Day, J., Schwarzschild, M. A., Cha, J.-H. J., Newell, K., Miller, D. W., Uéda, K., Young, A. B., Hyman, B. T., & Ashe, K. H. (2003). Motor dysfunction and gliosis with preserved dopaminergic markers in human alpha-synuclein A30P transgenic mice. *Neurobiology of Aging*, *24*(2), 245-258. https://doi.org/10.1016/s0197-4580(02)00091-x

González, S., Scorticati, C., García-Arencibia, M., de Miguel, R., Ramos, J. A., & Fernández-Ruiz, J. (2006). Effects of rimonabant, a selective cannabinoid CB1 receptor antagonist, in a rat model of Parkinson's disease. *Brain Research*, *1073-1074*, 209-219. https://doi.org/10.1016/j.brainres.2005.12.014

González-Mariscal, I., Carmona-Hidalgo, B., Winkler, M., Unciti-Broceta, J. D., Escamilla, A., Gómez-Cañas, M., Fernández-Ruiz, J., Fiebich, B. L., Romero-Zerbo, S.-Y., Bermúdez-Silva, F. J., Collado, J. A., & Muñoz, E. (2021). (+)-trans-Cannabidiol-2-hydroxy pentyl is a dual CB1R antagonist/CB2R agonist that prevents diabetic nephropathy in mice. *Pharmacological Research*, *169*, 105492. https://doi.org/10.1016/j.phrs.2021.105492

Gonzalo-Consuegra, C., Santos-García, I., García-Toscano, L., Martín-Baquero, R., Rodríguez-Cueto, C., Wittwer, M. B., Dzygiel, P., Grether, U., de Lago, E., & Fernández-Ruiz, J. (2024). Involvement of CB1 and CB2 receptors in neuroprotective

effects of cannabinoids in experimental TDP-43 related frontotemporal dementia using male mice. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *174*, 116473. https://doi.org/10.1016/j.biopha.2024.116473

Gorbatyuk, M. S., Shabashvili, A., Chen, W., Meyers, C., Sullivan, L. F., Salganik, M., Lin, J. H., Lewin, A. S., Muzyczka, N., & Gorbatyuk, O. S. (2012a). Glucose regulated protein 78 diminishes α-synuclein neurotoxicity in a rat model of Parkinson disease. *Molecular Therapy: The Journal of the American Society of Gene Therapy*, *20*(7), 1327-1337. https://doi.org/10.1038/mt.2012.28

Gorbatyuk, M. S., Shabashvili, A., Chen, W., Meyers, C., Sullivan, L. F., Salganik, M., Lin, J. H., Lewin, A. S., Muzyczka, N., & Gorbatyuk, O. S. (2012b). Glucose regulated protein 78 diminishes α-synuclein neurotoxicity in a rat model of Parkinson disease. *Molecular Therapy: The Journal of the American Society of Gene Therapy*, *20*(7), 1327-1337. https://doi.org/10.1038/mt.2012.28

Götz, M. R., Collado, J. A., Fernández-Ruiz, J., Fiebich, B. L., García-Toscano, L., Gómez-Cañas, M., Koch, O., Leha, A., Muñoz, E., Navarrete, C., Pazos, M. R., & Holzgrabe, U. (2019). Structure-Effect Relationships of Novel Semi-Synthetic Cannabinoid Derivatives. *Frontiers in Pharmacology*, *10*, 1284. https://doi.org/10.3389/fphar.2019.01284

Grabon, W., Ruiz, A., Gasmi, N., Degletagne, C., Georges, B., Belmeguenai, A., Bodennec, J., Rheims, S., Marcy, G., & Bezin, L. (2024). CB2 expression in mouse brain: From mapping to regulation in microglia under inflammatory conditions. *Journal of Neuroinflammation*, *21*(1), 206. https://doi.org/10.1186/s12974-024-03202-8

Granja, A. G., Carrillo-Salinas, F., Pagani, A., Gómez-Cañas, M., Negri, R., Navarrete, C., Mecha, M., Mestre, L., Fiebich, B. L., Cantarero, I., Calzado, M. A., Bellido, M. L., Fernandez-Ruiz, J., Appendino, G., Guaza, C., & Muñoz, E. (2012). A cannabigerol quinone alleviates neuroinflammation in a chronic model of multiple sclerosis. *Journal of Neuroimmune Pharmacology: The Official Journal of the Society on NeuroImmune Pharmacology*, *7*(4), 1002-1016. https://doi.org/10.1007/s11481-012-9399-3

Grealish, S., Mattsson, B., Draxler, P., & Björklund, A. (2010). Characterisation of behavioural and neurodegenerative changes induced by intranigral 6-hydroxydopamine lesions in a mouse model of Parkinson's disease. *The European Journal of Neuroscience*, *31*(12), 2266-2278. https://doi.org/10.1111/j.1460-9568.2010.07265.x

Greco, R., Demartini, C., Zanaboni, A., Tumelero, E., Elisa, C., Persico, A., Morotti, A., Amantea, D., & Tassorelli, C. (2021). Characterization of CB2 Receptor Expression in Peripheral Blood Monocytes of Acute Ischemic Stroke Patients. *Translational Stroke Research*, *12*(4), 550-558. https://doi.org/10.1007/s12975-020-00851-8

Greenbaum, E. A., Graves, C. L., Mishizen-Eberz, A. J., Lupoli, M. A., Lynch, D. R., Englander, S. W., Axelsen, P. H., & Giasson, B. I. (2005). The E46K mutation in alpha-synuclein increases amyloid fibril formation. *The Journal of Biological Chemistry*, *280*(9), 7800-7807. https://doi.org/10.1074/jbc.M411638200

Grigor'eva, E. V., Drozdova, E. S., Sorogina, D. A., Malakhova, A. A., Pavlova, S. V., Vyatkin, Y. V., Khabarova, E. A., Rzaev, J. A., Medvedev, S. P., & Zakian, S. M. (2022). Generation of induced pluripotent stem cell line, ICGi034-A, by reprogramming peripheral blood mononuclear cells from a patient with Parkinson's disease associated with GBA mutation. *Stem Cell Research*, *59*, 102651. https://doi.org/10.1016/j.scr.2021.102651

Grozdanov, V., Bliederhaeuser, C., Ruf, W. P., Roth, V., Fundel-Clemens, K., Zondler, L., Brenner, D., Martin-Villalba, A., Hengerer, B., Kassubek, J., Ludolph, A. C., Weishaupt, J. H., & Danzer, K. M. (2014). Inflammatory dysregulation of blood monocytes in Parkinson's disease patients. *Acta Neuropathologica*, *128*(5), 651-663. https://doi.org/10.1007/s00401-014-1345-4

Gubellini, P., Picconi, B., Bari, M., Battista, N., Calabresi, P., Centonze, D., Bernardi, G., Finazzi-Agrò, A., & Maccarrone, M. (2002). Experimental parkinsonism alters endocannabinoid degradation: Implications for striatal glutamatergic transmission. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *22*(16), 6900-6907. https://doi.org/10.1523/JNEUROSCI.22-16-06900.2002

Gugliandolo, A., Blando, S., Salamone, S., Pollastro, F., Mazzon, E., & D'Angiolini, S. (2023). Transcriptome Highlights Cannabinol Modulation of Mitophagy in a Parkinson's Disease In Vitro Model. *Biomolecules*, *13*(8), 1163. https://doi.org/10.3390/biom13081163

Gugliandolo, A., Pollastro, F., Bramanti, P., & Mazzon, E. (2020). Cannabidiol exerts protective effects in an in vitro model of Parkinson's disease activating AKT/mTOR pathway. *Fitoterapia*, *143*, 104553. https://doi.org/10.1016/j.fitote.2020.104553

Gugliandolo, A., Pollastro, F., Grassi, G., Bramanti, P., & Mazzon, E. (2018). In Vitro Model of Neuroinflammation: Efficacy of Cannabigerol, a Non-Psychoactive Cannabinoid. *International Journal of Molecular Sciences*, *19*(7), 1992. https://doi.org/10.3390/ijms19071992

Guindon, J., & Hohmann, A. G. (2008). A physiological role for endocannabinoid-derived products of cyclooxygenase-2-mediated oxidative metabolism. *British Journal of Pharmacology*, *153*(7), 1341-1343. https://doi.org/10.1038/bjp.2008.41

Guo, J., & Ikeda, S. R. (2004). Endocannabinoids modulate N-type calcium channels and G-protein-coupled inwardly rectifying potassium channels via CB1 cannabinoid receptors heterologously expressed in mammalian neurons. *Molecular Pharmacology*, *65*(3), 665-674. https://doi.org/10.1124/mol.65.3.665

Guo, Y., Guan, T., Shafiq, K., Yu, Q., Jiao, X., Na, D., Li, M., Zhang, G., & Kong, J. (2023). Mitochondrial dysfunction in aging. *Ageing Research Reviews*, *88*, 101955. https://doi.org/10.1016/j.arr.2023.101955

Gutiérrez-Valdez, A. L., García-Ruiz, R., Anaya-Martínez, V., Torres-Esquivel, C., Espinosa-Villanueva, J., Reynoso-Erazo, L., Tron-Alvarez, R., Aley-Medina, P., Sánchez-Betancourt, J., Montiel-Flores, E., & Avila-Costa, M. R. (2013). The combination of oral L-DOPA/rimonabant for effective dyskinesia treatment and cytological preservation in a rat model of Parkinson's disease and L-DOPA-induced dyskinesia. *Behavioural Pharmacology*, *24*(8), 640-652. https://doi.org/10.1097/FBP.0000000000000004

H

Hafida, E. G., Rachid, S., Halima, G., & Najib, K. (2024). CBD's potential impact on Parkinson's disease: An updated overview. *Open Medicine (Warsaw, Poland)*, *19*(1), 20241075. https://doi.org/10.1515/med-2024-1075

Han, K. H., Lim, S., Ryu, J., Lee, C.-W., Kim, Y., Kang, J.-H., Kang, S.-S., Ahn, Y. K., Park, C.-S., & Kim, J. J. (2009). CB1 and CB2 cannabinoid receptors differentially regulate the production of reactive oxygen species by macrophages. *Cardiovascular Research*, *84*(3), 378-386. https://doi.org/10.1093/cvr/cvp240

Hanus, L. O., Tchilibon, S., Ponde, D. E., Breuer, A., Fride, E., & Mechoulam, R. (2005a). Enantiomeric cannabidiol derivatives: Synthesis and binding to cannabinoid receptors. *Organic & Biomolecular Chemistry*, *3*(6), 1116-1123. https://doi.org/10.1039/b416943c

Hanus, L. O., Tchilibon, S., Ponde, D. E., Breuer, A., Fride, E., & Mechoulam, R. (2005b). Enantiomeric cannabidiol derivatives: Synthesis and binding to cannabinoid receptors. *Organic & Biomolecular Chemistry*, *3*(6), 1116-1123. https://doi.org/10.1039/b416943c

Haque, M. E., Mount, M. P., Safarpour, F., Abdel-Messih, E., Callaghan, S., Mazerolle, C., Kitada, T., Slack, R. S., Wallace, V., Shen, J., Anisman, H., & Park, D. S. (2012). Inactivation of Pink1 gene in vivo sensitizes dopamine-producing neurons to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and can be rescued by autosomal recessive Parkinson disease genes, Parkin or DJ-1. *The Journal of Biological Chemistry*, *287*(27), 23162-23170. https://doi.org/10.1074/jbc.M112.346437

Harms, A. S., Thome, A. D., Yan, Z., Schonhoff, A. M., Williams, G. P., Li, X., Liu, Y., Qin, H., Benveniste, E. N., & Standaert, D. G. (2018). Peripheral monocyte entry is required for alpha-Synuclein induced inflammation and Neurodegeneration in a model of Parkinson disease. *Experimental Neurology*, *300*, 179-187. https://doi.org/10.1016/j.expneurol.2017.11.010

Haspula, D., & Clark, M. A. (2021). Contrasting Roles of Ang II and ACEA in the Regulation of IL10 and IL1β Gene Expression in Primary SHR Astroglial Cultures. *Molecules (Basel, Switzerland)*, *26*(10), 3012. https://doi.org/10.3390/molecules26103012

Hawkes, C. H., Del Tredici, K., & Braak, H. (2007). Parkinson's disease: A dual-hit hypothesis. *Neuropathology and Applied Neurobiology*, *33*(6), 599-614. https://doi.org/10.1111/j.1365-2990.2007.00874.x

Hayes, M. T. (2019). Parkinson's Disease and Parkinsonism. *The American Journal of Medicine*, *132*(7), 802-807. https://doi.org/10.1016/j.amjmed.2019.03.001

He, J., Li, X., Yang, S., Li, Y., Lin, X., Xiu, M., Li, X., & Liu, Y. (2021). Gastrodin extends the lifespan and protects against neurodegeneration in the Drosophila PINK1 model of Parkinson's disease. *Food & Function*, *12*(17), 7816-7824. https://doi.org/10.1039/d1fo00847a

He, Y., Shen, H., Bi, G.-H., Zhang, H.-Y., Soler-Cedeño, O., Alton, H., Yang, Y., & Xi, Z.-X. (2024). GPR55 is expressed in glutamate neurons and functionally modulates drug taking and seeking in rats and mice. *Translational Psychiatry*, *14*(1), 101. https://doi.org/10.1038/s41398-024-02820-3

Henrich, M. T., Oertel, W. H., Surmeier, D. J., & Geibl, F. F. (2023). Mitochondrial dysfunction in Parkinson's disease—A key disease hallmark with therapeutic potential. *Molecular Neurodegeneration*, *18*(1), 83. https://doi.org/10.1186/s13024-023-00676-7

Henstridge, C. M., Balenga, N. A. B., Ford, L. A., Ross, R. A., Waldhoer, M., & Irving, A. J. (2009). The GPR55 ligand L-alpha-lysophosphatidylinositol promotes RhoA-dependent Ca2+ signaling and NFAT activation. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *23*(1), 183-193. https://doi.org/10.1096/fj.08-108670

Herkenham, M., Lynn, A. B., de Costa, B. R., & Richfield, E. K. (1991). Neuronal localization of cannabinoid receptors in the basal ganglia of the rat. *Brain Research*, *547*(2), 267-274. https://doi.org/10.1016/0006-8993(91)90970-7

Herkenham, M., Lynn, A. B., Johnson, M. R., Melvin, L. S., de Costa, B. R., & Rice, K. C. (1991). Characterization and localization of cannabinoid receptors in rat brain: A quantitative in vitro autoradiographic study. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *11*(2), 563-583. https://doi.org/10.1523/JNEUROSCI.11-02-00563.1991

Hewer, R. C., Christie, L. A., Doyle, K. J., Xu, X., Roberts, M. J., Dickson, L., Cheung, T., Cadwalladr, D. H., Pickford, P., Teall, M., Powell, J. A. C., Sheardown, S., Narayana, L., Brice, N. L., Dawson, L. A., Carlton, M., & Bürli, R. W. (2023). Discovery and Characterization of Novel CNS-Penetrant GPR55 Agonists. *Journal of Medicinal Chemistry*, *66*(18), 12858-12876. https://doi.org/10.1021/acs.jmedchem.3c00784

Heynen-Genel, S., Dahl, R., Shi, S., Milan, L., Hariharan, S., Sergienko, E., Hedrick, M., Dad, S., Stonich, D., Su, Y., Vicchiarelli, M., Mangravita-Novo, A., Smith, L. H., Chung, T. D., Sharir, H., Caron, M. G., Barak, L. S., & Abood, M. E. (2010). Screening for Selective Ligands for GPR55—Antagonists. En *Probe Reports from the NIH Molecular Libraries Program*. National Center for Biotechnology Information (US). http://www.ncbi.nlm.nih.gov/books/NBK66153/

Hill, J. D., Zuluaga-Ramirez, V., Gajghate, S., Winfield, M., & Persidsky, Y. (2018). Activation of GPR55 increases neural stem cell proliferation and promotes early adult hippocampal neurogenesis. *British Journal of Pharmacology*, *175*(16), 3407-3421. https://doi.org/10.1111/bph.14387

Hill, J. D., Zuluaga-Ramirez, V., Gajghate, S., Winfield, M., Sriram, U., Rom, S., & Persidsky, Y. (2019). Activation of GPR55 induces neuroprotection of hippocampal neurogenesis and immune responses of neural stem cells following chronic, systemic inflammation. *Brain, Behavior, and Immunity*, *76*, 165-181. https://doi.org/10.1016/j.bbi.2018.11.017

Hillard, C. J., Edgemond, W. S., Jarrahian, A., & Campbell, W. B. (1997). Accumulation of N-arachidonoylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. *Journal of Neurochemistry*, *69*(2), 631-638. https://doi.org/10.1046/j.1471-4159.1997.69020631.x

Hillard, C. J., Manna, S., Greenberg, M. J., DiCamelli, R., Ross, R. A., Stevenson, L. A., Murphy, V., Pertwee, R. G., & Campbell, W. B. (1999). Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). *The Journal of Pharmacology and Experimental Therapeutics*, *289*(3), 1427-1433.

Hindle, J. V. (2010). Ageing, neurodegeneration and Parkinson's disease. *Age and Ageing*, *39*(2), 156-161. https://doi.org/10.1093/ageing/afp223

Hirsch, L., Jette, N., Frolkis, A., Steeves, T., & Pringsheim, T. (2016). The Incidence of Parkinson's Disease: A Systematic Review and Meta-Analysis. *Neuroepidemiology*, *46*(4), 292-300. https://doi.org/10.1159/000445751

Hoenen, C., Gustin, A., Birck, C., Kirchmeyer, M., Beaume, N., Felten, P., Grandbarbe, L., Heuschling, P., & Heurtaux, T. (2016). Alpha-Synuclein Proteins Promote Pro-Inflammatory Cascades in Microglia: Stronger Effects of the A53T Mutant. *PloS One*, *11*(9), e0162717. https://doi.org/10.1371/journal.pone.0162717

Höglinger, G. U., Féger, J., Prigent, A., Michel, P. P., Parain, K., Champy, P., Ruberg, M., Oertel, W. H., & Hirsch, E. C. (2003). Chronic systemic complex I inhibition induces a hypokinetic multisystem degeneration in rats. *Journal of Neurochemistry*, *84*(3), 491-502. https://doi.org/10.1046/j.1471-4159.2003.01533.x

Holmqvist, S., Chutna, O., Bousset, L., Aldrin-Kirk, P., Li, W., Björklund, T., Wang, Z.-Y., Roybon, L., Melki, R., & Li, J.-Y. (2014). Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats. *Acta Neuropathologica*, *128*(6), 805-820. https://doi.org/10.1007/s00401-014-1343-6

Hosoi, T., Oba, T., & Ozawa, K. (2013). ER stress-mediated regulation of immune function under glucose-deprived condition in glial cells: Up- and down-regulation of PGE2 + IFNγ-induced IL-6 and iNOS expressions. *Biochemical and Biophysical Research Communications*, *441*(2), 525-528. https://doi.org/10.1016/j.bbrc.2013.10.109

Hou, X., Watzlawik, J. O., Fiesel, F. C., & Springer, W. (2020). Autophagy in Parkinson's Disease. *Journal of Molecular Biology*, *432*(8), 2651-2672. https://doi.org/10.1016/j.jmb.2020.01.037

Howland, D. S., Liu, J., She, Y., Goad, B., Maragakis, N. J., Kim, B., Erickson, J., Kulik, J., DeVito, L., Psaltis, G., DeGennaro, L. J., Cleveland, D. W., & Rothstein, J. D. (2002). Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proceedings of the National Academy of Sciences of the United States of America*, *99*(3), 1604-1609. https://doi.org/10.1073/pnas.032539299

Howlett, A. C., Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., Felder, C. C., Herkenham, M., Mackie, K., Martin, B. R., Mechoulam, R., & Pertwee, R. G. (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacological Reviews*, *54*(2), 161-202. https://doi.org/10.1124/pr.54.2.161

Howlett, A. C., Blume, L. C., & Dalton, G. D. (2010). CB(1) cannabinoid receptors and their associated proteins. *Current Medicinal Chemistry*, *17*(14), 1382-1393. https://doi.org/10.2174/092986710790980023

Hua, T., Vemuri, K., Pu, M., Qu, L., Han, G. W., Wu, Y., Zhao, S., Shui, W., Li, S., Korde, A., Laprairie, R. B., Stahl, E. L., Ho, J.-H., Zvonok, N., Zhou, H., Kufareva, I., Wu, B., Zhao, Q., Hanson, M. A., … Liu, Z.-J. (2016). Crystal Structure of the Human Cannabinoid Receptor CB1. *Cell*, *167*(3), 750-762.e14. https://doi.org/10.1016/j.cell.2016.10.004

Huang, C. C., Lo, S. W., & Hsu, K. S. (2001). Presynaptic mechanisms underlying cannabinoid inhibition of excitatory synaptic transmission in rat striatal neurons. *The Journal of Physiology*, *532*(Pt 3), 731-748. https://doi.org/10.1111/j.1469-7793.2001.0731e.x

Hunter, R. L., Cheng, B., Choi, D.-Y., Liu, M., Liu, S., Cass, W. A., & Bing, G. (2009). Intrastriatal lipopolysaccharide injection induces parkinsonism in C57/B6 mice. *Journal of Neuroscience Research*, *87*(8), 1913-1921. https://doi.org/10.1002/jnr.22012

Hunter, R., Ojha, U., Bhurtel, S., Bing, G., & Choi, D.-Y. (2017). Lipopolysaccharide-induced functional and structural injury of the mitochondria in the nigrostriatal pathway. *Neuroscience Research*, *114*, 62-69. https://doi.org/10.1016/j.neures.2016.09.007

Huo, J., Cui, Q., Yang, W., & Guo, W. (2018). LPS induces dopamine depletion and iron accumulation in substantia nigra in rat models of Parkinson's disease. *International Journal of Clinical and Experimental Pathology*, *11*(10), 4942-4949.

Hurley, S. D., Walter, S. A., Semple-Rowland, S. L., & Streit, W. J. (1999). Cytokine transcripts expressed by microglia in vitro are not expressed by ameboid microglia of the developing rat central nervous system. *Glia*, *25*(3), 304-309. https://doi.org/10.1002/(sici)1098-1136(19990201)25:3<304::aid-glia10>3.0.co;2-w

Hurst, K., Badgley, C., Ellsworth, T., Bell, S., Friend, L., Prince, B., Welch, J., Cowan, Z., Williamson, R., Lyon, C., Anderson, B., Poole, B., Christensen, M., McNeil, M., Call, J., & Edwards, J. G. (2017). A putative lysophosphatidylinositol receptor GPR55 modulates hippocampal synaptic plasticity. *Hippocampus*, *27*(9), 985-998. https://doi.org/10.1002/hipo.22747

I

Ibarra-Gutiérrez, M. T., Serrano-García, N., & Orozco-Ibarra, M. (2023). Rotenone-Induced Model of Parkinson's Disease: Beyond Mitochondrial Complex I Inhibition. *Molecular Neurobiology*, *60*(4), 1929-1948. https://doi.org/10.1007/s12035-022-03193-8

Iovino, L., Tremblay, M. E., & Civiero, L. (2020). Glutamate-induced excitotoxicity in Parkinson's disease: The role of glial cells. *Journal of Pharmacological Sciences*, *144*(3), 151-164. https://doi.org/10.1016/j.jphs.2020.07.011

Ito, G., & Utsunomiya-Tate, N. (2023). Overview of the Impact of Pathogenic LRRK2 Mutations in Parkinson's Disease. *Biomolecules*, *13*(5), 845. https://doi.org/10.3390/biom13050845

Iuvone, T., Esposito, G., De Filippis, D., Bisogno, T., Petrosino, S., Scuderi, C., Di Marzo, V., Steardo, L., & Endocannabinoid Research Group. (2007). Cannabinoid CB1 receptor stimulation affords neuroprotection in MPTP-induced neurotoxicity by attenuating S100B up-regulation in vitro. *Journal of Molecular Medicine (Berlin, Germany)*, *85*(12), 1379-1392. https://doi.org/10.1007/s00109-007-0233-y

Iuvone, T., Esposito, G., De Filippis, D., Scuderi, C., & Steardo, L. (2009). Cannabidiol: A promising drug for neurodegenerative disorders? *CNS Neuroscience & Therapeutics*, *15*(1), 65-75. https://doi.org/10.1111/j.1755-5949.2008.00065.x

J

Jan, T.-R., Rao, G. K., & Kaminski, N. E. (2002). Cannabinol enhancement of interleukin-2 (IL-2) expression by T cells is associated with an increase in IL-2 distal nuclear factor of activated T cell activity. *Molecular Pharmacology*, *61*(2), 446-454. https://doi.org/10.1124/mol.61.2.446

Janetzky, B., Hauck, S., Youdim, M. B., Riederer, P., Jellinger, K., Pantucek, F., Zöchling, R., Boissl, K. W., & Reichmann, H. (1994). Unaltered aconitase activity, but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. *Neuroscience Letters*, *169*(1-2), 126-128. https://doi.org/10.1016/0304-3940(94)90372-7

Jankovic, J., & Poewe, W. (2012). Therapies in Parkinson's disease. *Current Opinion in Neurology*, *25*(4), 433-447. https://doi.org/10.1097/WCO.0b013e3283542fc2

Jarvis, M. F., & Wagner, G. C. (1985). Age-dependent effects of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). *Neuropharmacology*, *24*(6), 581-583. https://doi.org/10.1016/0028-3908(85)90068-1

Javed, H., Kamal, M. A., & Ojha, S. (2016). An Overview on the Role of α -Synuclein in Experimental Models of Parkinson's Disease from Pathogenesis to Therapeutics. *CNS & Neurological Disorders Drug Targets*, *15*(10), 1240-1252. https://doi.org/10.2174/1871527315666160920160512

Jia, F., Fellner, A., & Kumar, K. R. (2022). Monogenic Parkinson's Disease: Genotype, Phenotype, Pathophysiology, and Genetic Testing. *Genes*, *13*(3), 471. https://doi.org/10.3390/genes13030471

Jiang, C., Hopfner, F., Katsikoudi, A., Hein, R., Catli, C., Evetts, S., Huang, Y., Wang, H., Ryder, J. W., Kuhlenbaeumer, G., Deuschl, G., Padovani, A., Berg, D., Borroni, B., Hu, M. T., Davis, J. J., & Tofaris, G. K. (2020). Serum neuronal exosomes predict and differentiate Parkinson's disease from atypical parkinsonism. *Journal of Neurology, Neurosurgery, and Psychiatry*, *91*(7), 720-729. https://doi.org/10.1136/jnnp-2019-322588

Jiang, M., Yun, Q., Shi, F., Niu, G., Gao, Y., Xie, S., & Yu, S. (2016). Downregulation of miR-384-5p attenuates rotenone-induced neurotoxicity in dopaminergic SH-SY5Y cells through inhibiting endoplasmic reticulum stress. *American Journal of Physiology. Cell Physiology*, *310*(9), C755-763. https://doi.org/10.1152/ajpcell.00226.2015

Jiang, P., Gan, M., Lin, W.-L., & Yen, S.-H. C. (2014). Nutrient deprivation induces α-synuclein aggregation through endoplasmic reticulum stress response and SREBP2 pathway. *Frontiers in Aging Neuroscience*, *6*, 268. https://doi.org/10.3389/fnagi.2014.00268

Joers, V., Murray, B. C., McLaughlin, C., Oliver, D., Staley, H. E., Coronado, J., Achat-Mendes, C., Golshani, S., Kelly, S. D., Goodson, M., Lee, D., Manfredsson, F. P., Moore Ii, B. M., & Tansey, M. G. (2024). Modulation of cannabinoid receptor 2 alters neuroinflammation and reduces formation of alpha-synuclein aggregates in a rat model of nigral synucleinopathy. *Journal of Neuroinflammation*, *21*(1), 240. https://doi.org/10.1186/s12974-024-03221-5

Johns, D. G., Behm, D. J., Walker, D. J., Ao, Z., Shapland, E. M., Daniels, D. A., Riddick, M., Dowell, S., Staton, P. C., Green, P., Shabon, U., Bao, W., Aiyar, N., Yue, T.-L., Brown, A. J., Morrison, A. D., & Douglas, S. A. (2007a). The novel endocannabinoid receptor GPR55 is activated by atypical cannabinoids but does not mediate their vasodilator effects. *British Journal of Pharmacology*, *152*(5), 825-831. https://doi.org/10.1038/sj.bjp.0707419

Johns, D. G., Behm, D. J., Walker, D. J., Ao, Z., Shapland, E. M., Daniels, D. A., Riddick, M., Dowell, S., Staton, P. C., Green, P., Shabon, U., Bao, W., Aiyar, N., Yue, T.-L., Brown, A. J., Morrison, A. D., & Douglas, S. A. (2007b). The novel endocannabinoid receptor GPR55 is activated by atypical cannabinoids but does not mediate their vasodilator effects. *British Journal of Pharmacology*, *152*(5), 825-831. https://doi.org/10.1038/sj.bjp.0707419

Johnson, J. R., Lossignol, D., Burnell-Nugent, M., & Fallon, M. T. (2013). An open-label extension study to investigate the long-term safety and tolerability of THC/CBD oromucosal spray and oromucosal THC spray in patients with terminal cancer-related pain refractory to strong opioid analgesics. *Journal of Pain and Symptom Management*, *46*(2), 207-218. https://doi.org/10.1016/j.jpainsymman.2012.07.014

Junior, N. C. F., Dos-Santos-Pereira, M., Guimarães, F. S., & Del Bel, E. (2020). Cannabidiol and Cannabinoid Compounds as Potential Strategies for Treating Parkinson's Disease and L-DOPA-Induced Dyskinesia. *Neurotoxicity Research*, *37*(1), 12-29. https://doi.org/10.1007/s12640-019-00109-8

K

Kaczocha, M., Hermann, A., Glaser, S. T., Bojesen, I. N., & Deutsch, D. G. (2006). Anandamide uptake is consistent with rate-limited diffusion and is regulated by the degree of its hydrolysis by fatty acid amide hydrolase. *The Journal of Biological Chemistry*, *281*(14), 9066-9075. https://doi.org/10.1074/jbc.M509721200

Kaiser, S., Zhang, L., Mollenhauer, B., Jacob, J., Longerich, S., Del-Aguila, J., Marcus, J., Raghavan, N., Stone, D., Fagboyegun, O., Galasko, D., Dakna, M., Bilican, B., Dovlatyan, M., Kostikova, A., Li, J., Peterson, B., Rotte, M., Sanz, V., … Serrano-Fernandez, P. (2023). A proteogenomic view of Parkinson's disease causality and heterogeneity. *NPJ Parkinson's Disease*, *9*(1), 24. https://doi.org/10.1038/s41531-023-00461-9

Kalia, L. V., & Lang, A. E. (2015). Parkinson's disease. *The Lancet*, *386*(9996), 896-912. https://doi.org/10.1016/S0140-6736(14)61393-3

Kallendrusch, S., Kremzow, S., Nowicki, M., Grabiec, U., Winkelmann, R., Benz, A., Kraft, R., Bechmann, I., Dehghani, F., & Koch, M. (2013). The G protein-coupled receptor 55 ligand l-α-lysophosphatidylinositol exerts microglia-dependent neuroprotection after excitotoxic lesion. *Glia*, *61*(11), 1822-1831. https://doi.org/10.1002/glia.22560

Kapur, A., Zhao, P., Sharir, H., Bai, Y., Caron, M. G., Barak, L. S., & Abood, M. E. (2009). Atypical responsiveness of the orphan receptor GPR55 to cannabinoid ligands. *The Journal of Biological Chemistry*, *284*(43), 29817-29827. https://doi.org/10.1074/jbc.M109.050187

Kargl, J., Balenga, N. A., Platzer, W., Martini, L., Whistler, J. L., & Waldhoer, M. (2012). The GPCR-associated sorting protein 1 regulates ligand-induced down-regulation of GPR55. *British Journal of Pharmacology*, *165*(8), 2611-2619. https://doi.org/10.1111/j.1476-5381.2011.01562.x

Kasatkina, L. A., Rittchen, S., & Sturm, E. M. (2021). Neuroprotective and Immunomodulatory Action of the Endocannabinoid System under Neuroinflammation. *International Journal of Molecular Sciences*, *22*(11), 5431. https://doi.org/10.3390/ijms22115431

Keener, A., & Bordelon, Y. (2016). Parkinsonism. *Seminars in Neurology*, *36*(04), 330-334. https://doi.org/10.1055/s-0036-1585097

Khan, E., Hasan, I., & Haque, M. E. (2023). Parkinson's Disease: Exploring Different Animal Model Systems. *International Journal of Molecular Sciences*, *24*(10), 9088. https://doi.org/10.3390/ijms24109088

Khaspekov, L. G., & Illarioshkin, S. N. (2024). Therapeutic Application of Modulators of Endogenous Cannabinoid System in Parkinson's Disease. *International Journal of Molecular Sciences*, *25*(15), 8520. https://doi.org/10.3390/ijms25158520

Kindred, J. H., Li, K., Ketelhut, N. B., Proessl, F., Fling, B. W., Honce, J. M., Shaffer, W. R., & Rudroff, T. (2017). Cannabis use in people with Parkinson's disease and Multiple Sclerosis: A web-based investigation. *Complementary Therapies in Medicine*, *33*, 99-104. https://doi.org/10.1016/j.ctim.2017.07.002

Kirik, D., Annett, L. E., Burger, C., Muzyczka, N., Mandel, R. J., & Björklund, A. (2003). Nigrostriatal alpha-synucleinopathy induced by viral vector-mediated overexpression of human alpha-synuclein: A new primate model of Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(5), 2884-2889. https://doi.org/10.1073/pnas.0536383100

Kitada, T., Tong, Y., Gautier, C. A., & Shen, J. (2009). Absence of nigral degeneration in aged parkin/DJ-1/PINK1 triple knockout mice. *Journal of Neurochemistry*, *111*(3), 696-702. https://doi.org/10.1111/j.1471-4159.2009.06350.x

Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., & Lehmann, J. M. (1997). Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(9), 4318-4323. https://doi.org/10.1073/pnas.94.9.4318

Kohno, M., Hasegawa, H., Inoue, A., Muraoka, M., Miyazaki, T., Oka, K., & Yasukawa, M. (2006). Identification of N-arachidonylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18. *Biochemical and Biophysical Research Communications*, *347*(3), 827-832. https://doi.org/10.1016/j.bbrc.2006.06.175

Komorowska-Müller, J. A., & Schmöle, A.-C. (2020). CB2 Receptor in Microglia: The Guardian of Self-Control. *International Journal of Molecular Sciences*, *22*(1), 19. https://doi.org/10.3390/ijms22010019

Kozela, E., Haj, C., Hanuš, L., Chourasia, M., Shurki, A., Juknat, A., Kaushansky, N., Mechoulam, R., & Vogel, Z. (2016). HU-446 and HU-465, Derivatives of the Non-psychoactive Cannabinoid Cannabidiol, Decrease the Activation of Encephalitogenic T Cells. *Chemical Biology & Drug Design*, *87*(1), 143-153. https://doi.org/10.1111/cbdd.12637

Krasowska, D., Gerkowicz, A., Mlak, R., Leziak, M., Małecka-Massalska, T., & Krasowska, D. (2021). Risk of Nonmelanoma Skin Cancers and Parkinson's Disease-Meta-Analysis and Systematic Review. *Cancers*, *13*(4), 587. https://doi.org/10.3390/cancers13040587

Kreitzer, A. C. (2009). Physiology and pharmacology of striatal neurons. *Annual Review of Neuroscience*, *32*, 127-147. https://doi.org/10.1146/annurev.neuro.051508.135422

Kumari, S., Kumaran, S. S., Goyal, V., Sharma, R. K., Sinha, N., Dwivedi, S. N., Srivastava, A. K., & Jagannathan, N. R. (2020). Identification of potential urine biomarkers in idiopathic parkinson's disease using NMR. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *510*, 442-449. https://doi.org/10.1016/j.cca.2020.08.005

Kurano, M., Kobayashi, T., Sakai, E., Tsukamoto, K., & Yatomi, Y. (2021). Lysophosphatidylinositol, especially albumin-bound form, induces inflammatory cytokines in macrophages. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *35*(6), e21673. https://doi.org/10.1096/fj.202100245R

L

Lanciego, J. L., Barroso-Chinea, P., Rico, A. J., Conte-Perales, L., Callén, L., Roda, E., Gómez-Bautista, V., López, I. P., Lluis, C., Labandeira-García, J. L., & Franco, R. (2011). Expression of the mRNA coding the cannabinoid receptor 2 in the pallidal complex of Macaca fascicularis. *Journal of Psychopharmacology (Oxford, England)*, *25*(1), 97-104. https://doi.org/10.1177/0269881110367732

Langston, J. W., Ballard, P., Tetrud, J. W., & Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science (New York, N.Y.)*, *219*(4587), 979-980. https://doi.org/10.1126/science.6823561

Lapointe, N., St-Hilaire, M., Martinoli, M.-G., Blanchet, J., Gould, P., Rouillard, C., & Cicchetti, F. (2004). Rotenone induces non-specific central nervous system and systemic toxicity. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *18*(6), 717-719. https://doi.org/10.1096/fj.03-0677fje

Laprairie, R. B., Bagher, A. M., Kelly, M. E. M., & Denovan-Wright, E. M. (2015a). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *British Journal of Pharmacology*, *172*(20), 4790-4805. https://doi.org/10.1111/bph.13250

Laprairie, R. B., Bagher, A. M., Kelly, M. E. M., & Denovan-Wright, E. M. (2015b). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *British Journal of Pharmacology*, *172*(20), 4790-4805. https://doi.org/10.1111/bph.13250

Lastres-Becker, I., Cebeira, M., de Ceballos, M. L., Zeng, B. Y., Jenner, P., Ramos, J. A., & Fernández-Ruiz, J. J. (2001). Increased cannabinoid CB1 receptor binding and activation of GTP-binding proteins in the basal ganglia of patients with Parkinson's syndrome and of MPTP-treated marmosets. *The European Journal of Neuroscience*, *14*(11), 1827-1832. https://doi.org/10.1046/j.0953-816x.2001.01812.x

Lastres-Becker, I., Molina-Holgado, F., Ramos, J. A., Mechoulam, R., & Fernández-Ruiz, J. (2005). Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: Relevance to Parkinson's disease. *Neurobiology of Disease*, *19*(1-2), 96-107. https://doi.org/10.1016/j.nbd.2004.11.009

Lastres-Becker, I., Ulusoy, A., Innamorato, N. G., Sahin, G., Rábano, A., Kirik, D., & Cuadrado, A. (2012). α-Synuclein expression and Nrf2 deficiency cooperate to aggravate protein aggregation, neuronal death and inflammation in early-stage Parkinson's disease. *Human Molecular Genetics*, *21*(14), 3173-3192. https://doi.org/10.1093/hmg/dds143

Lauckner, J. E., Hille, B., & Mackie, K. (2005). The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(52), 19144-19149. https://doi.org/10.1073/pnas.0509588102

Lauckner, J. E., Jensen, J. B., Chen, H.-Y., Lu, H.-C., Hille, B., & Mackie, K. (2008). GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(7), 2699-2704. https://doi.org/10.1073/pnas.0711278105

Lechner, S. A., Barnett, D. G. S., Gammie, S. C., & Kelm-Nelson, C. A. (2024). Prodromal Parkinson disease signs are predicted by a whole-blood inflammatory transcriptional signature in young Pink1-/- rats. *BMC Neuroscience*, *25*(1), 11. https://doi.org/10.1186/s12868-024-00857-0

Lee, B. D., Shin, J.-H., VanKampen, J., Petrucelli, L., West, A. B., Ko, H. S., Lee, Y.-I., Maguire-Zeiss, K. A., Bowers, W. J., Federoff, H. J., Dawson, V. L., & Dawson, T. M. (2010). Inhibitors of leucine-rich repeat kinase-2 protect against models of Parkinson's disease. *Nature Medicine*, *16*(9), 998-1000. https://doi.org/10.1038/nm.2199

Lee, C. S., Sauer, H., & Bjorklund, A. (1996). Dopaminergic neuronal degeneration and motor impairments following axon terminal lesion by instrastriatal 6-hydroxydopamine in the rat. *Neuroscience*, *72*(3), 641-653. https://doi.org/10.1016/0306-4522(95)00571-4

Lee, G., Elwood, F., McNally, J., Weiszmann, J., Lindstrom, M., Amaral, K., Nakamura, M., Miao, S., Cao, P., Learned, R. M., Chen, J.-L., & Li, Y. (2002). T0070907, a selective ligand for peroxisome proliferator-activated receptor gamma, functions as an antagonist of biochemical and cellular activities. *The Journal of Biological Chemistry*, *277*(22), 19649-19657. https://doi.org/10.1074/jbc.M200743200

Lee, J. H., Han, J., Kim, H., Park, S. M., Joe, E., & Jou, I. (2019). Parkinson's disease-associated LRRK2-G2019S mutant acts through regulation of SERCA activity to control ER stress in astrocytes. *Acta Neuropathologica Communications*, *7*, 68. https://doi.org/10.1186/s40478-019-0716-4

Leem, Y.-H., Park, J.-S., Park, J.-E., Kim, D.-Y., & Kim, H.-S. (2022). Neurogenic effects of rotarod walking exercise in subventricular zone, subgranular zone, and substantia nigra in MPTP-induced Parkinson's disease mice. *Scientific Reports*, *12*(1), 10544. https://doi.org/10.1038/s41598-022-14823-5

Leija-Salazar, M., Bermúdez de León, M., González-Horta, A., & González-Hernández, B. (2020). Arachidonyl-2'-chloroethylamide (ACEA), a synthetic agonist of cannabinoid receptor, increases CB1R gene expression and reduces dyskinesias in a rat model of Parkinson's disease. *Pharmacology, Biochemistry, and Behavior*, *194*, 172950. https://doi.org/10.1016/j.pbb.2020.172950

Levine, A., Liktor-Busa, E., Lipinski, A. A., Couture, S., Balasubramanian, S., Aicher, S. A., Langlais, P. R., Vanderah, T. W., & Largent-Milnes, T. M. (2021). Sex differences in the expression of the endocannabinoid system within V1M cortex and PAG of Sprague Dawley rats. *Biology of Sex Differences*, *12*(1), 60. https://doi.org/10.1186/s13293-021-00402-2

Levtova, N., Healy, L. M., Gonczi, C. M. C., Stopnicki, B., Blain, M., Kennedy, T. E., Moore, C. S., Antel, J. P., & Darlington, P. J. (2017). Comparative morphology and phagocytic capacity of primary human adult microglia with time-lapse imaging. *Journal of Neuroimmunology*, *310*, 143-149. https://doi.org/10.1016/j.jneuroim.2017.05.012

Li, X., Hua, T., Vemuri, K., Ho, J.-H., Wu, Y., Wu, L., Popov, P., Benchama, O., Zvonok, N., Locke, K., Qu, L., Han, G. W., Iyer, M. R., Cinar, R., Coffey, N. J., Wang, J., Wu, M., Katritch, V., Zhao, S., … Liu, Z.-J. (2019). Crystal Structure of the Human Cannabinoid Receptor CB2. *Cell*, *176*(3), 459-467.e13. https://doi.org/10.1016/j.cell.2018.12.011

Li, Y., Liu, W., Oo, T. F., Wang, L., Tang, Y., Jackson-Lewis, V., Zhou, C., Geghman, K., Bogdanov, M., Przedborski, S., Beal, M. F., Burke, R. E., & Li, C. (2009). Mutant LRRK2(R1441G) BAC transgenic mice recapitulate cardinal features of Parkinson's disease. *Nature Neuroscience*, *12*(7), 826-828. https://doi.org/10.1038/nn.2349

Li, Y.-H., He, Q., Yu, J., Liu, C., Feng, L., Chai, Z., Wang, Q., Zhang, H., Zhang, G.-X., Xiao, B., & Ma, C. (2015). Lipoic acid protects dopaminergic neurons in LPS-induced Parkinson's disease model. *Metabolic Brain Disease*, *30*(5), 1217-1226. https://doi.org/10.1007/s11011-015-9698-5

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., Bennett, M. L., Münch, A. E., Chung, W.-S., Peterson, T. C., Wilton, D. K., Frouin, A., Napier, B. A., Panicker, N., Kumar, M., Buckwalter, M. S., Rowitch, D. H., Dawson, V. L., Dawson, T. M., … Barres, B. A. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature*, *541*(7638), 481-487. https://doi.org/10.1038/nature21029

Lin, C.-H., Chen, C.-C., Chiang, H.-L., Liou, J.-M., Chang, C.-M., Lu, T.-P., Chuang, E. Y., Tai, Y.-C., Cheng, C., Lin, H.-Y., & Wu, M.-S. (2019). Altered gut microbiota and inflammatory cytokine responses in patients with Parkinson's disease. *Journal of Neuroinflammation*, *16*(1), 129. https://doi.org/10.1186/s12974-019-1528-y

Lin, Y.-C., Uang, H.-W., Lin, R.-J., Chen, I.-J., & Lo, Y.-C. (2007). Neuroprotective effects of glyceryl nonivamide against microglia-like cells and 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y human dopaminergic neuroblastoma cells. *The Journal of Pharmacology and Experimental Therapeutics*, *323*(3), 877-887. https://doi.org/10.1124/jpet.107.125955

Lindström, V., Gustafsson, G., Sanders, L. H., Howlett, E. H., Sigvardson, J., Kasrayan, A., Ingelsson, M., Bergström, J., & Erlandsson, A. (2017). Extensive uptake of α-synuclein oligomers in astrocytes results in sustained intracellular deposits and mitochondrial damage. *Molecular and Cellular Neurosciences*, *82*, 143-156. https://doi.org/10.1016/j.mcn.2017.04.009

Little, P. J., Compton, D. R., Johnson, M. R., Melvin, L. S., & Martin, B. R. (1988). Pharmacology and stereoselectivity of structurally novel cannabinoids in mice. *The Journal of Pharmacology and Experimental Therapeutics*, *247*(3), 1046-1051.

Litwiniuk, A., Baranowska-Bik, A., Domańska, A., Kalisz, M., & Bik, W. (2021). Contribution of Mitochondrial Dysfunction Combined with NLRP3 Inflammasome Activation in Selected Neurodegenerative Diseases. *Pharmaceuticals (Basel, Switzerland)*, *14*(12), 1221. https://doi.org/10.3390/ph14121221

Liu, G., Zhang, C., Yin, J., Li, X., Cheng, F., Li, Y., Yang, H., Uéda, K., Chan, P., & Yu, S. (2009). Alpha-Synuclein is differentially expressed in mitochondria from different rat brain regions and dose-dependently down-regulates complex I activity. *Neuroscience Letters*, *454*(3), 187-192. https://doi.org/10.1016/j.neulet.2009.02.056

Liu, M., Pan, D., Wang, M., Deng, H., & Ma, Z. (2024). JWH133 attenuates behavior deficits and iron accumulation in 6-OHDA-induced Parkinson's disease model rats. *Journal of Neurophysiology*, *132*(3), 733-743. https://doi.org/10.1152/jn.00137.2024

Liu, Y., Qin, L., Wilson, B., Wu, X., Qian, L., Granholm, A.-C., Crews, F. T., & Hong, J.-S. (2008). Endotoxin induces a delayed loss of TH-IR neurons in substantia nigra and motor behavioral deficits. *Neurotoxicology*, *29*(5), 864-870. https://doi.org/10.1016/j.neuro.2008.02.014

Liu, Z., Yang, N., Dong, J., Tian, W., Chang, L., Ma, J., Guo, J., Tan, J., Dong, A., He, K., Zhou, J., Cinar, R., Wu, J., Salinas, A. G., Sun, L., Kumar, M., Sullivan, B. T., Oldham, B. B., Pitz, V., … Tang, B. (2022). Deficiency in endocannabinoid synthase DAGLB contributes to early onset Parkinsonism and murine nigral dopaminergic neuron dysfunction. *Nature Communications*, *13*(1), 3490. https://doi.org/10.1038/s41467-022-31168-9

Lo Bianco, C., Ridet, J.-L., Schneider, B. L., Deglon, N., & Aebischer, P. (2002). Alpha -Synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(16), 10813-10818. https://doi.org/10.1073/pnas.152339799

Long, J. Z., Nomura, D. K., & Cravatt, B. F. (2009). Characterization of monoacylglycerol lipase inhibition reveals differences in central and peripheral endocannabinoid metabolism. *Chemistry & Biology*, *16*(7), 744-753. https://doi.org/10.1016/j.chembiol.2009.05.009

Lopes, K. de P., Snijders, G. J. L., Humphrey, J., Allan, A., Sneeboer, M. A. M., Navarro, E., Schilder, B. M., Vialle, R. A., Parks, M., Missall, R., van Zuiden, W., Gigase, F. A. J., Kübler, R., van Berlekom, A. B., Hicks, E. M., Böttcher, C., Priller, J., Kahn, R. S., de Witte, L. D., & Raj, T. (2022). Genetic analysis of the human microglial transcriptome across brain regions, aging and disease pathologies. *Nature Genetics*, *54*(1), 4-17. https://doi.org/10.1038/s41588-021-00976-y

López, A., Aparicio, N., Pazos, M. R., Grande, M. T., Barreda-Manso, M. A., Benito-Cuesta, I., Vázquez, C., Amores, M., Ruiz-Pérez, G., García-García, E., Beatka, M., Tolón, R. M., Dittel, B. N., Hillard, C. J., & Romero, J. (2018). Cannabinoid CB2 receptors in the mouse brain: Relevance for Alzheimer's disease. *Journal of Neuroinflammation*, *15*(1), 158. https://doi.org/10.1186/s12974-018-1174-9

López-Rodríguez, M. L., Viso, A., Ortega-Gutiérrez, S., Fowler, C. J., Tiger, G., de Lago, E., Fernández-Ruiz, J., & Ramos, J. A. (2003). Design, synthesis, and biological evaluation of new inhibitors of the endocannabinoid uptake: Comparison with effects on fatty acid amidohydrolase. *Journal of Medicinal Chemistry*, *46*(8), 1512-1522. https://doi.org/10.1021/jm0210818

Loria, F., Vargas, J. Y., Bousset, L., Syan, S., Salles, A., Melki, R., & Zurzolo, C. (2017). α-Synuclein transfer between neurons and astrocytes indicates that astrocytes play a role in degradation rather than in spreading. *Acta Neuropathologica*, *134*(5), 789-808. https://doi.org/10.1007/s00401-017-1746-2

Lotan, I., Treves, T. A., Roditi, Y., & Djaldetti, R. (2014). Cannabis (medical marijuana) treatment for motor and non-motor symptoms of Parkinson disease: An open-label observational study. *Clinical Neuropharmacology*, *37*(2), 41-44. https://doi.org/10.1097/WNF.0000000000000016

Lozano, C. S., Tam, J., & Lozano, A. M. (2018). The changing landscape of surgery for Parkinson's Disease. *Movement Disorders: Official Journal of the Movement Disorder Society*, *33*(1), 36-47. https://doi.org/10.1002/mds.27228

Lu, H.-C., & Mackie, K. (2016). An Introduction to the Endogenous Cannabinoid System. *Biological Psychiatry*, *79*(7), 516-525. https://doi.org/10.1016/j.biopsych.2015.07.028

Lu, H.-C., & Mackie, K. (2021). Review of the Endocannabinoid System. *Biological Psychiatry. Cognitive Neuroscience and Neuroimaging*, *6*(6), 607-615. https://doi.org/10.1016/j.bpsc.2020.07.016

Luk, K. C., Kehm, V., Carroll, J., Zhang, B., O'Brien, P., Trojanowski, J. Q., & Lee, V. M.-Y. (2012). Pathological α-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. *Science (New York, N.Y.)*, *338*(6109), 949-953. https://doi.org/10.1126/science.1227157

Luk, K. C., Kehm, V. M., Zhang, B., O'Brien, P., Trojanowski, J. Q., & Lee, V. M. Y. (2012). Intracerebral inoculation of pathological α-synuclein initiates a rapidly progressive neurodegenerative α-synucleinopathy in mice. *The Journal of Experimental Medicine*, *209*(5), 975-986. https://doi.org/10.1084/jem.20112457

Luthman, J., Fredriksson, A., Sundström, E., Jonsson, G., & Archer, T. (1989). Selective lesion of central dopamine or noradrenaline neuron systems in the neonatal rat: Motor behavior and monoamine alterations at adult stage. *Behavioural Brain Research*, *33*(3), 267-277. https://doi.org/10.1016/s0166-4328(89)80121-4

M

Maccarrone, M., Gubellini, P., Bari, M., Picconi, B., Battista, N., Centonze, D., Bernardi, G., Finazzi-Agrò, A., & Calabresi, P. (2003). Levodopa treatment reverses endocannabinoid system abnormalities in experimental parkinsonism. *Journal of Neurochemistry*, *85*(4), 1018-1025. https://doi.org/10.1046/j.1471-4159.2003.01759.x

Maccarrone, M., Rossi, S., Bari, M., De Chiara, V., Fezza, F., Musella, A., Gasperi, V., Prosperetti, C., Bernardi, G., Finazzi-Agrò, A., Cravatt, B. F., & Centonze, D. (2008). Anandamide inhibits metabolism and physiological actions of 2-arachidonoylglycerol in the striatum. *Nature Neuroscience*, *11*(2), 152-159. https://doi.org/10.1038/nn2042

Mackie, K. (2005a). Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handbook of Experimental Pharmacology*, *168*, 299-325. https://doi.org/10.1007/3-540-26573-2_10

Mackie, K. (2005b). Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handbook of Experimental Pharmacology*, *168*, 299-325. https://doi.org/10.1007/3-540-26573-2_10

Magnus, T., Chan, A., Grauer, O., Toyka, K. V., & Gold, R. (2001). Microglial phagocytosis of apoptotic inflammatory T cells leads to down-regulation of microglial immune activation. *Journal of Immunology (Baltimore, Md.: 1950)*, *167*(9), 5004-5010. https://doi.org/10.4049/jimmunol.167.9.5004

Malek, N. (2019). Deep Brain Stimulation in Parkinson's Disease. *Neurology India*, *67*(4), 968. https://doi.org/10.4103/0028-3886.266268

Malek, N., Kanavou, S., Lawton, M. A., Pitz, V., Grosset, K. A., Bajaj, N., Barker, R. A., Ben-Shlomo, Y., Burn, D. J., Foltynie, T., Hardy, J., Williams, N. M., Wood, N., Morris, H. R., Grosset, D. G., & PRoBaND clinical consortium. (2019). L-dopa responsiveness in early Parkinson's disease is associated with the rate of motor progression. *Parkinsonism & Related Disorders*, *65*, 55-61. https://doi.org/10.1016/j.parkreldis.2019.05.022

Mao, Y., Huang, Y., Zhang, Y., Wang, C., Wu, H., Tian, X., Liu, Y., Hou, B., Liang, Y., Rong, H., Gu, X., & Ma, Z. (2019). Cannabinoid receptor 2-selective agonist JWH015 attenuates bone cancer pain through the amelioration of impaired autophagy flux induced by inflammatory mediators in the spinal cord. *Molecular Medicine Reports*, *20*(6), 5100-5110. https://doi.org/10.3892/mmr.2019.10772

Marichal-Cancino, B. A., Fajardo-Valdez, A., Ruiz-Contreras, A. E., Mendez-Díaz, M., & Prospero-García, O. (2017). Advances in the Physiology of GPR55 in the Central Nervous System. *Current Neuropharmacology*, *15*(5), 771-778. https://doi.org/10.2174/1570159X14666160729155441

Marin, C., & Aguilar, E. (2011). In vivo 6-OHDA-induced neurodegeneration and nigral autophagic markers expression. *Neurochemistry International*, *58*(4), 521-526. https://doi.org/10.1016/j.neuint.2011.01.010

Marsicano, G., Goodenough, S., Monory, K., Hermann, H., Eder, M., Cannich, A., Azad, S. C., Cascio, M. G., Gutiérrez, S. O., van der Stelt, M., López-Rodriguez, M. L., Casanova, E., Schütz, G., Zieglgänsberger, W., Di Marzo, V., Behl, C., & Lutz, B. (2003). CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science (New York, N.Y.)*, *302*(5642), 84-88. https://doi.org/10.1126/science.1088208

Marsicano, G., & Lafenêtre, P. (2009). Roles of the endocannabinoid system in learning and memory. *Current Topics in Behavioral Neurosciences*, *1*, 201-230. https://doi.org/10.1007/978-3-540-88955-7_8

Marsicano, G., Moosmann, B., Hermann, H., Lutz, B., & Behl, C. (2002). Neuroprotective properties of cannabinoids against oxidative stress: Role of the cannabinoid receptor CB1. *Journal of Neurochemistry*, *80*(3), 448-456. https://doi.org/10.1046/j.0022-3042.2001.00716.x

Martella, G., Madeo, G., Maltese, M., Vanni, V., Puglisi, F., Ferraro, E., Schirinzi, T., Valente, E. M., Bonanni, L., Shen, J., Mandolesi, G., Mercuri, N. B., Bonsi, P., & Pisani, A. (2016). Exposure to low-dose rotenone precipitates synaptic plasticity alterations in PINK1 heterozygous knockout mice. *Neurobiology of Disease*, *91*, 21-36. https://doi.org/10.1016/j.nbd.2015.12.020

Martin, B. R., Compton, D. R., Thomas, B. F., Prescott, W. R., Little, P. J., Razdan, R. K., Johnson, M. R., Melvin, L. S., Mechoulam, R., & Ward, S. J. (1991). Behavioral, biochemical, and molecular modeling evaluations of cannabinoid analogs. *Pharmacology, Biochemistry, and Behavior*, *40*(3), 471-478. https://doi.org/10.1016/0091-3057(91)90349-7

Martin, M., Ledent, C., Parmentier, M., Maldonado, R., & Valverde, O. (2002). Involvement of CB1 cannabinoid receptors in emotional behaviour. *Psychopharmacology*, *159*(4), 379-387. https://doi.org/10.1007/s00213-001-0946-5

Martínez-Fernández, R., Natera-Villalba, E., Máñez Miró, J. U., Rodriguez-Rojas, R., Marta Del Álamo, M., Pineda-Pardo, J. Á., Ammann, C., Obeso, I., Mata-Marín, D., Hernández-Fernández, F., Gasca-Salas, C., Matarazzo, M., Alonso-Frech, F., & Obeso, J. A. (2023). Prospective Long-term Follow-up of Focused Ultrasound Unilateral Subthalamotomy for Parkinson Disease. *Neurology*, *100*(13), e1395-e1405. https://doi.org/10.1212/WNL.0000000000206771

Martínez-Pinilla, E., Reyes-Resina, I., Oñatibia-Astibia, A., Zamarbide, M., Ricobaraza, A., Navarro, G., Moreno, E., Dopeso-Reyes, I. G., Sierra, S., Rico, A. J., Roda, E., Lanciego, J. L., & Franco, R. (2014a). CB1 and GPR55 receptors are co-expressed and form heteromers in rat and monkey striatum. *Experimental Neurology*, *261*, 44-52. https://doi.org/10.1016/j.expneurol.2014.06.017

Martínez-Pinilla, E., Reyes-Resina, I., Oñatibia-Astibia, A., Zamarbide, M., Ricobaraza, A., Navarro, G., Moreno, E., Dopeso-Reyes, I. G., Sierra, S., Rico, A. J., Roda, E., Lanciego, J. L., & Franco, R. (2014b). CB1 and GPR55 receptors are co-expressed and form heteromers in rat and monkey striatum. *Experimental Neurology*, *261*, 44-52. https://doi.org/10.1016/j.expneurol.2014.06.017

Martínez-Pinilla, E., Rico, A. J., Rivas-Santisteban, R., Lillo, J., Roda, E., Navarro, G., Franco, R., & Lanciego, J. L. (2020). Expression of cannabinoid CB1 R-GPR55 heteromers in neuronal subtypes of the Macaca fascicularis striatum. *Annals of the New York Academy of Sciences*, *1475*(1), 34-42. https://doi.org/10.1111/nyas.14413

Martínez-Pinilla, E., Varani, K., Reyes-Resina, I., Angelats, E., Vincenzi, F., Ferreiro-Vera, C., Oyarzabal, J., Canela, E. I., Lanciego, J. L., Nadal, X., Navarro, G., Borea, P. A., & Franco, R. (2017a). Binding and Signaling Studies Disclose a Potential Allosteric Site for Cannabidiol in Cannabinoid CB2 Receptors. *Frontiers in Pharmacology*, *8*, 744. https://doi.org/10.3389/fphar.2017.00744

Martínez-Pinilla, E., Varani, K., Reyes-Resina, I., Angelats, E., Vincenzi, F., Ferreiro-Vera, C., Oyarzabal, J., Canela, E. I., Lanciego, J. L., Nadal, X., Navarro, G., Borea, P. A., & Franco, R. (2017b). Binding and Signaling Studies Disclose a Potential Allosteric Site for Cannabidiol in Cannabinoid CB2 Receptors. *Frontiers in Pharmacology*, *8*, 744. https://doi.org/10.3389/fphar.2017.00744

Martin-Santos, R., Crippa, J. A., Batalla, A., Bhattacharyya, S., Atakan, Z., Borgwardt, S., Allen, P., Seal, M., Langohr, K., Farré, M., Zuardi, A. W., & McGuire, P. K. (2012). Acute effects of a single, oral dose of d9-tetrahydrocannabinol (THC) and cannabidiol (CBD) administration in healthy volunteers. *Current Pharmaceutical Design*, *18*(32), 4966-4979. https://doi.org/10.2174/138161212802884780

Matouk, A. I., Taye, A., El-Moselhy, M. A., Heeba, G. H., & Abdel-Rahman, A. A. (2018). Abnormal cannabidiol confers cardioprotection in diabetic rats independent of glycemic control. *European Journal of Pharmacology*, *820*, 256-264. https://doi.org/10.1016/j.ejphar.2017.12.039

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., & Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, *346*(6284), 561-564. https://doi.org/10.1038/346561a0

Matsui, H., Ito, J., Matsui, N., Uechi, T., Onodera, O., & Kakita, A. (2021). Cytosolic dsDNA of mitochondrial origin induces cytotoxicity and neurodegeneration in cellular and zebrafish models of Parkinson's disease. *Nature Communications*, *12*(1), 3101. https://doi.org/10.1038/s41467-021-23452-x

Matsuoka, Y., Vila, M., Lincoln, S., McCormack, A., Picciano, M., LaFrancois, J., Yu, X., Dickson, D., Langston, W. J., McGowan, E., Farrer, M., Hardy, J., Duff, K., Przedborski, S., & Di Monte, D. A. (2001). Lack of nigral pathology in transgenic mice expressing human alpha-synuclein driven by the tyrosine hydroxylase promoter. *Neurobiology of Disease*, *8*(3), 535-539. https://doi.org/10.1006/nbdi.2001.0392

McCarthy, R. C., Lu, D.-Y., Alkhateeb, A., Gardeck, A. M., Lee, C.-H., & Wessling-Resnick, M. (2016). Characterization of a novel adult murine immortalized microglial cell line and its activation by amyloid-beta. *Journal of Neuroinflammation*, *13*, 21. https://doi.org/10.1186/s12974-016-0484-z

McFarthing, K., Buff, S., Rafaloff, G., Pitzer, K., Fiske, B., Navangul, A., Beissert, K., Pilcicka, A., Fuest, R., Wyse, R. K., & Stott, S. R. W. (2024). Parkinson's Disease Drug Therapies in the Clinical Trial Pipeline: 2024 Update. *Journal of Parkinson's Disease*, *14*(5), 899-912. https://doi.org/10.3233/JPD-240272

McGregor, M. M., & Nelson, A. B. (2019). Circuit Mechanisms of Parkinson's Disease. *Neuron*, *101*(6), 1042-1056. https://doi.org/10.1016/j.neuron.2019.03.004

McKinnon, C., De Snoo, M. L., Gondard, E., Neudorfer, C., Chau, H., Ngana, S. G., O'Hara, D. M., Brotchie, J. M., Koprich, J. B., Lozano, A. M., Kalia, L. V., & Kalia, S. K. (2020). Early-onset impairment of the ubiquitin-proteasome system in dopaminergic neurons caused by α-synuclein. *Acta Neuropathologica Communications*, *8*(1), 17. https://doi.org/10.1186/s40478-020-0894-0

McMahon, J. M., McQuaid, S., Reynolds, R., & FitzGerald, U. F. (2012). Increased expression of ER stress- and hypoxia-associated molecules in grey matter lesions in multiple sclerosis. *Multiple Sclerosis (Houndmills, Basingstoke, England)*, *18*(10), 1437-1447. https://doi.org/10.1177/1352458512438455

Meares, G. P., Liu, Y., Rajbhandari, R., Qin, H., Nozell, S. E., Mobley, J. A., Corbett, J. A., & Benveniste, E. N. (2014). PERK-dependent activation of JAK1 and STAT3 contributes to endoplasmic reticulum stress-induced inflammation. *Molecular and Cellular Biology*, *34*(20), 3911-3925. https://doi.org/10.1128/MCB.00980-14

Mechoulam, R. (2000). Looking back at Cannabis research. *Current Pharmaceutical Design*, *6*(13), 1313-1322. https://doi.org/10.2174/1381612003399509

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., Gopher, A., Almog, S., Martin, B. R., & Compton, D. R. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochemical Pharmacology*, *50*(1), 83-90. https://doi.org/10.1016/0006-2952(95)00109-d

Mechoulam, R., Lander, N., Srebnik, M., Breuer, A., Segal, M., Feigenbaum, J. J., Jarbe, T. U., & Consroe, P. (1987). Stereochemical requirements for cannabimimetic activity. *NIDA Research Monograph*, *79*, 15-30.

Mechoulam, R., Lander, N., Varkony, T. H., Kimmel, I., Becker, O., Ben-Zvi, Z., Edery, H., & Porath, G. (1980). Stereochemical requirements for cannabinoid activity. *Journal of Medicinal Chemistry*, *23*(10), 1068-1072. https://doi.org/10.1021/jm00184a002

Mechoulam, R., & Shvo, Y. (1963). Hashish. I. The structure of cannabidiol. *Tetrahedron*, *19*(12), 2073-2078. https://doi.org/10.1016/0040-4020(63)85022-x

Melo, E. P., Konno, T., Farace, I., Awadelkareem, M. A., Skov, L. R., Teodoro, F., Sancho, T. P., Paton, A. W., Paton, J. C., Fares, M., Paulo, P. M. R., Zhang, X., & Avezov, E. (2022). Stress-induced protein disaggregation in the endoplasmic reticulum catalysed by BiP. *Nature Communications*, *13*(1), 2501. https://doi.org/10.1038/s41467-022-30238-2

Méndez-Díaz, M., Ruiz-Contreras, A. E., Cortés-Morelos, J., & Prospéro-García, O. (2021). Cannabinoids and Sleep/Wake Control. *Advances in Experimental Medicine and Biology*, *1297*, 83-95. https://doi.org/10.1007/978-3-030-61663-2_6

Mendivil-Perez, M., Felizardo-Otalvaro, A. A., Jimenez-Del-Rio, M., & Velez-Pardo, C. (2023). Cannabidiol Protects Dopaminergic-like Neurons against Paraquat- and Maneb-Induced Cell Death through Safeguarding DJ-1CYS106 and Caspase 3 Independently of Cannabinoid Receptors: Relevance in Parkinson's Disease. *ACS Chemical Neuroscience*, *14*(11), 2159-2171. https://doi.org/10.1021/acschemneuro.3c00176

Mesnage, V., Houeto, J. L., Bonnet, A. M., Clavier, I., Arnulf, I., Cattelin, F., Le Fur, G., Damier, P., Welter, M. L., & Agid, Y. (2004). Neurokinin B, neurotensin, and cannabinoid receptor antagonists and Parkinson disease. *Clinical Neuropharmacology*, *27*(3), 108-110. https://doi.org/10.1097/00002826-200405000-00003

Minakawa, E. N., & Nagai, Y. (2021). Protein Aggregation Inhibitors as Disease-Modifying Therapies for Polyglutamine Diseases. *Frontiers in Neuroscience*, *15*, 621996. https://doi.org/10.3389/fnins.2021.621996

Mirza, A. Z., Althagafi, I. I., & Shamshad, H. (2019). Role of PPAR receptor in different diseases and their ligands: Physiological importance and clinical implications. *European Journal of Medicinal Chemistry*, *166*, 502-513. https://doi.org/10.1016/j.ejmech.2019.01.067

Molina-Holgado, E., Esteban, P. F., Arevalo-Martin, Á., Moreno-Luna, R., Molina-Holgado, F., & Garcia-Ovejero, D. (2023). Endocannabinoid signaling in oligodendroglia. *Glia*, *71*(1), 91-102. https://doi.org/10.1002/glia.24180

Montero, C., Campillo, N. E., Goya, P., & Páez, J. A. (2005). Homology models of the cannabinoid CB1 and CB2 receptors. A docking analysis study. *European Journal of Medicinal Chemistry*, *40*(1), 75-83. https://doi.org/10.1016/j.ejmech.2004.10.002

Moors, T. E., Hoozemans, J. J. M., Ingrassia, A., Beccari, T., Parnetti, L., Chartier-Harlin, M.-C., & van de Berg, W. D. J. (2017). Therapeutic potential of autophagy-enhancing agents in Parkinson's disease. *Molecular Neurodegeneration*, *12*(1), 11. https://doi.org/10.1186/s13024-017-0154-3

Moradi Vastegani, S., Nasrolahi, A., Ghaderi, S., Belali, R., Rashno, M., Farzaneh, M., & Khoshnam, S. E. (2023). Mitochondrial Dysfunction and Parkinson's Disease: Pathogenesis and Therapeutic Strategies. *Neurochemical Research*, *48*(8), 2285-2308. https://doi.org/10.1007/s11064-023-03904-0

Morale, M. C., Serra, P. A., L'episcopo, F., Tirolo, C., Caniglia, S., Testa, N., Gennuso, F., Giaquinta, G., Rocchitta, G., Desole, M. S., Miele, E., & Marchetti, B. (2006). Estrogen, neuroinflammation and neuroprotection in Parkinson's disease: Glia dictates resistance versus vulnerability to neurodegeneration. *Neuroscience*, *138*(3), 869-878. https://doi.org/10.1016/j.neuroscience.2005.07.060

Morales, P., Reggio, P. H., & Jagerovic, N. (2017). An Overview on Medicinal Chemistry of Synthetic and Natural Derivatives of Cannabidiol. *Frontiers in Pharmacology*, *8*, 422. https://doi.org/10.3389/fphar.2017.00422

Morales, P., Whyte, L. S., Chicharro, R., Gómez-Cañas, M., Pazos, M. R., Goya, P., Irving, A. J., Fernández-Ruiz, J., Ross, R. A., & Jagerovic, N. (2016). Identification of Novel GPR55 Modulators Using Cell-Impedance-Based Label-Free Technology. *Journal of Medicinal Chemistry*, *59*(5), 1840-1853. https://doi.org/10.1021/acs.jmedchem.5b01331

Moreno, E., Andradas, C., Medrano, M., Caffarel, M. M., Pérez-Gómez, E., Blasco-Benito, S., Gómez-Cañas, M., Pazos, M. R., Irving, A. J., Lluís, C., Canela, E. I., Fernández-Ruiz, J., Guzmán, M., McCormick, P. J., & Sánchez, C. (2014). Targeting CB2-GPR55 receptor heteromers modulates cancer cell signaling. *The Journal of Biological Chemistry*, *289*(32), 21960-21972. https://doi.org/10.1074/jbc.M114.561761

Morgese, M. G., Cassano, T., Cuomo, V., & Giuffrida, A. (2007). Anti-dyskinetic effects of cannabinoids in a rat model of Parkinson's disease: Role of CB(1) and TRPV1 receptors. *Experimental Neurology*, *208*(1), 110-119. https://doi.org/10.1016/j.expneurol.2007.07.021

Moriconi, A., Cerbara, I., Maccarrone, M., & Topai, A. (2010). GPR55: Current knowledge and future perspectives of a purported «Type-3» cannabinoid receptor. *Current Medicinal Chemistry*, *17*(14), 1411-1429. https://doi.org/10.2174/092986710790980069

Muller, C., Morales, P., & Reggio, P. H. (2018). Cannabinoid Ligands Targeting TRP Channels. *Frontiers in Molecular Neuroscience*, *11*, 487. https://doi.org/10.3389/fnmol.2018.00487

Müller-Nedebock, A. C., Brennan, R. R., Venter, M., Pienaar, I. S., van der Westhuizen, F. H., Elson, J. L., Ross, O. A., & Bardien, S. (2019). The unresolved role of mitochondrial DNA in Parkinson's disease: An overview of published studies, their limitations, and future prospects. *Neurochemistry International*, *129*, 104495. https://doi.org/10.1016/j.neuint.2019.104495

Munro, S., Thomas, K. L., & Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, *365*(6441), 61-65. https://doi.org/10.1038/365061a0

Muñoz-Delgado, L., Macías-García, D., Jesús, S., Martín-Rodríguez, J. F., Labrador-Espinosa, M. Á., Jiménez-Jaraba, M. V., Adarmes-Gómez, A., Carrillo, F., & Mir, P. (2021). Peripheral Immune Profile and Neutrophil-to-Lymphocyte Ratio in Parkinson's Disease. *Movement Disorders*, *36*(10), 2426-2430. https://doi.org/10.1002/mds.28685

Muñoz-Manchado, A. B., Villadiego, J., Romo-Madero, S., Suárez-Luna, N., Bermejo-Navas, A., Rodríguez-Gómez, J. A., Garrido-Gil, P., Labandeira-García, J. L., Echevarría, M., López-Barneo, J., & Toledo-Aral, J. J. (2016). Chronic and progressive Parkinson's disease MPTP model in adult and aged mice. *Journal of Neurochemistry*, *136*(2), 373-387. https://doi.org/10.1111/jnc.13409

N

Nalls, M. A., Blauwendraat, C., Vallerga, C. L., Heilbron, K., Bandres-Ciga, S., Chang, D., Tan, M., Kia, D. A., Noyce, A. J., Xue, A., Bras, J., Young, E., von Coelln, R., Simón-Sánchez, J., Schulte, C., Sharma, M., Krohn, L., Pihlstrøm, L., Siitonen, A., … International Parkinson's Disease Genomics Consortium. (2019). Identification of novel risk loci, causal insights, and heritable risk for Parkinson's disease: A meta-analysis of genome-wide association studies. *The Lancet. Neurology*, *18*(12), 1091-1102. https://doi.org/10.1016/S1474-4422(19)30320-5

Nambu, A., Tokuno, H., & Takada, M. (2002). Functional significance of the cortico-subthalamo-pallidal «hyperdirect» pathway. *Neuroscience Research*, *43*(2), 111-117. https://doi.org/10.1016/s0168-0102(02)00027-5

Navarrete, F., García-Gutiérrez, M. S., Aracil-Fernández, A., Lanciego, J. L., & Manzanares, J. (2018). Cannabinoid CB1 and CB2 Receptors, and Monoacylglycerol Lipase Gene Expression Alterations in the Basal Ganglia of Patients with Parkinson's Disease. *Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics*, *15*(2), 459-469. https://doi.org/10.1007/s13311-018-0603-x

Navarrete, M., & Araque, A. (2008). Endocannabinoids mediate neuron-astrocyte communication. *Neuron*, *57*(6), 883-893. https://doi.org/10.1016/j.neuron.2008.01.029

Navarro, E., Udine, E., de Paiva Lopes, K., Parks, M., Riboldi, G., Schilder, B. M., Humphrey, J., Snijders, G. J. L., Vialle, R. A., Zhuang, M., Sikder, T., Argyrou, C., Allan, A., Chao, M. J., Farrell, K., Henderson, B., Simon, S., Raymond, D., Elango, S., … Raj, T. (2021). Dysregulation of mitochondrial and proteolysosomal genes in Parkinson's disease myeloid cells. *Nature aging*, *1*(9), 850-863. https://doi.org/10.1038/s43587-021-00110-x

Navarro, E., Udine, E., Lopes, K. de P., Parks, M., Riboldi, G., Schilder, B. M., Humphrey, J., Snijders, G. J. L., Vialle, R. A., Zhuang, M., Sikder, T., Argyrou, C., Allan, A., Chao, M. J., Farrell, K., Henderson, B., Simon, S., Raymond, D., Elango, S., … Raj, T. (2021). Dysregulation of mitochondrial and proteolysosomal genes in Parkinson's disease myeloid cells. *Nature Aging*, *1*(9), 850-863. https://doi.org/10.1038/s43587-021-00110-x

Navia-Paldanius, D., Savinainen, J. R., & Laitinen, J. T. (2012). Biochemical and pharmacological characterization of human α/β-hydrolase domain containing 6 (ABHD6) and 12 (ABHD12). *Journal of Lipid Research*, *53*(11), 2413-2424. https://doi.org/10.1194/jlr.M030411

Nicoletti, A., Baschi, R., Cicero, C. E., Iacono, S., Re, V. L., Luca, A., Schirò, G., Monastero, R., & Gender Neurology Study Group of the Italian Society of Neurology. (2023). Sex and gender differences in Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis: A narrative review. *Mechanisms of Ageing and Development*, *212*, 111821. https://doi.org/10.1016/j.mad.2023.111821

Nilsson, J., Constantinescu, J., Nellgård, B., Jakobsson, P., Brum, W. S., Gobom, J., Forsgren, L., Dalla, K., Constantinescu, R., Zetterberg, H., Hansson, O., Blennow, K., Bäckström, D., & Brinkmalm, A. (2023). Cerebrospinal Fluid Biomarkers of Synaptic Dysfunction are Altered in Parkinson's Disease and Related Disorders. *Movement Disorders: Official Journal of the Movement Disorder Society*, *38*(2), 267-277. https://doi.org/10.1002/mds.29287

Niso-Santano, M., González-Polo, R. A., Bravo-San Pedro, J. M., Gómez-Sánchez, R., Lastres-Becker, I., Ortiz-Ortiz, M. A., Soler, G., Morán, J. M., Cuadrado, A., Fuentes, J. M., & Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED). (2010). Activation of apoptosis signal-regulating kinase 1 is a key factor in paraquat-induced cell death: Modulation by the Nrf2/Trx axis. *Free Radical Biology & Medicine*, *48*(10), 1370-1381. https://doi.org/10.1016/j.freeradbiomed.2010.02.024

Nixon, A. M., Meadowcroft, M. D., Neely, E. B., Snyder, A. M., Purnell, C. J., Wright, J., Lamendella, R., Nandar, W., Huang, X., & Connor, J. R. (2018). HFE Genotype Restricts the Response to Paraquat in a Mouse Model of Neurotoxicity. *Journal of Neurochemistry*, *145*(4), 299-311. https://doi.org/10.1111/jnc.14299

Noyce, A. J., Bestwick, J. P., Silveira-Moriyama, L., Hawkes, C. H., Giovannoni, G., Lees, A. J., & Schrag, A. (2012). Meta-analysis of early nonmotor features and risk factors for Parkinson disease. *Annals of Neurology*, *72*(6), 893-901. https://doi.org/10.1002/ana.23687

Nuber, S., Rajsombath, M., Minakaki, G., Winkler, J., Müller, C. P., Ericsson, M., Caldarone, B., Dettmer, U., & Selkoe, D. J. (2018). Abrogating Native α-Synuclein Tetramers in Mice Causes a L-DOPA-Responsive Motor Syndrome Closely Resembling Parkinson's Disease. *Neuron*, *100*(1), 75-90.e5. https://doi.org/10.1016/j.neuron.2018.09.014

O

Obeso, J. A., Stamelou, M., Goetz, C. G., Poewe, W., Lang, A. E., Weintraub, D., Burn, D., Halliday, G. M., Bezard, E., Przedborski, S., Lehericy, S., Brooks, D. J., Rothwell, J. C., Hallett, M., DeLong, M. R., Marras, C., Tanner, C. M., Ross, G. W., Langston, J. W., … Stoessl, A. J. (2017). Past, present, and future of Parkinson's disease: A special essay on the 200th Anniversary of the Shaking Palsy. *Movement Disorders: Official Journal of the Movement Disorder Society*, *32*(9), 1264-1310. https://doi.org/10.1002/mds.27115

Oh, T., Daadi, E. S., Kim, J., Daadi, E. W., Chen, P.-J., Roy-Choudhury, G., Bohmann, J., Blass, B. E., & Daadi, M. M. (2022). Dopamine D3 receptor ligand suppresses the expression of levodopa-induced dyskinesia in nonhuman primate model of parkinson's disease. *Experimental Neurology*, *347*, 113920. https://doi.org/10.1016/j.expneurol.2021.113920

Oka, S., Nakajima, K., Yamashita, A., Kishimoto, S., & Sugiura, T. (2007). Identification of GPR55 as a lysophosphatidylinositol receptor. *Biochemical and Biophysical Research Communications*, *362*(4), 928-934. https://doi.org/10.1016/j.bbrc.2007.08.078

Orso, B., Brosse, S., Frasnelli, J., & Arnaldi, D. (2024). Opportunities and Pitfalls of REM Sleep Behavior Disorder and Olfactory Dysfunction as Early Markers in Parkinson's Disease. *Journal of Parkinson's Disease*, *14*(s2), S275-S285. https://doi.org/10.3233/JPD-230348

Ossowska, K., Wardas, J., Smiałowska, M., Kuter, K., Lenda, T., Wierońska, J. M., Zieba, B., Nowak, P., Dabrowska, J., Bortel, A., Kwieciński, A., & Wolfarth, S. (2005). A slowly developing dysfunction of dopaminergic nigrostriatal neurons induced by long-term paraquat administration in rats: An animal model of preclinical stages of Parkinson's disease? *The European Journal of Neuroscience*, *22*(6), 1294-1304. https://doi.org/10.1111/j.1460-9568.2005.04301.x

O'Sullivan, S. E. (2016). An update on PPAR activation by cannabinoids. *British Journal of Pharmacology*, *173*(12), 1899-1910. https://doi.org/10.1111/bph.13497

Oubraim, S., Fauzan, M., Studholme, K., Gordon, C., Glaser, S. T., Shen, R.-Y., Ojima, I., Kaczocha, M., & Haj-Dahmane, S. (2025). Astrocytic FABP5 mediates retrograde endocannabinoid transport at central synapses. *iScience*, *28*(5), 112342. https://doi.org/10.1016/j.isci.2025.112342

Overton, H. A., Babbs, A. J., Doel, S. M., Fyfe, M. C. T., Gardner, L. S., Griffin, G., Jackson, H. C., Procter, M. J., Rasamison, C. M., Tang-Christensen, M., Widdowson, P. S., Williams, G. M., & Reynet, C. (2006). Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. *Cell Metabolism*, *3*(3), 167-175. https://doi.org/10.1016/j.cmet.2006.02.004

P

Pacher, P., & Mechoulam, R. (2011). Is lipid signaling through cannabinoid 2 receptors part of a protective system? *Progress in Lipid Research*, *50*(2), 193-211. https://doi.org/10.1016/j.plipres.2011.01.001

Palazuelos, J., Aguado, T., Pazos, M. R., Julien, B., Carrasco, C., Resel, E., Sagredo, O., Benito, C., Romero, J., Azcoitia, I., Fernández-Ruiz, J., Guzmán, M., & Galve-Roperh, I. (2009a). Microglial CB2 cannabinoid receptors are neuroprotective in Huntington's disease excitotoxicity. *Brain: A Journal of Neurology*, *132*(Pt 11), 3152-3164. https://doi.org/10.1093/brain/awp239

Palazuelos, J., Aguado, T., Pazos, M. R., Julien, B., Carrasco, C., Resel, E., Sagredo, O., Benito, C., Romero, J., Azcoitia, I., Fernández-Ruiz, J., Guzmán, M., & Galve-Roperh, I. (2009b). Microglial CB2 cannabinoid receptors are neuroprotective in Huntington's disease excitotoxicity. *Brain: A Journal of Neurology*, *132*(Pt 11), 3152-3164. https://doi.org/10.1093/brain/awp239

Palomo-Garo, C., Gómez-Gálvez, Y., García, C., & Fernández-Ruiz, J. (2016). Targeting the cannabinoid CB2 receptor to attenuate the progression of motor deficits in LRRK2-transgenic mice. *Pharmacological Research*, *110*, 181-192. https://doi.org/10.1016/j.phrs.2016.04.004

Panicker, N., Sarkar, S., Harischandra, D. S., Neal, M., Kam, T.-I., Jin, H., Saminathan, H., Langley, M., Charli, A., Samidurai, M., Rokad, D., Ghaisas, S., Pletnikova, O., Dawson, V. L., Dawson, T. M., Anantharam, V., Kanthasamy, A. G., & Kanthasamy, A. (2019). Fyn kinase regulates misfolded α-synuclein uptake and NLRP3 inflammasome activation in microglia. *The Journal of Experimental Medicine*, *216*(6), 1411-1430. https://doi.org/10.1084/jem.20182191

Paolicelli, R. C., Sierra, A., Stevens, B., Tremblay, M.-E., Aguzzi, A., Ajami, B., Amit, I., Audinat, E., Bechmann, I., Bennett, M., Bennett, F., Bessis, A., Biber, K., Bilbo, S., Blurton-Jones, M., Boddeke, E., Brites, D., Brône, B., Brown, G. C., … Wyss-Coray, T. (2022). Microglia states and nomenclature: A field at its crossroads. *Neuron*, *110*(21), 3458-3483. https://doi.org/10.1016/j.neuron.2022.10.020

Parkinson, J. (2002). An essay on the shaking palsy. 1817. *The Journal of Neuropsychiatry and Clinical Neurosciences*, *14*(2). https://doi.org/10.1176/jnp.14.2.223

Parkinson Progression Marker Initiative. (2011). The Parkinson Progression Marker Initiative (PPMI). *Progress in Neurobiology*, *95*(4), 629-635. https://doi.org/10.1016/j.pneurobio.2011.09.005

Parlar, A., Arslan, S. O., Doğan, M. F., Çam, S. A., Yalçin, A., Elibol, E., Özer, M. K., Üçkardeş, F., & Kara, H. (2018). The exogenous administration of CB2 specific agonist, GW405833, inhibits inflammation by reducing cytokine production and oxidative stress. *Experimental and Therapeutic Medicine*, *16*(6), 4900-4908. https://doi.org/10.3892/etm.2018.6753

Patricio, F., Morales Dávila, E., Patricio-Martínez, A., Arana Del Carmen, N., Martínez, I., Aguilera, J., Perez-Aguilar, J. M., & Limón, I. D. (2022). Intrapallidal injection of cannabidiol or a selective GPR55 antagonist decreases motor asymmetry and improves fine motor skills in hemiparkinsonian rats. *Frontiers in Pharmacology*, *13*, 945836. https://doi.org/10.3389/fphar.2022.945836

Patti, F., Messina, S., Solaro, C., Amato, M. P., Bergamaschi, R., Bonavita, S., Bruno Bossio, R., Brescia Morra, V., Costantino, G. F., Cavalla, P., Centonze, D., Comi, G., Cottone, S., Danni, M., Francia, A., Gajofatto, A., Gasperini, C., Ghezzi, A., Iudice, A., … SA.FE. study group. (2016). Efficacy and safety of cannabinoid oromucosal spray for multiple sclerosis spasticity. *Journal of Neurology, Neurosurgery, and Psychiatry*, *87*(9), 944-951. https://doi.org/10.1136/jnnp-2015-312591

Pazi, M. B., Belan, D. V., Komarova, E. Y., & Ekimova, I. V. (2024). Intranasal Administration of GRP78 Protein (HSPA5) Confers Neuroprotection in a Lactacystin-Induced Rat Model of Parkinson's Disease. *International Journal of Molecular Sciences*, *25*(7), 3951. https://doi.org/10.3390/ijms25073951

Peball, M., Krismer, F., Knaus, H.-G., Djamshidian, A., Werkmann, M., Carbone, F., Ellmerer, P., Heim, B., Marini, K., Valent, D., Goebel, G., Ulmer, H., Stockner, H., Wenning, G. K., Stolz, R., Krejcy, K., Poewe, W., Seppi, K., & Collaborators of the Parkinson's Disease Working Group Innsbruck. (2020). Non-Motor Symptoms in Parkinson's Disease are Reduced by Nabilone. *Annals of Neurology*, *88*(4), 712-722. https://doi.org/10.1002/ana.25864

Peball, M., Seppi, K., Krismer, F., Knaus, H.-G., Spielberger, S., Heim, B., Ellmerer, P., Werkmann, M., Poewe, W., & Djamshidian, A. (2022). Effects of Nabilone on Sleep Outcomes in Patients with Parkinson's Disease: A Post-hoc Analysis of NMS-Nab Study. *Movement Disorders Clinical Practice*, *9*(6), 751-758. https://doi.org/10.1002/mdc3.13471

Pérez-Rial, S., García-Gutiérrez, M. S., Molina, J. A., Pérez-Nievas, B. G., Ledent, C., Leiva, C., Leza, J. C., & Manzanares, J. (2011). Increased vulnerability to 6-hydroxydopamine lesion and reduced development of dyskinesias in mice lacking CB1 cannabinoid receptors. *Neurobiology of Aging*, *32*(4), 631-645. https://doi.org/10.1016/j.neurobiolaging.2009.03.017

Pertwee, R. G. (2007). GPR55: A new member of the cannabinoid receptor clan? *British Journal of Pharmacology*, *152*(7), 984-986. https://doi.org/10.1038/sj.bjp.0707464

Pertwee, R. G. (2008a). The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. *British Journal of Pharmacology*, *153*(2), 199-215. https://doi.org/10.1038/sj.bjp.0707442

Pertwee, R. G. (2008b). The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. *British Journal of Pharmacology*, *153*(2), 199-215. https://doi.org/10.1038/sj.bjp.0707442

Pertwee, R. G. (2015). Endocannabinoids and Their Pharmacological Actions. *Handbook of Experimental Pharmacology*, *231*, 1-37. https://doi.org/10.1007/978-3-319-20825-1_1

Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P. H., Di Marzo, V., Elphick, M. R., Greasley, P. J., Hansen, H. S., Kunos, G., Mackie, K., Mechoulam, R., & Ross, R. A. (2010). International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: Beyond CB_1 and CB_2. *Pharmacological Reviews*, *62*(4), 588-631. https://doi.org/10.1124/pr.110.003004

Pettorruso, M., Fasano, A., De Risio, L., Ricciardi, L., Di Nicola, M., Martinotti, G., Janiri, L., & Bentivoglio, A. R. (2016). Punding in non-demented Parkinson's disease patients: Relationship with psychiatric and addiction spectrum comorbidity. *Journal of the Neurological Sciences*, *362*, 344-347. https://doi.org/10.1016/j.jns.2016.02.016

Pietr, M., Kozela, E., Levy, R., Rimmerman, N., Lin, Y. H., Stella, N., Vogel, Z., & Juknat, A. (2009). Differential changes in GPR55 during microglial cell activation. *FEBS Letters*, *583*(12), 2071-2076. https://doi.org/10.1016/j.febslet.2009.05.028

Pintor, A., Tebano, M. T., Martire, A., Grieco, R., Galluzzo, M., Scattoni, M. L., Pèzzola, A., Coccurello, R., Felici, F., Cuomo, V., Piomelli, D., Calamandrei, G., & Popoli, P. (2006). The cannabinoid receptor agonist WIN 55,212-2 attenuates the effects induced by quinolinic acid in the rat striatum. *Neuropharmacology*, *51*(5), 1004-1012. https://doi.org/10.1016/j.neuropharm.2006.06.013

Piomelli, D. (2003). The molecular logic of endocannabinoid signalling. *Nature Reviews. Neuroscience*, *4*(11), 873-884. https://doi.org/10.1038/nrn1247

Piomelli, D., Tarzia, G., Duranti, A., Tontini, A., Mor, M., Compton, T. R., Dasse, O., Monaghan, E. P., Parrott, J. A., & Putman, D. (2006). Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597). *CNS Drug Reviews*, *12*(1), 21-38. https://doi.org/10.1111/j.1527-3458.2006.00021.x

Pisani, V., Moschella, V., Bari, M., Fezza, F., Galati, S., Bernardi, G., Stanzione, P., Pisani, A., & Maccarrone, M. (2010). Dynamic changes of anandamide in the cerebrospinal fluid of Parkinson's disease patients. *Movement Disorders: Official Journal of the Movement Disorder Society*, *25*(7), 920-924. https://doi.org/10.1002/mds.23014

Pissadaki, E. K., & Bolam, J. P. (2013). The energy cost of action potential propagation in dopamine neurons: Clues to susceptibility in Parkinson's disease. *Frontiers in Computational Neuroscience*, *7*, 13. https://doi.org/10.3389/fncom.2013.00013

Pizcueta, P., Vergara, C., Emanuele, M., Vilalta, A., Rodríguez-Pascau, L., & Martinell, M. (2023). Development of PPARγ Agonists for the Treatment of Neuroinflammatory and Neurodegenerative Diseases: Leriglitazone as a Promising Candidate. *International Journal of Molecular Sciences*, *24*(4), 3201. https://doi.org/10.3390/ijms24043201

Poewe, W., Seppi, K., Tanner, C. M., Halliday, G. M., Brundin, P., Volkmann, J., Schrag, A.-E., & Lang, A. E. (2017). Parkinson disease. *Nature Reviews. Disease Primers*, *3*, 17013. https://doi.org/10.1038/nrdp.2017.13

Polissidis, A., Koronaiou, E., Nikolopoulou, G., Viel, C., Nikatou, M., Bogiongko, M., Sardi, S. P., Xilouri, M., Vekrellis, K., & Stefanis, L. (2022). A double-hit in vivo model of *GBA* viral microRNA-mediated downregulation and human alpha-synuclein overexpression demonstrates nigrostriatal degeneration. *Neurobiology of Disease*, *163*, 105612. https://doi.org/10.1016/j.nbd.2022.105612

Polito, L., Greco, A., & Seripa, D. (2016). Genetic Profile, Environmental Exposure, and Their Interaction in Parkinson's Disease. *Parkinson's Disease*, *2016*, 6465793. https://doi.org/10.1155/2016/6465793

Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., & Nussbaum, R. L. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science (New York, N.Y.)*, *276*(5321), 2045-2047. https://doi.org/10.1126/science.276.5321.2045

Price, D. A., Martinez, A. A., Seillier, A., Koek, W., Acosta, Y., Fernandez, E., Strong, R., Lutz, B., Marsicano, G., Roberts, J. L., & Giuffrida, A. (2009). WIN55,212-2, a cannabinoid receptor agonist, protects against nigrostriatal cell loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *The European Journal of Neuroscience*, *29*(11), 2177-2186. https://doi.org/10.1111/j.1460-9568.2009.06764.x

Price, M. R., Baillie, G. L., Thomas, A., Stevenson, L. A., Easson, M., Goodwin, R., McLean, A., McIntosh, L., Goodwin, G., Walker, G., Westwood, P., Marrs, J., Thomson, F., Cowley, P., Christopoulos, A., Pertwee, R. G., & Ross, R. A. (2005). Allosteric modulation of the cannabinoid CB1 receptor. *Molecular Pharmacology*, *68*(5), 1484-1495. https://doi.org/10.1124/mol.105.016162

Priyadarshi, A., Khuder, S. A., Schaub, E. A., & Priyadarshi, S. S. (2001). Environmental risk factors and Parkinson's disease: A metaanalysis. *Environmental Research*, *86*(2), 122-127. https://doi.org/10.1006/enrs.2001.4264

Przedborski, S., Levivier, M., Jiang, H., Ferreira, M., Jackson-Lewis, V., Donaldson, D., & Togasaki, D. M. (1995). Dose-dependent lesions of the dopaminergic nigrostriatal pathway induced by intrastriatal injection of 6-hydroxydopamine. *Neuroscience*, *67*(3), 631-647. https://doi.org/10.1016/0306-4522(95)00066-r

Q

Qiao, Z., Kumar, A., Kumar, P., & Song, Z.-H. (2012). Involvement of a non-CB1/CB2 cannabinoid receptor in the aqueous humor outflow-enhancing effects of abnormal-cannabidiol. *Experimental Eye Research*, *100*, 59-64. https://doi.org/10.1016/j.exer.2012.05.001

Quintanilla, R. A., Utreras, E., & Cabezas-Opazo, F. A. (2014). Role of PPAR γ in the Differentiation and Function of Neurons. *PPAR Research*, *2014*, 768594. https://doi.org/10.1155/2014/768594

R

Raffa, R. B., & Ward, S. J. (2012). CB_1-independent mechanisms of Δ^9-THCV, AM251 and SR141716 (rimonabant). *Journal of Clinical Pharmacy and Therapeutics*, *37*(3), 260-265. https://doi.org/10.1111/j.1365-2710.2011.01284.x

Ramos Atance, J. A., & Fernández Ruiz, J. (2000). Cannabinoides: Propiedades químicas y aspectos metabólicos. *Adicciones: Revista de socidrogalcohol*, *12*(2), 41-58.

Rao, V. K., Lewis-Bakker, M. M., Wasilewski, E., Clarke, H. A., & Kotra, L. P. (2025). Stereoisomers of cannabidiols and their pharmacological activities—A potentially novel direction for cannabinoids. *Bioorganic & Medicinal Chemistry*, *117*, 118019. https://doi.org/10.1016/j.bmc.2024.118019

Raphael, B., & Gabet, Y. (2016). The skeletal endocannabinoid system: Clinical and experimental insights. *Journal of Basic and Clinical Physiology and Pharmacology*, *27*(3), 237-245. https://doi.org/10.1515/jbcpp-2015-0073

Recasens, A., Dehay, B., Bové, J., Carballo-Carbajal, I., Dovero, S., Pérez-Villalba, A., Fernagut, P.-O., Blesa, J., Parent, A., Perier, C., Fariñas, I., Obeso, J. A., Bezard, E., & Vila, M. (2014). Lewy body extracts from Parkinson disease brains trigger α-synuclein pathology and neurodegeneration in mice and monkeys. *Annals of Neurology*, *75*(3), 351-362. https://doi.org/10.1002/ana.24066

Reich, C. G., Taylor, M. E., & McCarthy, M. M. (2009). Differential effects of chronic unpredictable stress on hippocampal CB1 receptors in male and female rats. *Behavioural Brain Research*, *203*(2), 264-269. https://doi.org/10.1016/j.bbr.2009.05.013

Riboldi, G. M., Vialle, R. A., Navarro, E., Udine, E., de Paiva Lopes, K., Humphrey, J., Allan, A., Parks, M., Henderson, B., Astudillo, K., Argyrou, C., Zhuang, M., Sikder, T., Oriol Narcis, J., Kumar, S. D., Janssen, W., Sowa, A., Comi, G. P., Di Fonzo, A., … Raj, T. (2022). Transcriptome deregulation of peripheral monocytes and whole blood in GBA-related Parkinson's disease. *Molecular Neurodegeneration*, *17*(1), 52. https://doi.org/10.1186/s13024-022-00554-8

Ritzel, R. M., Doran, S. J., Glaser, E. P., Meadows, V. E., Faden, A. I., Stoica, B. A., & Loane, D. J. (2019). Old age increases microglial senescence, exacerbates secondary neuroinflammation, and worsens neurological outcomes after acute traumatic brain injury in mice. *Neurobiology of Aging*, *77*, 194-206. https://doi.org/10.1016/j.neurobiolaging.2019.02.010

Rivas-Santisteban, R., Lillo, A., Lillo, J., Rebassa, J.-B., Contestí, J. S., Saura, C. A., Franco, R., & Navarro, G. (2021). N-Methyl-D-aspartate (NMDA) and cannabinoid CB2 receptors form functional complexes in cells of the central nervous system: Insights into the therapeutic potential of neuronal and microglial NMDA receptors. *Alzheimer's Research & Therapy*, *13*(1), 184. https://doi.org/10.1186/s13195-021-00920-6

Rocha, E. M., De Miranda, B., & Sanders, L. H. (2018). Alpha-synuclein: Pathology, mitochondrial dysfunction and neuroinflammation in Parkinson's disease. *Neurobiology of Disease*, *109*(Pt B), 249-257. https://doi.org/10.1016/j.nbd.2017.04.004

Rodríguez-Carreiro, S., Gómez-Cañas, M., Lubrini, F., Gonzalo-Consuegra, C., Winkler, M., Caprioglio, D., Appendino, G., García, C., Morales, P., Jagerovic, N., Fischer, J. T., Fiebich, B. L., Goetz, M. R., Muñoz, E., & Fernández-Ruiz, J. (2025). Investigation in the CB1 and CB2 receptor binding profile and intrinsic activity of (−) and (+)-enantiomers of some naturally occurring phytocannabinoids or synthetic derivatives. *European Journal of Medicinal Chemistry Reports*, *14*, 100262. https://doi.org/10.1016/j.ejmcr.2025.100262

Rodríguez-Carreiro, S., Navarro, E., Muñoz, E., & Fernández-Ruiz, J. (2023). The Cannabigerol Derivative VCE-003.2 Exerts Therapeutic Effects in 6-Hydroxydopamine-Lesioned Mice: Comparison with The Classic Dopaminergic Replacement Therapy. *Brain Sciences*, *13*(9), 1272. https://doi.org/10.3390/brainsci13091272

Rodríguez-Cueto, C., Benito, C., Fernández-Ruiz, J., Romero, J., Hernández-Gálvez, M., & Gómez-Ruiz, M. (2014). Changes in CB(1) and CB(2) receptors in the post-mortem cerebellum of humans affected by spinocerebellar ataxias. *British Journal of Pharmacology*, *171*(6), 1472-1489. https://doi.org/10.1111/bph.12283

Rodríguez-Cueto, C., García-Toscano, L., Santos-García, I., Gómez-Almería, M., Gonzalo-Consuegra, C., Espejo-Porras, F., Fernández-Ruiz, J., & de Lago, E. (2021). Targeting the CB2 receptor and other endocannabinoid elements to delay disease progression in amyotrophic lateral sclerosis. *British Journal of Pharmacology*, *178*(6), 1373-1387. https://doi.org/10.1111/bph.15386

Rodríguez-Cueto, C., Gómez-Almería, M., García Toscano, L., Romero, J., Hillard, C. J., de Lago, E., & Fernández-Ruiz, J. (2021). Inactivation of the CB2 receptor accelerated the neuropathological deterioration in TDP-43 transgenic mice, a model of amyotrophic lateral sclerosis. *Brain Pathology (Zurich, Switzerland)*, *31*(6), e12972. https://doi.org/10.1111/bpa.12972

Rodríguez-Cueto, C., Santos-García, I., García-Toscano, L., Espejo-Porras, F., Bellido, Ml., Fernández-Ruiz, J., Muñoz, E., & de Lago, E. (2018). Neuroprotective effects of the cannabigerol quinone derivative VCE-003.2 in SOD1G93A transgenic mice, an experimental model of amyotrophic lateral sclerosis. *Biochemical Pharmacology*, *157*, 217-226. https://doi.org/10.1016/j.bcp.2018.07.049

Ross, R. A. (2003). Anandamide and vanilloid TRPV1 receptors. *British Journal of Pharmacology*, *140*(5), 790-801. https://doi.org/10.1038/sj.bjp.0705467

Ross, R. A. (2009). The enigmatic pharmacology of GPR55. *Trends in Pharmacological Sciences*, *30*(3), 156-163. https://doi.org/10.1016/j.tips.2008.12.004

Rubino, T., & Parolaro, D. (2011). Sexually dimorphic effects of cannabinoid compounds on emotion and cognition. *Frontiers in Behavioral Neuroscience*, *5*, 64. https://doi.org/10.3389/fnbeh.2011.00064

Ryberg, E., Larsson, N., Sjögren, S., Hjorth, S., Hermansson, N.-O., Leonova, J., Elebring, T., Nilsson, K., Drmota, T., & Greasley, P. J. (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. *British Journal of Pharmacology*, *152*(7), 1092-1101. https://doi.org/10.1038/sj.bjp.0707460

S

Sacino, A. N., Brooks, M., Thomas, M. A., McKinney, A. B., Lee, S., Regenhardt, R. W., McGarvey, N. H., Ayers, J. I., Notterpek, L., Borchelt, D. R., Golde, T. E., & Giasson, B. I. (2014). Intramuscular injection of α-synuclein induces CNS α-synuclein pathology and a rapid-onset motor phenotype in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(29), 10732-10737. https://doi.org/10.1073/pnas.1321785111

Sagredo, O., González, S., Aroyo, I., Pazos, M. R., Benito, C., Lastres-Becker, I., Romero, J. P., Tolón, R. M., Mechoulam, R., Brouillet, E., Romero, J., & Fernández-Ruiz, J. (2009). Cannabinoid CB2 receptor agonists protect the striatum against malonate toxicity: Relevance for Huntington's disease. *Glia*, *57*(11), 1154-1167. https://doi.org/10.1002/glia.20838

Salazar, M., Carracedo, A., Salanueva, I. J., Hernández-Tiedra, S., Lorente, M., Egia, A., Vázquez, P., Blázquez, C., Torres, S., García, S., Nowak, J., Fimia, G. M., Piacentini, M., Cecconi, F., Pandolfi, P. P., González-Feria, L., Iovanna, J. L., Guzmán, M., Boya, P., & Velasco, G. (2009). Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. *The Journal of Clinical Investigation*, *119*(5), 1359-1372. https://doi.org/10.1172/jci37948

Salganik, M., Sergeyev, V. G., Shinde, V., Meyers, C. A., Gorbatyuk, M. S., Lin, J. H., Zolotukhin, S., & Gorbatyuk, O. S. (2015). The loss of glucose-regulated protein 78 (GRP78) during normal aging or from siRNA knockdown augments human alpha-synuclein (α-syn) toxicity to rat nigral neurons. *Neurobiology of Aging*, *36*(6), 2213-2223. https://doi.org/10.1016/j.neurobiolaging.2015.02.018

Saliba, S. W., Gläser, F., Deckers, A., Keil, A., Hurrle, T., Apweiler, M., Ferver, F., Volz, N., Endres, D., Bräse, S., & Fiebich, B. L. (2021). Effects of a Novel GPR55 Antagonist on the Arachidonic Acid Cascade in LPS-Activated Primary Microglial Cells. *International Journal of Molecular Sciences*, *22*(5), 2503. https://doi.org/10.3390/ijms22052503

Saliba, S. W., Jauch, H., Gargouri, B., Keil, A., Hurrle, T., Volz, N., Mohr, F., van der Stelt, M., Bräse, S., & Fiebich, B. L. (2018). Anti-neuroinflammatory effects of GPR55 antagonists in LPS-activated primary microglial cells. *Journal of Neuroinflammation*, *15*(1), 322. https://doi.org/10.1186/s12974-018-1362-7

Sánchez-Fernández, N., Gómez-Acero, L., Sarasola, L. I., Argerich, J., Chevigné, A., Jacobson, K. A., Ciruela, F., Fernández-Dueñas, V., & Aso, E. (2024). Cannabidiol negatively modulates adenosine A2A receptor functioning in living cells. *Acta Neuropsychiatrica*, *36*(5), 320-324. https://doi.org/10.1017/neu.2023.30

Sánchez-Zavaleta, R., Ávalos-Fuentes, J. A., González-Hernández, A. V., Recillas-Morales, S., Paz-Bermúdez, F. J., Leyva-Gómez, G., Cortés, H., & Florán, B. (2022). Presynaptic nigral GPR55 receptors stimulate [3 H]-GABA release through [3

H]-cAMP production and PKA activation and promote motor behavior. *Synapse (New York, N.Y.)*, *76*(11-12), e22246. https://doi.org/10.1002/syn.22246

Sanmarco, L. M., Wheeler, M. A., Gutiérrez-Vázquez, C., Polonio, C. M., Linnerbauer, M., Pinho-Ribeiro, F. A., Li, Z., Giovannoni, F., Batterman, K. V., Scalisi, G., Zandee, S. E. J., Heck, E. S., Alsuwailm, M., Rosene, D. L., Becher, B., Chiu, I. M., Prat, A., & Quintana, F. J. (2021). Gut-licensed IFNγ+ NK cells drive LAMP1+TRAIL+ anti-inflammatory astrocytes. *Nature*, *590*(7846), 473-479. https://doi.org/10.1038/s41586-020-03116-4

Santos, N. A. G., Martins, N. M., Sisti, F. M., Fernandes, L. S., Ferreira, R. S., Queiroz, R. H. C., & Santos, A. C. (2015). The neuroprotection of cannabidiol against MPP^+-induced toxicity in PC12 cells involves trkA receptors, upregulation of axonal and synaptic proteins, neuritogenesis, and might be relevant to Parkinson's disease. *Toxicology in Vitro: An International Journal Published in Association with BIBRA*, *30*(1 Pt B), 231-240. https://doi.org/10.1016/j.tiv.2015.11.004

Sañudo-Peña, M. C., Patrick, S. L., Khen, S., Patrick, R. L., Tsou, K., & Walker, J. M. (1998). Cannabinoid effects in basal ganglia in a rat model of Parkinson's disease. *Neuroscience Letters*, *248*(3), 171-174. https://doi.org/10.1016/s0304-3940(98)00368-1

Sañudo-Peña, M. C., Romero, J., Seale, G. E., Fernandez-Ruiz, J. J., & Walker, J. M. (2000). Activational role of cannabinoids on movement. *European Journal of Pharmacology*, *391*(3), 269-274. https://doi.org/10.1016/s0014-2999(00)00044-3

Sawzdargo, M., Nguyen, T., Lee, D. K., Lynch, K. R., Cheng, R., Heng, H. H., George, S. R., & O'Dowd, B. F. (1999). Identification and cloning of three novel human G protein-coupled receptor genes GPR52, PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain. *Brain Research. Molecular Brain Research*, *64*(2), 193-198. https://doi.org/10.1016/s0169-328x(98)00277-0

Schenkman, M., Moore, C. G., Kohrt, W. M., Hall, D. A., Delitto, A., Comella, C. L., Josbeno, D. A., Christiansen, C. L., Berman, B. D., Kluger, B. M., Melanson, E. L., Jain, S., Robichaud, J. A., Poon, C., & Corcos, D. M. (2018). Effect of High-Intensity Treadmill Exercise on Motor Symptoms in Patients With De Novo Parkinson Disease: A Phase 2 Randomized Clinical Trial. *JAMA Neurology*, *75*(2), 219-226. https://doi.org/10.1001/jamaneurol.2017.3517

Schildknecht, S., Di Monte, D. A., Pape, R., Tieu, K., & Leist, M. (2017). Tipping Points and Endogenous Determinants of Nigrostriatal Degeneration by MPTP. *Trends in Pharmacological Sciences*, *38*(6), 541-555. https://doi.org/10.1016/j.tips.2017.03.010

Schopohl, B., Kohlhaas, M., Nickel, A. G., Schiuma, A.-F., Maas, S. L., van der Vorst, E. P. C., Shia, Y. X., Maack, C., Steffens, S., & Puhl, S.-L. (2025). Gpr55 deficiency crucially alters cardiomyocyte homeostasis and counteracts angiotensin II induced maladaption in female mice. *British Journal of Pharmacology*, *182*(3), 670-691. https://doi.org/10.1111/bph.17350

Schüle, L.-L., Glasmacher, S., Gertsch, J., Roggan, M. D., Transfeld, J.-L., Bindila, L., Lutz, B., Kolbe, C.-C., Bilkei-Gorzo, A., Zimmer, A., & Leidmaa, E. (2021). Diacylglycerol lipase alpha in astrocytes is involved in maternal care and affective behaviors. *Glia*, *69*(2), 377-391. https://doi.org/10.1002/glia.23903

Seppi, K., Weintraub, D., Coelho, M., Perez-Lloret, S., Fox, S. H., Katzenschlager, R., Hametner, E.-M., Poewe, W., Rascol, O., Goetz, C. G., & Sampaio, C. (2011). The Movement Disorder Society Evidence-Based Medicine Review Update: Treatments for the non-motor symptoms of Parkinson's disease. *Movement Disorders: Official Journal of the Movement Disorder Society*, *26 Suppl 3*(0 3), S42-80. https://doi.org/10.1002/mds.23884

Shao, B.-Z., Wei, W., Ke, P., Xu, Z.-Q., Zhou, J.-X., & Liu, C. (2014). Activating cannabinoid receptor 2 alleviates pathogenesis of experimental autoimmune encephalomyelitis via activation of autophagy and inhibiting NLRP3 inflammasome. *CNS Neuroscience & Therapeutics*, *20*(12), 1021-1028. https://doi.org/10.1111/cns.12349

Sharir, H., Console-Bram, L., Mundy, C., Popoff, S. N., Kapur, A., & Abood, M. E. (2012). The endocannabinoids anandamide and virodhamine modulate the activity of the candidate cannabinoid receptor GPR55. *Journal of Neuroimmune Pharmacology: The Official Journal of the Society on NeuroImmune Pharmacology*, *7*(4), 856-865. https://doi.org/10.1007/s11481-012-9351-6

Shen, S.-Y., Yu, R., Li, W., Liang, L.-F., Han, Q.-Q., Huang, H.-J., Li, B., Xu, S.-F., Wu, G.-C., Zhang, Y.-Q., & Yu, J. (2022). The neuroprotective effects of GPR55 against hippocampal neuroinflammation and impaired adult neurogenesis in CSDS mice. *Neurobiology of Disease*, *169*, 105743. https://doi.org/10.1016/j.nbd.2022.105743

Shi, J., Cai, Q., Zhang, J., He, X., Liu, Y., Zhu, R., & Jin, L. (2017). AM1241 alleviates MPTP-induced Parkinson's disease and promotes the regeneration of DA neurons in PD mice. *Oncotarget*, *8*(40), 67837-67850. https://doi.org/10.18632/oncotarget.18871

Shohami, E., & Mechoulam, R. (2000). Dexanabinol (HU-211): A nonpsychotropic cannabinoid with neuroprotective properties. *Drug Development Research*, *50*(3-4), 211-215. https://doi.org/10.1002/1098-2299(200007/08)50:3/4<211::AID-DDR3>3.0.CO;2-G

Shohet, A., Khlebtovsky, A., Roizen, N., Roditi, Y., & Djaldetti, R. (2017). Effect of medical cannabis on thermal quantitative measurements of pain in patients with Parkinson's disease. *European Journal of Pain (London, England)*, *21*(3), 486-493. https://doi.org/10.1002/ejp.942

Shore, D. M., & Reggio, P. H. (2015). The therapeutic potential of orphan GPCRs, GPR35 and GPR55. *Frontiers in Pharmacology*, *6*, 69. https://doi.org/10.3389/fphar.2015.00069

Sieradzan, K. A., Fox, S. H., Hill, M., Dick, J. P., Crossman, A. R., & Brotchie, J. M. (2001). Cannabinoids reduce levodopa-induced dyskinesia in Parkinson's disease: A pilot study. *Neurology*, *57*(11), 2108-2111. https://doi.org/10.1212/wnl.57.11.2108

Sierra, S., Luquin, N., & Navarro-Otano, J. (2018). The endocannabinoid system in cardiovascular function: Novel insights and clinical implications. *Clinical Autonomic Research: Official Journal of the Clinical Autonomic Research Society*, *28*(1), 35-52. https://doi.org/10.1007/s10286-017-0488-5

Sierra, S., Luquin, N., Rico, A. J., Gómez-Bautista, V., Roda, E., Dopeso-Reyes, I. G., Vázquez, A., Martínez-Pinilla, E., Labandeira-García, J. L., Franco, R., & Lanciego, J. L. (2015). Detection of cannabinoid receptors CB1 and CB2 within basal ganglia output neurons in macaques: Changes following experimental parkinsonism. *Brain Structure & Function*, *220*(5), 2721-2738. https://doi.org/10.1007/s00429-014-0823-8

Simola, N., Morelli, M., & Carta, A. R. (2007). The 6-hydroxydopamine model of Parkinson's disease. *Neurotoxicity Research*, *11*(3-4), 151-167. https://doi.org/10.1007/BF03033565

Simon, D. K., Tanner, C. M., & Brundin, P. (2020). Parkinson Disease Epidemiology, Pathology, Genetics, and Pathophysiology. *Clinics in Geriatric Medicine*, *36*(1), 1-12. https://doi.org/10.1016/j.cger.2019.08.002

Sims, S. G., Cisney, R. N., Lipscomb, M. M., & Meares, G. P. (2022). The role of endoplasmic reticulum stress in astrocytes. *Glia*, *70*(1), 5-19. https://doi.org/10.1002/glia.24082

Singh, U. P., Singh, N. P., Singh, B., Price, R. L., Nagarkatti, M., & Nagarkatti, P. S. (2012). Cannabinoid receptor-2 (CB2) agonist ameliorates colitis in IL-10(-/-) mice by attenuating the activation of T cells and promoting their apoptosis. *Toxicology and Applied Pharmacology*, *258*(2), 256-267. https://doi.org/10.1016/j.taap.2011.11.005

Sisay, S., Pryce, G., Jackson, S. J., Tanner, C., Ross, R. A., Michael, G. J., Selwood, D. L., Giovannoni, G., & Baker, D. (2013). Genetic background can result in a marked or minimal effect of gene knockout (GPR55 and CB2 receptor) in experimental autoimmune encephalomyelitis models of multiple sclerosis. *PloS One*, *8*(10), e76907. https://doi.org/10.1371/journal.pone.0076907

Skou, L. D., Johansen, S. K., Okarmus, J., & Meyer, M. (2024). Pathogenesis of DJ-1/PARK7-Mediated Parkinson's Disease. *Cells*, *13*(4), 296. https://doi.org/10.3390/cells13040296

Smajić, S., Prada-Medina, C. A., Landoulsi, Z., Ghelfi, J., Delcambre, S., Dietrich, C., Jarazo, J., Henck, J., Balachandran, S., Pachchek, S., Morris, C. M., Antony, P., Timmermann, B., Sauer, S., Pereira, S. L., Schwamborn, J. C., May, P., Grünewald, A., & Spielmann, M. (2021). Single-cell sequencing of human midbrain reveals glial activation and a Parkinson-specific neuronal state. *Brain*, *145*(3), 964-978. https://doi.org/10.1093/brain/awab446

Sokołowska, P., Wiktorowska-Owczarek, A., Tambor, J., Gawlak-Socka, S., Kowalczyk, E., & Jóźwiak-Bębenista, M. (2024). Endoplasmic Reticulum Stress Differently Modulates the Release of IL-6 and IL-8 Cytokines in Human Glial Cells. *International Journal of Molecular Sciences*, *25*(16), 8687. https://doi.org/10.3390/ijms25168687

Solinas, M., Justinova, Z., Goldberg, S. R., & Tanda, G. (2006). Anandamide administration alone and after inhibition of fatty acid amide hydrolase (FAAH) increases dopamine levels in the nucleus accumbens shell in rats. *Journal of Neurochemistry*, *98*(2), 408-419. https://doi.org/10.1111/j.1471-4159.2006.03880.x

Song, H., Chen, J., Huang, J., Sun, P., Liu, Y., Xu, L., Wei, C., Mu, X., Lu, X., Wang, W., Zhang, N., Shang, M., Mo, M., Zhang, W., Zhao, H., & Han, F. (2023). Epigenetic modification in Parkinson's disease. *Frontiers in Cell and Developmental Biology*, *11*, 1123621. https://doi.org/10.3389/fcell.2023.1123621

Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., & Goedert, M. (1997). Alpha-synuclein in Lewy bodies. *Nature*, *388*(6645), 839-840. https://doi.org/10.1038/42166

Sprenkle, N. T., Sims, S. G., Sánchez, C. L., & Meares, G. P. (2017). Endoplasmic reticulum stress and inflammation in the central nervous system. *Molecular Neurodegeneration*, *12*(1), 42. https://doi.org/10.1186/s13024-017-0183-y

Staton, P. C., Hatcher, J. P., Walker, D. J., Morrison, A. D., Shapland, E. M., Hughes, J. P., Chong, E., Mander, P. K., Green, P. J., Billinton, A., Fulleylove, M., Lancaster, H. C., Smith, J. C., Bailey, L. T., Wise, A., Brown, A. J., Richardson, J. C., & Chessell, I. P. (2008). The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain. *PAIN*, *139*(1), 225-236. https://doi.org/10.1016/j.pain.2008.04.006

Staubo, S. C., Fuskevåg, O. M., Toft, M., Lie, I. H., Alvik, K. M. J., Jostad, P., Tingvoll, S. H., Lilleng, H., Rosqvist, K., Størset, E., Odin, P., Dietrichs, E., & Dietrichs, E. S. (2024). Dopamine agonist serum concentrations and impulse control disorders in Parkinson's disease. *European Journal of Neurology*, *31*(2), e16144. https://doi.org/10.1111/ene.16144

Stempel, A. V., Stumpf, A., Zhang, H.-Y., Özdoğan, T., Pannasch, U., Theis, A.-K., Otte, D.-M., Wojtalla, A., Rácz, I., Ponomarenko, A., Xi, Z.-X., Zimmer, A., & Schmitz, D. (2016). Cannabinoid Type 2 Receptors Mediate a Cell Type-Specific Plasticity in the Hippocampus. *Neuron*, *90*(4), 795-809. https://doi.org/10.1016/j.neuron.2016.03.034

Stone, S., & Lin, W. (2015). The unfolded protein response in multiple sclerosis. *Frontiers in Neuroscience*, *9*, 264. https://doi.org/10.3389/fnins.2015.00264

Strougo, A., Zuurman, L., Roy, C., Pinquier, J. L., van Gerven, J. M. A., Cohen, A. F., & Schoemaker, R. C. (2008). Modelling of the concentration—Effect relationship of THC on central nervous system parameters and heart rate—Insight into its mechanisms of action and a tool for clinical research and development of cannabinoids. *Journal of Psychopharmacology (Oxford, England)*, *22*(7), 717-726. https://doi.org/10.1177/0269881108089870

Sulzer, D., Alcalay, R. N., Garretti, F., Cote, L., Kanter, E., Agin-Liebes, J., Liong, C., McMurtrey, C., Hildebrand, W. H., Mao, X., Dawson, V. L., Dawson, T. M., Oseroff, C., Pham, J., Sidney, J., Dillon, M. B., Carpenter, C., Weiskopf, D., Phillips, E., … Sette, A. (2017). T cells from patients with Parkinson's disease recognize α-synuclein peptides. *Nature*, *546*(7660), 656-661. https://doi.org/10.1038/nature22815

Sun, H., Wang, Z., Zhang, Q., Liu, Y., Yang, J., Liu, H., Shi, C., Wang, Y., & Xu, Y. (2021). Generation of induced pluripotent stem cell line (ZZUi027-A) derived from skin fibroblasts from a Parkinson's disease patient with RAB39B gene mutation. *Stem Cell Research*, *55*, 102454. https://doi.org/10.1016/j.scr.2021.102454

Sun, L., Apweiler, M., Normann, C., Grathwol, C. W., Hurrle, T., Gräßle, S., Jung, N., Bräse, S., & Fiebich, B. L. (2024). Anti-Inflammatory Effects of GPR55 Agonists and Antagonists in LPS-Treated BV2 Microglial Cells. *Pharmaceuticals (Basel, Switzerland)*, *17*(6), 674. https://doi.org/10.3390/ph17060674

Suyama, K., Watanabe, M., Sakabe, K., Okada, Y., Matsuyama, D., Kuroiwa, M., & Mochida, J. (2011). Overexpression of GRP78 protects glial cells from endoplasmic reticulum stress. *Neuroscience Letters*, *504*(3), 271-276. https://doi.org/10.1016/j.neulet.2011.09.045

Sveinbjornsdottir, S. (2016). The clinical symptoms of Parkinson's disease. *Journal of Neurochemistry*, *139 Suppl 1*, 318-324. https://doi.org/10.1111/jnc.13691

T

Talebi, M., Sadoughi, M. M., Ayatollahi, S. A., Ainy, E., Kiani, R., Zali, A., & Miri, M. (2023). Therapeutic potentials of cannabidiol: Focus on the Nrf2 signaling pathway. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *168*, 115805. https://doi.org/10.1016/j.biopha.2023.115805

Tarragon, E., & Moreno, J. J. (2019). Cannabinoids, Chemical Senses, and Regulation of Feeding Behavior. *Chemical Senses*, *44*(2), 73-89. https://doi.org/10.1093/chemse/bjy068

Terada, T., Yokokura, M., Yoshikawa, E., Futatsubashi, M., Kono, S., Konishi, T., Miyajima, H., Hashizume, T., & Ouchi, Y. (2016). Extrastriatal spreading of microglial activation in Parkinson's disease: A positron emission tomography study. *Annals of Nuclear Medicine*, *30*(8), 579-587. https://doi.org/10.1007/s12149-016-1099-2

Ternianov, A., Pérez-Ortiz, J. M., Solesio, M. E., García-Gutiérrez, M. S., Ortega-Álvaro, A., Navarrete, F., Leiva, C., Galindo, M. F., & Manzanares, J. (2012). Overexpression of CB2 cannabinoid receptors results in neuroprotection against behavioral and neurochemical alterations induced by intracaudate administration of 6-hydroxydopamine. *Neurobiology of Aging*, *33*(2), 421.e1-16. https://doi.org/10.1016/j.neurobiolaging.2010.09.012

Thomas, A., Stevenson, L. A., Wease, K. N., Price, M. R., Baillie, G., Ross, R. A., & Pertwee, R. G. (2005). Evidence that the plant cannabinoid Delta9-tetrahydrocannabivarin is a cannabinoid CB1 and CB2 receptor antagonist. *British Journal of Pharmacology*, *146*(7), 917-926. https://doi.org/10.1038/sj.bjp.0706414

Tiberi, M., Evron, T., Saracini, S., Boffa, L., Mercuri, N. B., Chintalacharuvu, S. R., Atamas, S. P., & Chiurchiù, V. (2022). Potent T cell-mediated anti-inflammatory role of the selective CB2 agonist lenabasum in multiple sclerosis. *Neuropathology and Applied Neurobiology*, *48*(2), e12768. https://doi.org/10.1111/nan.12768

Tolón, R. M., Núñez, E., Pazos, M. R., Benito, C., Castillo, A. I., Martínez-Orgado, J. A., & Romero, J. (2009). The activation of cannabinoid CB2 receptors stimulates in situ and in vitro beta-amyloid removal by human macrophages. *Brain Research*, *1283*, 148-154. https://doi.org/10.1016/j.brainres.2009.05.098

Trempe, J.-F., & Fon, E. A. (2013). Structure and Function of Parkin, PINK1, and DJ-1, the Three Musketeers of Neuroprotection. *Frontiers in Neurology*, *4*, 38. https://doi.org/10.3389/fneur.2013.00038

Trétiakoff, C. (1919). *Contribution a l'etude l'anatomie pathologique du locus Niger de soemmering: Avec quelques déductions relatives à la pathogénie des troubles du tonus musculaire et de la maladie de Parkinson*. Jouve.

Tretter, L., Sipos, I., & Adam-Vizi, V. (2004). Initiation of neuronal damage by complex I deficiency and oxidative stress in Parkinson's disease. *Neurochemical Research*, *29*(3), 569-577. https://doi.org/10.1023/b:nere.0000014827.94562.4b

Tsou, K., Brown, S., Sañudo-Peña, M. C., Mackie, K., & Walker, J. M. (1998). Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience*, *83*(2), 393-411. https://doi.org/10.1016/s0306-4522(97)00436-3

Tsukita, K., Sakamaki-Tsukita, H., & Takahashi, R. (2022). Long-term Effect of Regular Physical Activity and Exercise Habits in Patients With Early Parkinson Disease. *Neurology*, *98*(8), e859-e871. https://doi.org/10.1212/WNL.0000000000013218

Tu, H., Zhang, Z. W., Qiu, L., Lin, Y., Jiang, M., Chia, S.-Y., Wei, Y., Ng, A. S. L., Reynolds, R., Tan, E.-K., & Zeng, L. (2022). Increased expression of pathological markers in Parkinson's disease dementia post-mortem brains compared to dementia with Lewy bodies. *BMC Neuroscience*, *23*(1), 3. https://doi.org/10.1186/s12868-021-00687-4

U

Usmani, A., Shavarebi, F., & Hiniker, A. (2021). The Cell Biology of LRRK2 in Parkinson's Disease. *Molecular and Cellular Biology*, *41*(5), e00660-20. https://doi.org/10.1128/MCB.00660-20

V

Vallée, A., Vallée, J.-N., & Lecarpentier, Y. (2021). Potential role of cannabidiol in Parkinson's disease by targeting the WNT/β-catenin pathway, oxidative stress and inflammation. *Aging*, *13*(7), 10796-10813. https://doi.org/10.18632/aging.202951

Van Den Eeden, S. K., Tanner, C. M., Bernstein, A. L., Fross, R. D., Leimpeter, A., Bloch, D. A., & Nelson, L. M. (2003). Incidence of Parkinson's disease: Variation by age, gender, and race/ethnicity. *American Journal of Epidemiology*, *157*(11), 1015-1022. https://doi.org/10.1093/aje/kwg068

van der Stelt, M., & Di Marzo, V. (2005). Cannabinoid receptors and their role in neuroprotection. *Neuromolecular Medicine*, *7*(1-2), 37-50. https://doi.org/10.1385/NMM:7:1-2:037

van der Stelt, M., Fox, S. H., Hill, M., Crossman, A. R., Petrosino, S., Di Marzo, V., & Brotchie, J. M. (2005). A role for endocannabinoids in the generation of parkinsonism and levodopa-induced dyskinesia in MPTP-lesioned non-human primate models of Parkinson's disease. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *19*(9), 1140-1142. https://doi.org/10.1096/fj.04-3010fje

Van Laere, K., Casteels, C., Lunskens, S., Goffin, K., Grachev, I. D., Bormans, G., & Vandenberghe, W. (2012). Regional changes in type 1 cannabinoid receptor availability in Parkinson's disease in vivo. *Neurobiology of Aging*, *33*(3), 620.e1-8. https://doi.org/10.1016/j.neurobiolaging.2011.02.009

Varshney, K., Patel, A., Ansari, S., Shet, P., & Panag, S. S. (2023). Cannabinoids in Treating Parkinson's Disease Symptoms: A Systematic Review of Clinical Studies. *Cannabis and Cannabinoid Research*, *8*(5), 716-730. https://doi.org/10.1089/can.2023.0023

Velasco, G., Hernández-Tiedra, S., Dávila, D., & Lorente, M. (2016). The use of cannabinoids as anticancer agents. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *64*, 259-266. https://doi.org/10.1016/j.pnpbp.2015.05.010

Verkhratsky, A., & Nedergaard, M. (2018). Physiology of Astroglia. *Physiological Reviews*, *98*(1), 239-389. https://doi.org/10.1152/physrev.00042.2016

Vidović, M., & Rikalovic, M. G. (2022). Alpha-Synuclein Aggregation Pathway in Parkinson's Disease: Current Status and Novel Therapeutic Approaches. *Cells*, *11*(11), 1732. https://doi.org/10.3390/cells11111732

Villa, A., Vegeto, E., Poletti, A., & Maggi, A. (2016). Estrogens, Neuroinflammation, and Neurodegeneration. *Endocrine Reviews*, *37*(4), 372-402. https://doi.org/10.1210/er.2016-1007

Villapol, S. (2018). Roles of Peroxisome Proliferator-Activated Receptor Gamma on Brain and Peripheral Inflammation. *Cellular and Molecular Neurobiology*, *38*(1), 121-132. https://doi.org/10.1007/s10571-017-0554-5

Vingill, S., Connor-Robson, N., & Wade-Martins, R. (2018). Are rodent models of Parkinson's disease behaving as they should? *Behavioural Brain Research*, *352*, 133-141. https://doi.org/10.1016/j.bbr.2017.10.021

Viñals, X., Moreno, E., Lanfumey, L., Cordomí, A., Pastor, A., de La Torre, R., Gasperini, P., Navarro, G., Howell, L. A., Pardo, L., Lluís, C., Canela, E. I., McCormick, P. J., Maldonado, R., & Robledo, P. (2015). Cognitive Impairment Induced by Delta9-tetrahydrocannabinol Occurs through Heteromers between Cannabinoid CB1 and Serotonin 5-HT2A Receptors. *PLoS Biology*, *13*(7), e1002194. https://doi.org/10.1371/journal.pbio.1002194

Viveros-Paredes, J. M., González-Castañeda, R. E., Gertsch, J., Chaparro-Huerta, V., López-Roa, R. I., Vázquez-Valls, E., Beas-Zarate, C., Camins-Espuny, A., & Flores-Soto, M. E. (2017). Neuroprotective Effects of β-Caryophyllene against Dopaminergic Neuron Injury in a Murine Model of Parkinson's Disease Induced by MPTP. *Pharmaceuticals (Basel, Switzerland)*, *10*(3), 60. https://doi.org/10.3390/ph10030060

Volpicelli-Daley, L. A., Gamble, K. L., Schultheiss, C. E., Riddle, D. M., West, A. B., & Lee, V. M.-Y. (2014). Formation of α-synuclein Lewy neurite-like aggregates in axons impedes the transport of distinct endosomes. *Molecular Biology of the Cell*, *25*(25), 4010-4023. https://doi.org/10.1091/mbc.E14-02-0741

Vrechi, T. A. M., Guarache, G. C., Oliveira, R. B., Guedes, E. da C., Erustes, A. G., Leão, A. H. F. F., Abílio, V. C., Zuardi, A. W., Hallak, J. E. C., Crippa, J. A., Bincoletto, C., Ureshino, R. P., Smaili, S. S., & Pereira, G. J. S. (2025). Cannabidiol-Induced Autophagy Ameliorates Tau Protein Clearance. *Neurotoxicity Research*, *43*(1), 8. https://doi.org/10.1007/s12640-025-00729-3

Vrechi, T. A. M., Leão, A. H. F. F., Morais, I. B. M., Abílio, V. C., Zuardi, A. W., Hallak, J. E. C., Crippa, J. A., Bincoletto, C., Ureshino, R. P., Smaili, S. S., & Pereira, G. J. S. (2021). Cannabidiol induces autophagy via ERK1/2 activation in neural cells. *Scientific Reports*, *11*(1), 5434. https://doi.org/10.1038/s41598-021-84879-2

W

Wager-Miller, J., Westenbroek, R., & Mackie, K. (2002). Dimerization of G protein-coupled receptors: CB1 cannabinoid receptors as an example. *Chemistry and Physics of Lipids*, *121*(1-2), 83-89. https://doi.org/10.1016/s0009-3084(02)00151-2

Wahner, A. D., Bronstein, J. M., Bordelon, Y. M., & Ritz, B. (2007). Nonsteroidal anti-inflammatory drugs may protect against Parkinson disease. *Neurology*, *69*(19), 1836-1842. https://doi.org/10.1212/01.wnl.0000279519.99344.ad

Walsh, S. K., Hector, E. E., Andréasson, A.-C., Jönsson-Rylander, A.-C., & Wainwright, C. L. (2014). GPR55 deletion in mice leads to age-related ventricular dysfunction and impaired adrenoceptor-mediated inotropic responses. *PloS One*, *9*(9), e108999. https://doi.org/10.1371/journal.pone.0108999

Walsh, S., Mnich, K., Mackie, K., Gorman, A. M., Finn, D. P., & Dowd, E. (2010). Loss of cannabinoid CB1 receptor expression in the 6-hydroxydopamine-induced nigrostriatal terminal lesion model of Parkinson's disease in the rat. *Brain Research Bulletin*, *81*(6), 543-548. https://doi.org/10.1016/j.brainresbull.2010.01.009

Wang, D., Qu, S., Zhang, Z., Tan, L., Chen, X., Zhong, H.-J., & Chong, C.-M. (2023). Strategies targeting endoplasmic reticulum stress to improve Parkinson's disease. *Frontiers in Pharmacology*, *14*, 1288894. https://doi.org/10.3389/fphar.2023.1288894

Wang, L., Cui, Y., Han, B., Du, Y., Salewala, K. S., Wang, S., Zhao, W., Zhang, H., Wang, S., Xu, X., Ma, J., Zhu, Y., & Tuo, H. (2025). Gut microbiota and Parkinson's disease. *Chinese Medical Journal*, *138*(3), 289-297. https://doi.org/10.1097/CM9.0000000000003318

Wang, L., Wu, X., Yang, G., Hu, N., Zhao, Z., Zhao, L., & Li, S. (2022). Cannabidiol Alleviates the Damage to Dopaminergic Neurons in 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Induced Parkinson's Disease Mice Via Regulating Neuronal Apoptosis and Neuroinflammation. *Neuroscience*, *498*, 64-72. https://doi.org/10.1016/j.neuroscience.2022.06.036

Wang, M., Liu, M., & Ma, Z. (2023). Cannabinoid type 2 receptor activation inhibits MPP+-induced M1 differentiation of microglia through activating PI3K/Akt/Nrf2 signal pathway. *Molecular Biology Reports*, *50*(5), 4423-4433. https://doi.org/10.1007/s11033-023-08395-4

Wang, T., Shi, C., Luo, H., Zheng, H., Fan, L., Tang, M., Su, Y., Yang, J., Mao, C., & Xu, Y. (2022). Neuroinflammation in Parkinson's Disease: Triggers, Mechanisms, and Immunotherapies. *The Neuroscientist: A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *28*(4), 364-381. https://doi.org/10.1177/1073858421991066

Wang, X., Xiang, X., Hu, J., Wu, Y., Li, Y., Jin, S., & Wu, X. (2022). Pharmacological Activation of GPR55 Improved Cognitive Impairment Induced by Lipopolysaccharide in Mice. *Journal of Molecular Neuroscience*, *72*(8), 1656-1669. https://doi.org/10.1007/s12031-022-02020-y

Wang, Z., Li, Y., Cai, S., Li, R., & Cao, G. (2018). Cannabinoid receptor 2 agonist attenuates blood-brain barrier damage in a rat model of intracerebral hemorrhage by activating the Rac1 pathway. *International Journal of Molecular Medicine*, *42*(5), 2914-2922. https://doi.org/10.3892/ijmm.2018.3834

Warden, A., Truitt, J., Merriman, M., Ponomareva, O., Jameson, K., Ferguson, L. B., Mayfield, R. D., & Harris, R. A. (2016). Localization of PPAR isotypes in the adult mouse and human brain. *Scientific Reports*, *6*, 27618. https://doi.org/10.1038/srep27618

Winslow, A. R., Chen, C.-W., Corrochano, S., Acevedo-Arozena, A., Gordon, D. E., Peden, A. A., Lichtenberg, M., Menzies, F. M., Ravikumar, B., Imarisio, S., Brown, S., O'Kane, C. J., & Rubinsztein, D. C. (2010). α-Synuclein impairs macroautophagy: Implications for Parkinson's disease. *The Journal of Cell Biology*, *190*(6), 1023-1037. https://doi.org/10.1083/jcb.201003122

Wolf, P., Schoeniger, A., & Edlich, F. (2022). Pro-apoptotic complexes of BAX and BAK on the outer mitochondrial membrane. *Biochimica Et Biophysica Acta. Molecular Cell Research*, *1869*(10), 119317. https://doi.org/10.1016/j.bbamcr.2022.119317

World Health Organization. (2023). *Parkinson disease*. https://www.who.int/news-room/fact-sheets/detail/parkinson-disease

Wouters, E., Walraed, J., Banister, S. D., & Stove, C. P. (2019). Insights into biased signaling at cannabinoid receptors: Synthetic cannabinoid receptor agonists. *Biochemical Pharmacology*, *169*, 113623. https://doi.org/10.1016/j.bcp.2019.08.025

Wu, C.-S., Chen, H., Sun, H., Zhu, J., Jew, C. P., Wager-Miller, J., Straiker, A., Spencer, C., Bradshaw, H., Mackie, K., & Lu, H.-C. (2013). GPR55, a G-protein coupled receptor for lysophosphatidylinositol, plays a role in motor coordination. *PloS One*, *8*(4), e60314. https://doi.org/10.1371/journal.pone.0060314

X

Xiang, X., Wang, X., Jin, S., Hu, J., Wu, Y., Li, Y., & Wu, X. (2022). Activation of GPR55 attenuates cognitive impairment and neurotoxicity in a mouse model of Alzheimer's disease induced by Aβ1-42 through inhibiting RhoA/ROCK2 pathway. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *112*, 110423. https://doi.org/10.1016/j.pnpbp.2021.110423

Xiang, X., Wang, X., Wu, Y., Hu, J., Li, Y., Jin, S., & Wu, X. (2022). Activation of GPR55 attenuates cognitive impairment, oxidative stress, neuroinflammation, and synaptic dysfunction in a streptozotocin-induced Alzheimer's mouse model. *Pharmacology, Biochemistry, and Behavior*, *214*, 173340. https://doi.org/10.1016/j.pbb.2022.173340

Xilouri, M., Brekk, O. R., & Stefanis, L. (2013). α-Synuclein and protein degradation systems: A reciprocal relationship. *Molecular Neurobiology*, *47*(2), 537-551. https://doi.org/10.1007/s12035-012-8341-2

Xiong, N., Xiong, J., Jia, M., Liu, L., Zhang, X., Chen, Z., Huang, J., Zhang, Z., Hou, L., Luo, Z., Ghoorah, D., Lin, Z., & Wang, T. (2013). The role of autophagy in Parkinson's disease: Rotenone-based modeling. *Behavioral and Brain Functions: BBF*, *9*, 13. https://doi.org/10.1186/1744-9081-9-13

Xu, Q., Park, Y., Huang, X., Hollenbeck, A., Blair, A., Schatzkin, A., & Chen, H. (2010). Physical activities and future risk of Parkinson disease. *Neurology*, *75*(4), 341-348. https://doi.org/10.1212/WNL.0b013e3181ea1597

Y

Yang, W., Wang, G., Wang, C.-E., Guo, X., Yin, P., Gao, J., Tu, Z., Wang, Z., Wu, J., Hu, X., Li, S., & Li, X.-J. (2015). Mutant alpha-synuclein causes age-dependent neuropathology in monkey brain. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *35*(21), 8345-8358. https://doi.org/10.1523/JNEUROSCI.0772-15.2015

Yee, R. E., Huang, S. C., Stout, D. B., Irwin, I., Shoghi-Jadid, K., Togaski, D. M., DeLanney, L. E., Langston, J. W., Satyamurthy, N., Farahani, K. F., Phelps, M. E., & Barrio, J. R. (2000). Nigrostriatal reduction of aromatic L-amino acid decarboxylase

activity in MPTP-treated squirrel monkeys: In vivo and in vitro investigations. *Journal of Neurochemistry*, *74*(3), 1147-1157. https://doi.org/10.1046/j.1471-4159.2000.741147.x

Youssef, D. A., El-Fayoumi, H. M., & Mahmoud, M. F. (2019). Beta-caryophyllene alleviates diet-induced neurobehavioral changes in rats: The role of CB2 and PPAR-γ receptors. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *110*, 145-154. https://doi.org/10.1016/j.biopha.2018.11.039

Yu, H., Liu, X., Chen, B., Vickstrom, C. R., Friedman, V., Kelly, T. J., Bai, X., Zhao, L., Hillard, C. J., & Liu, Q.-S. (2021). The Neuroprotective Effects of the CB2 Agonist GW842166x in the 6-OHDA Mouse Model of Parkinson's Disease. *Cells*, *10*(12), 3548. https://doi.org/10.3390/cells10123548

Yuan, H., Sarre, S., Ebinger, G., & Michotte, Y. (2005). Histological, behavioural and neurochemical evaluation of medial forebrain bundle and striatal 6-OHDA lesions as rat models of Parkinson's disease. *Journal of Neuroscience Methods*, *144*(1), 35-45. https://doi.org/10.1016/j.jneumeth.2004.10.004

Yuan, J., Jiang, C., Wang, J., Chen, C.-J., Hao, Y., Zhao, G., Feng, Z., & Xie, X.-Q. (2022). In Silico Prediction and Validation of CB2 Allosteric Binding Sites to Aid the Design of Allosteric Modulators. *Molecules (Basel, Switzerland)*, *27*(2), 453. https://doi.org/10.3390/molecules27020453

Yue, M., Hinkle, K. M., Davies, P., Trushina, E., Fiesel, F. C., Christenson, T. A., Schroeder, A. S., Zhang, L., Bowles, E., Behrouz, B., Lincoln, S. J., Beevers, J. E., Milnerwood, A. J., Kurti, A., McLean, P. J., Fryer, J. D., Springer, W., Dickson, D. W., Farrer, M. J., & Melrose, H. L. (2015). Progressive dopaminergic alterations and mitochondrial abnormalities in LRRK2 G2019S knock-in mice. *Neurobiology of Disease*, *78*, 172-195. https://doi.org/10.1016/j.nbd.2015.02.031

Yuste, J. E., Echeverry, M. B., Ros-Bernal, F., Gomez, A., Ros, C. M., Campuzano, C. M., Fernandez-Villalba, E., & Herrero, M. T. (2012). 7-Nitroindazole down-regulates dopamine/DARPP-32 signaling in neostriatal neurons in a rat model of Parkinson's disease. *Neuropharmacology*, *63*(7), 1258-1267. https://doi.org/10.1016/j.neuropharm.2012.07.031

Z

Zhang, H.-Y., Bi, G.-H., Li, X., Li, J., Qu, H., Zhang, S.-J., Li, C.-Y., Onaivi, E. S., Gardner, E. L., Xi, Z.-X., & Liu, Q.-R. (2015). Species differences in cannabinoid receptor 2 and receptor responses to cocaine self-administration in mice and rats. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *40*(4), 1037-1051. https://doi.org/10.1038/npp.2014.297

Zhang, W., Xiao, D., Mao, Q., & Xia, H. (2023). Role of neuroinflammation in neurodegeneration development. *Signal Transduction and Targeted Therapy*, *8*(1), 267. https://doi.org/10.1038/s41392-023-01486-5

Zhang, Y., Chen, K., Sloan, S. A., Bennett, M. L., Scholze, A. R., O'Keeffe, S., Phatnani, H. P., Guarnieri, P., Caneda, C., Ruderisch, N., Deng, S., Liddelow, S. A., Zhang, C., Daneman, R., Maniatis, T., Barres, B. A., & Wu, J. Q. (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *34*(36), 11929-11947. https://doi.org/10.1523/JNEUROSCI.1860-14.2014

Zhang, Z., Yang, X., Song, Y.-Q., & Tu, J. (2021). Autophagy in Alzheimer's disease pathogenesis: Therapeutic potential and future perspectives. *Ageing Research Reviews*, *72*, 101464. https://doi.org/10.1016/j.arr.2021.101464

Zhou, J., Deng, Y., Li, F., Yin, C., Shi, J., & Gong, Q. (2019). Icariside II attenuates lipopolysaccharide-induced neuroinflammation through inhibiting TLR4/MyD88/NF-κB pathway in rats. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *111*, 315-324. https://doi.org/10.1016/j.biopha.2018.10.201

Zhu, H., Xiao, F., Xiao, Y., Guo, Y., Shan, X., Zhang, Z., Zhang, L., & Guo, H. (2023). Targeting CB2R in astrocytes for Parkinson's disease therapy: Unraveling the Foxg1-mediated neuroprotective mechanism through autophagy-mediated NLRP3 degradation. *Journal of Neuroinflammation*, *20*(1), 304. https://doi.org/10.1186/s12974-023-02989-2

Zoerner, A. A., Gutzki, F.-M., Batkai, S., May, M., Rakers, C., Engeli, S., Jordan, J., & Tsikas, D. (2011). Quantification of endocannabinoids in biological systems by chromatography and mass spectrometry: A comprehensive review from an analytical and biological perspective. *Biochimica Et Biophysica Acta*, *1811*(11), 706-723. https://doi.org/10.1016/j.bbalip.2011.08.004

Zoratti, C., Kipmen-Korgun, D., Osibow, K., Malli, R., & Graier, W. F. (2003). Anandamide initiates Ca(2+) signaling via CB2 receptor linked to phospholipase C in calf pulmonary endothelial cells. *British Journal of Pharmacology*, *140*(8), 1351-1362. https://doi.org/10.1038/sj.bjp.0705529

Zou, S., & Kumar, U. (2018). Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. *International Journal of Molecular Sciences*, *19*(3), 833. https://doi.org/10.3390/ijms19030833

Zuardi, A. W., Crippa, J. a. S., Hallak, J. E. C., Pinto, J. P., Chagas, M. H. N., Rodrigues, G. G. R., Dursun, S. M., & Tumas, V. (2009). Cannabidiol for the treatment of psychosis in Parkinson's disease. *Journal of Psychopharmacology (Oxford, England)*, *23*(8), 979-983. https://doi.org/10.1177/0269881108096519

APPENDIX

This doctoral thesis has so far resulted in a first-author scientific publication:

Rodríguez-Carreiro, S., Gómez-Cañas, M., Lubrini, F., Gonzalo-Consuegra, C., Winkler, M., Caprioglio, D., Appendino, G., García, C., Morales, P., Jagerovic, N., Fischer, J. T., Fiebich, B. L., Goetz, M. R., Muñoz, E., & Fernández-Ruiz, J. (2025). Investigation in the CB1 and CB2 receptor binding profile and intrinsic activity of (−) and (+)-enantiomers of some naturally occurring phytocannabinoids or synthetic derivatives. European Journal of Medicinal Chemistry Reports, 14, 100262. https://doi.org/10.1016/j.ejmcr.2025.100262

Additional publications, not directly linked to the thesis project, have also been generated in parallel:

Rodríguez-Carreiro, S., Navarro, E., Muñoz, E., & Fernández-Ruiz, J. (2023). The Cannabigerol Derivative VCE-003.2 Exerts Therapeutic Effects in 6-Hydroxydopamine-Lesioned Mice: Comparison with The Classic Dopaminergic Replacement Therapy. *Brain Sciences*, *13*(9), 1272. https://doi.org/10.3390/brainsci13091272

Rodríguez-Carreiro, S., Nogales, M. del C., Jiménez-Galán, D., Carmona-Lorenzo, S., Caro-Martín, A., Navarro, E., & Esteras, N. (2024). Role of non-neuronal cells in neurorepair: A focus on proteinopathy and neurodegeneration. *Regenerative Medicine Reports*, *1*(1), 31. https://doi.org/10.4103/REGENMED.REGENMED-D-24-00005

Burgaz, S., Navarro, E., **Rodríguez-Carreiro, S.**, Navarrete, C., Garrido-Rodríguez, M., Lastres-Becker, I., Chocarro, J., Lanciego, J. L., Muñoz, E., & Fernández-Ruiz, J. (2024). Investigation in the cannabigerol derivative VCE-003.2 as a disease-modifying agent in a mouse model of experimental synucleinopathy. *Behavioral and Brain Functions: BBF*, *20*(1), 28. https://doi.org/10.1186/s12993-024-00256-9

European Journal of Medicinal Chemistry Reports 14 (2025) 100262

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry Reports

journal homepage: www.editorialmanager.com/ejmcr/default.aspx

Investigation in the CB_1 and CB_2 receptor binding profile and intrinsic activity of (−) and (+)-enantiomers of some naturally occurring phytocannabinoids or synthetic derivatives

Santiago Rodríguez-Carreiro [a,b,c], María Gómez-Cañas [a,b,c], Francesca Lubrini [a], Claudia Gonzalo-Consuegra [a,b,c], Matthias Winkler [d,1], Diego Caprioglio [e], Giovanni Appendino [e], Concepción García [a,b,c], Paula Morales [f], Nadine Jagerovic [f], Joerg T. Fischer [d], Bernd L. Fiebich [g], Marcus R. Goetz [d,2], Eduardo Muñoz [h,i,j], Javier Fernández-Ruiz [a,b,c,*]

[a] Instituto Universitario de Investigación en Neuroquímica, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, Madrid, Spain
[b] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain
[c] Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain
[d] Symrise AG, Holzminden, Germany
[e] Dipartimento di Scienze del Farmaco, Università degli Study del Piamonte Orientale, Novara, Italy
[f] Instituto de Química Médica-CSIC, Madrid, Spain
[g] VivaCell Biotechnology GmbH, Denzlingen, Germany
[h] Maimonides Biomedical Research Institute of Córdoba, Spain
[i] Department of Cellular Biology, Physiology and Immunology, University of Córdoba, Spain
[j] University Hospital Reina Sofía, Córdoba, Spain

ARTICLE INFO

Keywords:
CBD
CBDV
CBDA-Derivatives
(−)- and (+)-enantiomers
CB_1 receptor
CB_2 receptor
Binding
Agonist
Inverse agonist
Antagonist
Anti-inflammatory and neuroprotective effects

ABSTRACT

Cannabidiol (CBD) and cannabidivarin (CBDV) have shown promising clinical efficacy for the management of epilepsy, and beneficial effects have been demonstrated for CBD in a diversity of other pathologies (pain, schizophrenia, Tourette syndrome, anxiety). However, the mechanism(s) involved are still largely elusive, as are the molecular target(s) involved. CBD and CBDV do not orthosterically bind the cannabinoid type-1 (CB_1) and type-2 (CB_2) receptors, showing only modest allosteric modulation of both end-points. CBD and CBDV are biosynthesized as optically highly pure (−)-enantiomers, and most bioactivity data refer to these forms. (+)-CBD and related analogues [(+)-cannabidiolic acid (CBDA), its esters, and (+)-CBDV] can be obtained by chemical synthesis, and we present evidence that the (+)- and (−)-enantiomers of CBD, CBDV and of a selection of derivatives of CBDA have distinct binding profiles and functional activity at the CB_1/CB_2 receptors. Thus, with the single exception of the methyl ester of CBDA, all the (+)-enantiomers showed higher affinities than the (−)-isomers for both receptors, in particular for the CB_2 receptors. The affinity of the (+)-enantiomers for both CB_1 and CB_2 receptors showed a marked dependence on the nature of the alkyl residue on the aromatic ring and the esterification pattern of CBDA. Potency was rarely in the low nM value for CB_1, but generally so for CB_2. Enantiomers showing low nM activity were further investigated for their intrinsic activity using GTPγS binding assays. This proved that (+)-CBD, (+)-CBDV and the methyl ester of (+)-CBDA are agonists at the CB_2 receptor, with the β-hydroxyethyl ester of (+)-CBDA being an inverse agonist, and its β-hydroxypentyl ester behaving as an agonist at CB_1 and an inverse agonist at CB_2. Finally, we assayed *in vitro* the anti-inflammatory and neuroprotective properties of three compounds [(+)-CBD, (+)-CBDV and (+)-CBDA methyl ester] strongly activating CB_2, showing their ability to reduce the production of proinflammatory factors and protecting neurons against their toxicity. Remarkably, these benefits were eliminated by the selective blockade of the CB_2 receptor,

* Corresponding author. Department of Biochemistry and Molecular Biology, Faculty of Medicine, Complutense University, 28040-Madrid, Spain.
E-mail address: jjfr@med.ucm.es (J. Fernández-Ruiz).
[1] Current address: Matthias Winkler, Dr. Pfleger Arzneimittel GmbH, Dr.-Robert-Pfleger-Str. 12, Bamberg, Germany.
[2] Current address: Marcus R. Goetz, Dr. Goetz GmbH, Kampstr. 18, Wesertal, Germany.

https://doi.org/10.1016/j.ejmcr.2025.100262
Received 1 March 2025; Received in revised form 1 April 2025; Accepted 1 April 2025
Available online 2 April 2025
2772-4174/© 2025 The Authors. Published by Elsevier Masson SAS. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

highlighting its role as a (+)-CBD target. In summary, our data show that remarkable differences between (−)- and (+)-enantiomers of CBD, CBDV and related compounds exist in terms of CB_1/CB_2 receptor binding profile and intrinsic activity. The observation that the natural (−)-enantiomers do not bind CB_2 receptors suggests that their effects are associated with different targets.

1. Introduction

Cannabidiol (CBD) is a major non-psychotropic cannabis-derived cannabinoid, and has been shown to be endowed with pleiotropic properties potentially beneficial for numerous central and peripheral human disorders [1–5]. As regards CNS pathologies, CBD treatment has been associated to anticonvulsant [6,7], antiemetic [8,9], anxiolytic [10,11], antipsychotic [12,13], antioxidant [14,15], anti-inflammatory [16,17] and neuroprotective [1,18–20] activity in experimental models of epilepsy, vomiting and nausea, anxiety, schizophrenia, oxidative injury, neuroinflammation, and neurodegeneration, respectively (reviewed in Ref. [1,3–5]). However, these well-known broad--spectrum therapeutic properties of CBD contrast with the fact that its underlying cellular and molecular mechanism(s) are not yet completely clarified [1,3–5,21]. Certain activities have been related to endocannabinoid-mediated mechanisms: e.g., inhibition of endocannabinoid inactivation [22,23], allosteric modulation of classic cannabinoid receptors [24–27], activity at atypical cannabinoid receptors as G protein-coupled 55 receptor (GPR 55) [28,29] and members of the transient receptor potential (TRP) family, e.g., TRP vanilloid type-1 ($TRPV_1$) [30]. Conversely, others seem to be the result of endocannabinoid-independent processes: e.g., binding to serotonin receptor types [31–33], adenosine uptake inhibition [2,34], targeting nuclear receptors of the peroxisome proliferator-activated receptor (PPAR) family [35], modulation of ion channels [1], among others.

None of these potential mechanisms links CBD with a direct interaction with classic cannabinoid receptors, at least with their orthosteric sites, and this is why CBD has been frequently viewed as an atypical cannabinoid as concerns its pharmacodynamics profile [1,3–5]. The only and recent exception is the possibility that CBD could bind the allosteric site in the cannabinoid type-1 (CB_1) receptor acting as a negative allosteric modulator (NAM) [24–27], then inhibiting the activity of Δ^9-tetrahydrocannabinol (Δ^9-THC) [36], something that has been recently extended to the cannabinoid type-2 (CB_2) receptor [25,37]. In both cases, the binding of CBD to classic cannabinoid receptors would be at regulatory rather than orthosteric sites, for which it shows negligible affinity [1,3–5], as confirmed by its lack of psychoactivity. The NAM activity of CBD was also demonstrated in endogenous and heterologous cell culture expression systems for ERK1/2 phosphorylation and β-arrestin-1 recruitment in the presence of orthosteric ligands such as Δ^9-THC and 2-arachydonoyl glycerol [24]. Subsequent modelling studies showed that CBD binds to putative CB_1 receptor allosteric sites [38,39]. In addition, some CBD derivatives (see Ref. [40] for a recent review) as its classic, mostly synthetic, metabolic analogues (e.g., desoxy-CBD, 7-hydroxy-CBD, cannabidiolic acid (CBDA)) [41,42], fluorinated CBD [43], CBD hydroxyquinones [44,45], abnormal CBD [46], or the (+)-CBD stereoisomers investigated in this study, have extended the pharmacodynamics profile of CBD (e.g., more potency (fluorinated CBD), activity at novel sites as GPR18 (abnormal CBD) or PPARs (CBD hydroquinones)), as well as its therapeutic potential [42–46], and could be useful also to better understand the mechanisms of action of CBD itself.

One of these derivatives is cannabidivarin (CBDV), which shows a shortened alkyl residue (3- *versus* 5-carbon length), and occurs naturally in the cannabis plant [47]. Its mechanism of action has long remained elusive (reviewed in Ref. [48]), despite its demonstrated anticonvulsant [49–51], antioxidant [52] and antitumoral [53] properties, with also beneficial effects for the management of autism-related disorders, like the Rett syndrome [51,54], and neurodegenerative disorders [55]. Overall, the biological profile of CBDV might well be independent of classic cannabinoid receptors, e.g., antioxidant effects associated to reactive oxygen species scavenging activity (also exerted by CBD [56]), activity at $TRPV_1$, $TRPV_2$ and transient receptor potential ankyrin type 1 ($TRPA_1$) receptors, as well as GPR55 antagonism (reviewed in Ref. [48]).

In the cannabis plant, CBD and CBDV exist in the (−)-enantiomer form, and the study of their biological profile has been based on these enantiomers (reviewed in Ref. [5,57]). Conversely, CBD and CBDV can be obtained by synthesis, which can generate also the non-natural (+)-enantiomeric form or the racemate [5,57]. It was early suggested that stereochemistry strongly affected the binding of Δ^9-THC and analogues to CB_1 and CB_2 receptors ([41,58–62]; reviewed in Ref. [2,5]), with the (−)-forms being more active than the (+)-forms [63]. Thus, compared to (−)-*trans*-Δ^9-THC (the major naturally-occurring isomer), (−)-*cis*-Δ^9-THC was less potent as a CB_1/CB_2 receptor partial agonist *in vitro*, although activity in the cannabinoid tetrad was retained [64]. However, (+)-*cis*-Δ^9-THC was almost inactive ($K_i > 10$ μM) at both CB_1 and CB_2 receptors, compared with the (−)-*trans*-Δ^9-THC and (−)-*cis*-Δ^9-THC [61,65,66]. The same trend was observed in Δ^9-THC derivatives, like those with a dimethylheptyl side chain, for which the (−)-enantiomer (HU-210) is highly active at CB_1/CB_2 receptors, whereas the (+)-enantiomer (HU-211 or dexanabinol) is devoid of any activity at these receptors [67], yet exhibiting interesting pharmacological properties as a non-competitive antagonist for NMDA receptors, showing potential for the management of brain trauma [68].

Overall, few studies have investigated the influence of chirality on the activity of CBD and its derivatives, and this provided the rationale for this study, where various analogues of the natural product and its native acid form (CBDA), including CBDV, CBDA-methyl ester (CBDA-Me), CBDA-glycol ester (CBDA-Gly), CBDA-hydroxypentyl ester (CBDA-Hyp), CBDA-ethyl ester (CBDA-Et) and dimethylheptyl-CBD (DMH-CBD), were investigated, also investigating if differences existed in our assays between natural and synthetic CBD, a controversial issue [69].

2. Materials and methods

2.1. Chemistry

CBD, CBDV, CBDA-Me, CBDA-Gly and CBDA-Hyp were synthesized in (−)- and (+)-*trans*-enantiomeric forms in Symrise AG, Holzminden, Germany following procedures described in Fiebich et al. [70]. CBDA-Et and DMH-CBD were synthesized also in the two enantiomeric forms at the University of Eastern Piedmont, Italy, as described in Mattoteia et al. [71].

2.2. CB_1/CB_2 receptor binding affinity

All compounds under investigation in this study were analyzed by competition studies that allow the determination of their affinity (K_i values in a range of concentrations between 10^{-11}-10^{-4} M) for both receptors against the classical cannabinoid ligand [^{3}H]-CP55940 (164.5 Ci/mmol, PerkinElmer, Boston, MA, USA). The competition studies were carried out with commercially available membranes prepared from CB_1 or CB_2 receptor-stably transfected HEK-293 cells (RBHCB1M400UA and RBXCB2M400UA; PerkinElmer Life and Analytical Sciences, Boston, MA, USA) following procedures previously published [72]. Briefly, membranes were added in assay buffer (for CB_1: 50 mM Tris-Cl, 5 mM $MgCl_2.H_2O$, 2.5 mM EDTA, 0.5 mg/ml bovine serum albumin, pH 7.4, or

for CB_2: 50 mM Tris-Cl, 5 mM $MgCl_2.H_2O$, 2.5 mM EGTA, 1 mg/ml bovine serum albumin, pH 7.5) at a final concentration of 8 μg/well and 4 μg/well for CB_1 and for CB_2 receptors, respectively. The radioligand was used at 0.4 nM for CB_1 receptors or 0.53 nM for CB_2 receptors, always in a final volume of 200 μl for both receptors. The membranes were incubated for 90 min at 30 °C with the radioligand and the different concentrations of (−)- and (+)-CBD and derivatives. Non-specific binding was determined with non-radiolabeled WIN55,212-2 (Sigma Aldrich, Madrid, Spain, 10 μM) in the presence of radioligand. 100 % binding of the [^{3}H]-CP55940 was determined by incubation of the membranes with radioligand in the absence of the compound under investigation. All plastic materials employed were siliconized with Sigmacote (Sigma-Aldrich, Madrid, Spain) to prevent possible adhesion of the compounds. After incubation, free radioligand was separated from bound radioligand, by filtration in GF/C filters, previously treated with a 0.05 % (v/v) polyethylenimine solution. Then, filters were washed nine times with cold assay buffer, using the Harvester® filtermate equipment (PerkinElmer, Boston, MA, USA). Radioactivity was measured using a liquid scintillation spectrometer (Microbeta Trilux 1450 LSC & Luminescence Counter (PerkinElmer, Boston, MA, USA)). Data were expressed as percentage of [^{3}H]-CP55940 binding and were analyzed by using GraphPad Prism® version 7 (GraphPad Software Inc., San Diego, CA, USA), for the calculation of K_i values for each receptor. They were expressed as mean ± SEM of at least three experiments performed in triplicate for each point.

2.3. [^{35}S]-GTPγS binding analysis

[^{35}S]-GTPγS binding was analyzed using CB_1 or CB_2 receptor-containing membranes (HTS020M2, Eurofins Discovery Services, St. Charles, MO, USA). To this end, membranes (5 μg/well) were permeabilized by addition of saponin (Sigma-Aldrich, Madrid, Spain), then mixed with 0.3 nM [^{35}S]-GTPγS (PerkinElmer, Boston, MA, USA) and 10 μM GDP (Sigma-Aldrich, Madrid, Spain) in 20 mM HEPES (Sigma-Aldrich, Madrid, Spain) buffer containing 100 mM NaCl (Merck, Madrid, Spain) and 10 mM $MgCl_2$ (Merck, Madrid, Spain), at pH 7.4. Increasing concentrations of the compounds of interest: (+)-CBD, (+)-CBDV, (+)-CBDA-Me, (+)-CBDA-Gly and (+)-CBDA-Hyp (from 10^{-12} to 10^{-5} M) were added in a final volume of 100 μl and incubated for 30 min at 30 °C. The non-specific signal was measured with 10 μM GTPγS (Sigma-Aldrich, Madrid, Spain). All 96-well plates and the tubes necessary for the experiment were previously siliconized with Sigmacote® (Sigma-Aldrich, Madrid, Spain). The reaction was terminated by rapid vacuum filtration with a filter mate Harvester apparatus (PerkinElmer, Boston, MA, USA) through Filtermat A GF/C filters. The filters were washed nine times with ice-cold filtration buffer (10 mM sodium phosphate, pH 7.4), and bound radioactivity was measured with a 1450 LSC & Luminiscence counter Wallac MicroBeta TriLux (PerkinElmer, Boston, MA, USA). [^{35}S]-GTPγS binding data were analyzed to determine the EC_{50} or IC_{50}, as well as the E_{max} values by using an iterative curve-fitting procedure with the GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego, CA, USA). Values are expressed as mean ± SEM of at least three experiments performed in triplicate for each point.

2.4. Cell-based assays for analyzing anti-inflammatory and neuroprotective effects

The anti-inflammatory and neuroprotective properties of some of the (−)- and (+)-enantiomers were investigated in two cell-based assays using cultures of the mouse induced-(or immortalized) microglial (iMG) cell line (generously provided by Dr. Isabel Lastres-Becker, Universidad Autónoma de Madrid, Spain), and of the mouse striatal neuronal cell line M213-2O (generously donated by Dr. WJ Freed, National Institute on Drug Abuse, Bethesda, MD, USA). In the first experiment, cultured iMG cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Verviers, Belgium) supplemented with 10 % fetal bovine serum (FBS, Sigma-Aldrich, Madrid, Spain), 2 mM Ultraglutamine and antibiotics (Lonza, Verviers, Belgium) in a humidified atmosphere of 5 % CO_2 at 37 °C. For experiments, cells were plated at a density of 2.25 x 10^5 cells *per* well in 12-well culture plates and incubated in DMEM with a reduction of FBS to 1 %. Three hours later, cells were treated for 24 h with 100 ng/ml LPS (from Escherichia coli 055:B5, Sigma-Aldrich, Madrid, Spain), alone or in combination with some of the (−)- and (+)-enantiomers, used at three different concentrations: 0.01, 0.1 and 1 μM, and added 1 h before LPS. Twenty-four hours after the addition of LPS, media were removed, whereas cell pellets were collected for analysing mRNA levels of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) by qPCR. In a second experiment, cultured iMG cells were again maintained in DMEM (Lonza, Verviers, Belgium) supplemented with 10 % FBS (Sigma-Aldrich, Madrid, Spain), 2 mM Ultraglutamine and antibiotics (Lonza, Verviers, Belgium) in a humidified atmosphere of 5 % CO_2 at 37 °C. Again, they were plated at a density of 2.25 x 10^5 cells *per* well in 6-well culture plates and incubated in DMEM with a reduction of FBS to 1 %. Three hours later, cells were treated for 24 h with 100 ng/ml LPS (from Escherichia coli 055:B5, Sigma-Aldrich, Madrid, Spain), alone or in combination with (+)-CBD, (+)-CBDV and (+)-CBDA-Me at the concentration of 1 μM (this was the concentration most active in reducing LPS-induced elevation of proinflammatory markers in the previous experiment) added 1 h before LPS. This experiment also included two additional groups for each (+)-enantiomer which consisted in co-treatment with the selective CB_2 receptor antagonist SR144528 (Cayman Chemical Co., Ann Arbor, MI, USA) or the selective PPAR-γ inhibitor T0070907 (Cayman Chemical, Ann Arbor, MI, USA), both at 10 μM, as well as control cells only treated with vehicle (0.1 % DMSO). Twenty-four hours after the addition of LPS, media were removed to be added to cultures of the rat M-213-2O striatal cells to induce cell death following a procedure described previously [72]. To this end, M-213-2O cells were maintained in DMEM supplemented with 10 % FBS, 2 mM Ultraglutamine, and 1 % antibiotics (Lonza, Verviers, Belgium) and under a humidified 5 % CO_2 atmosphere at 37 °C. For cytotoxicity experiments, cells were seeded at 50000 cells/well in 24-well plates and maintained under a humidified atmosphere (5 % CO_2) at 37 °C overnight. Afterwards, normal medium was completely replaced by the different conditioned media generated in iMG cell experiments. Cells were incubated for 40 h before the neuronal death was analyzed with the MTT assay (Panreac AppliChem., Barcelona, Spain). The data were normalized in relation with a control group consisting in M-213-2O cells exposed to those conditioned media generated by iMG cells in absence of LPS.

2.5. Real time qRT-PCR analysis

Cell pellets from the *in vitro* experiments were used for qRT-PCR analysis. Total RNA was isolated using TRI Reagent® (Sigma-Aldrich, Madrid, Spain) following the procedure described by manufacturers. The total amount of RNA extracted was measured by spectrometry at 260 nm and its purity from the ratio between the absorbance values at 260 and 280 nm. After genomic DNA was removed (to eliminate DNA contamination), single-stranded complementary DNA was synthesized from up to 1 μg of total RNA using the commercial kit Rneasy Mini Quantitect Reverse Transcription (Qiagen, Hilgen, Germany). The reaction mixture was kept frozen at −20 °C until enzymatic amplification. Quantitative RT-PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) to quantify mRNA levels for TNF-α (ref. Mm99999064_m1), IL-1β (ref. Mm00434228_m1), iNOS (ref. Mm01309902_m1), COX-2 (ref. Mm00478372_m1), using GAPDH expression (ref. Mm99999915_g1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied

Biosystems, Foster City, CA, USA). Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Calculation of in silico ADME parameters

A set of 34 physicochemical descriptors was computed using QikProp version 3.5 integrated in Maestro (Schrödinger, LLC, New York, USA). The QikProp descriptors are shown in Supplementary Material 1. The 3D conformations used in the calculation of QikProp descriptors were generated using the program Spartan'08 (Wave function, Inc., Irvine, CA, USA) as follows: The structure of each molecule was built from the fragment library available in the program. Then, *ab initio* energy minimizations of each structure at the Hartree−Fock 6-31G* level were performed. A conformational search was next implemented using Molecular Mechanics, followed by a minimization of the energy of each conformer calculated at the Hartree−Fock 6-31G* level. The global minimum energy conformer of each compound was used as input for ADME studies with QikProp.

2.7. Statistical analysis

All statistical analyses were carried out using GraphPad Prism 10.2.0 for Windows (GraphPad Software, San Diego, CA, USA). Values were expressed as mean ± standard error of the mean (SEM). Data in cell-based assays were assessed for multiple comparison using the one-way analysis of variance followed by the Tukey's (data of cell viability) or Dunnett's (data of proinflammatory mediators) test.

3. Results

3.1. Binding affinity of (−)- and (+)-cannabinoids in receptor binding assays

Competition studies using CB_1 or CB_2 receptor-transfected membranes, [^{3}H]-CP55940 as a radioactive probe, and different concentrations of the compounds (always within the interval 10^{-4}-10^{-12} M) under investigation were conducted with the different (−)- and/or (+)-cannabinoids. This served to calculate their K_i values as an index of their CB_1 and CB_2 receptor affinities. In our first analysis, we compared naturally-occurring (−)-CBD (isolated from cannabis plant) and synthetic (−)-CBD for their affinity (K_i) at the CB_1 and CB_2 receptors. In both cases, (−)-CBD showed similar very low affinity values for both receptors ($K_i > 3$ μM) (Fig. 1), with an expected lack of selectivity that was visible even despite the very low affinity values (CB_1/CB_2 K_i ratio = 2.3 for synthetic (−)-CBD and 1.9 for natural (−)-CBD). This occurred in both (−)-CBD forms, then being irrespective of their natural or synthetic origin, with binding curves in Fig. 1 that reflect the characteristic hyperbole profile of those compounds having a small or negligible affinity (K_i in the μM range), which contrasts with the classic sigmoid pattern of those compounds having a high affinity (K_i in the nM range) (see below). Therefore, we can conclude that there is not any significant difference when synthetic and plant-derived (−)-CBD are analyzed for their binding at both CB_1 and CB_2 receptors (Fig. 1). This confirms that chemical synthesis *per se* does not affect the binding profile of (−)-CBD that was previously described using naturally-occurring (−)-CBD [69] and was not the reason for the differences that will be presented below when (−)-enantiomers are compared with (+)-enantiomers.

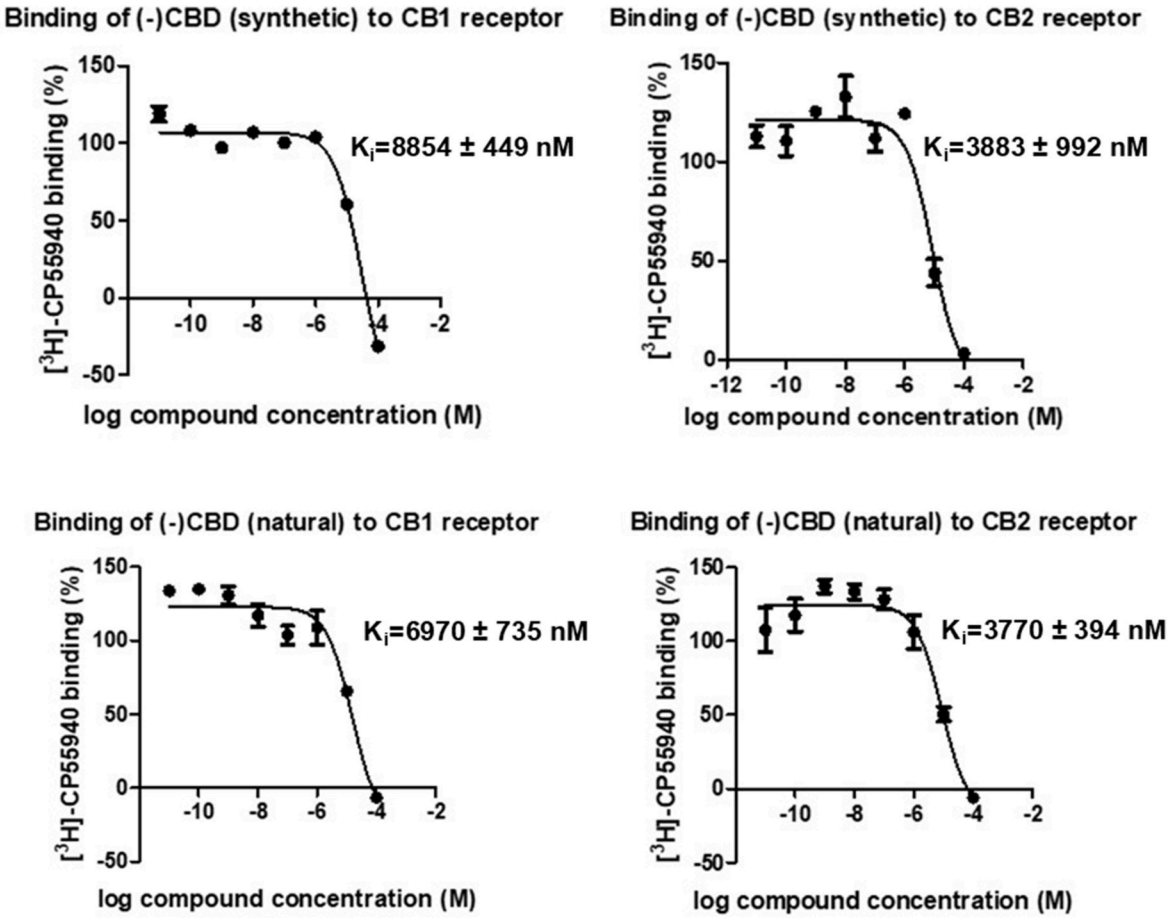


Fig. 1. Representative competition curves for the binding of plant-derived (natural) and synthetic (−)-CBD at the CB_1 and CB_2 receptors. K_i values were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

Next, we compared the two enantiomers for the seven cannabinoid derivatives under investigation in this study, all of them, (−)- and (+)-enantiomers generated always by chemical synthesis. Our data revealed, in general, a greater affinity of (+)- *versus* (−)-enantiomers of these derivatives at the CB_1 and, in particular, CB_2 receptors (Figs. 2–7). Thus, in the case of CBD, the (+)-enantiomer reached an affinity in the nM range (~100 fold higher *versus* (−)-CBD) for the CB_2 receptor, whereas the increase was lower and in the high nM range (~10 fold *versus* (−)-CBD) for the CB_1 receptor (Fig. 2). These different increases in affinity between both receptors affected the CB_1/CB_2 K_i ratio, which informs about receptor selectivity, being 2.3 for the (−)-CBD, but elevated up to 24.3 for (+)-CBD. In the case of CBDV, the (+)-enantiomer also reached an affinity in the nM range (~100 fold higher *versus* (−)-CBDV) for the CB_2 receptor, again with a lower increase, but sufficient to situate the affinity in a medium nM range (~40 fold *versus* (−)-CBD), for the CB_1 receptor (Fig. 3). The CB_1/CB_2 K_i ratio of (−)-CBDV was 3.5, whereas it elevated to 8.9 in the case of (+)-CBDV, again indicating that the (+)-enantiomer was more active at the CB_2 receptor.

CBDA-Me was the exception as both (−) and (+) enantiomer forms showed relatively similar affinities, which are in the nM range (<50 nM) for the CB_2 receptor and in a much more moderate nM range (>300 nM) for the CB_1 receptor (Fig. 4). The CB_1/CB_2 K_i ratios were 12.3 for the (+)-enantiomer and 8.1 for the (−)-enantiomer. As for CBDA-Gly, (−)- and (+)-enantiomers bind the CB_2 receptor, but the affinity of the (+)-enantiomer was again in a low nM range (~30 fold higher *versus* (−)-CBDA-Gly), whereas their affinities for the CB_1 receptor were lower, in particular in the case of the (−)-enantiomer (~10 fold lower *versus* (+)-CBDA-Gly) whose binding ability was almost negligible (K_i in the μM range) (Fig. 5). Again, the (+)-enantiomer displayed a much higher preference for the CB_2 receptor (CB_1/CB_2 K_i ratio = 27.8) compared with the (−)-enantiomer (CB_1/CB_2 K_i ratio = 10.5). CBDA-Hyp resulted in being one of the derivatives having the highest affinities for both receptors, in particular in the case of the (+)-enantiomer (~330 fold and ~80 fold higher *versus* (−)-CBDA-Hyp for the CB_1 and CB_2 receptor, respectively), which showed K_i values in a low nM range (K_i < 3 nM) (Fig. 6). Contrarily to the above compounds, the (−)-enantiomer showed much more preference for the CB_2 receptor (CB_1/CB_2 K_i ratio = 10.9) compared with the (+)-enantiomer (CB_1/CB_2 K_i ratio = 2.6), although in this case the differences turned out to be lower. All (−)- and (+)-CBDA-Hyp data (including similar data to those obtained here for (−)- and (+)-CBD) agree with previous results already published for this CBD derivative in a study addressed to investigate its benefits for the treatment of diabetic nephropathy in mice [73].

Two additional derivatives (CBDA-Et and DMH-CBD) were also analyzed for their affinity at the CB_1 and CB_2 receptors and showed again that the (+)-enantiomers resulted in being more active at these receptors than (−)-enantiomers (Figs. 7 and 8). Thus, (+)-CBDA-Et showed higher affinities (~5 fold higher *versus* (−)-CBDA-Et) for both receptors, with a CB_1/CB_2 K_i ratio also similar for the (−)- and (+)-enantiomers (13.7 and 12.6, respectively) (Fig. 7), strongly indicating their preference for the CB_2 receptor. As regards to (−)- and (+)-enantiomers of DMH-CBD, they were all highly active (K_i < 10 nM) at both receptors, with the (+)-enantiomer having a higher affinity (~20 fold and ~3 fold higher *versus* (−)-DMH-CBD for the CB_1 and CB_2 receptors, respectively) (Fig. 8). Their CB_1/CB_2 K_i ratios were 1.3 for the (−)-enantiomer, indicating no preference, but lower (0.2) for the (+)-enantiomer. This may indicate a certain preference for the CB_2 receptor despite the low K_i values which enable the (+)-enantiomer to be active at both receptors. It is important to remark that the high affinity and potency of DMH-CBD for cannabinoid receptors was already disclosed in the early 90s, showing that the presence of a dimethyl-heptyl side chain, both in Δ^9-

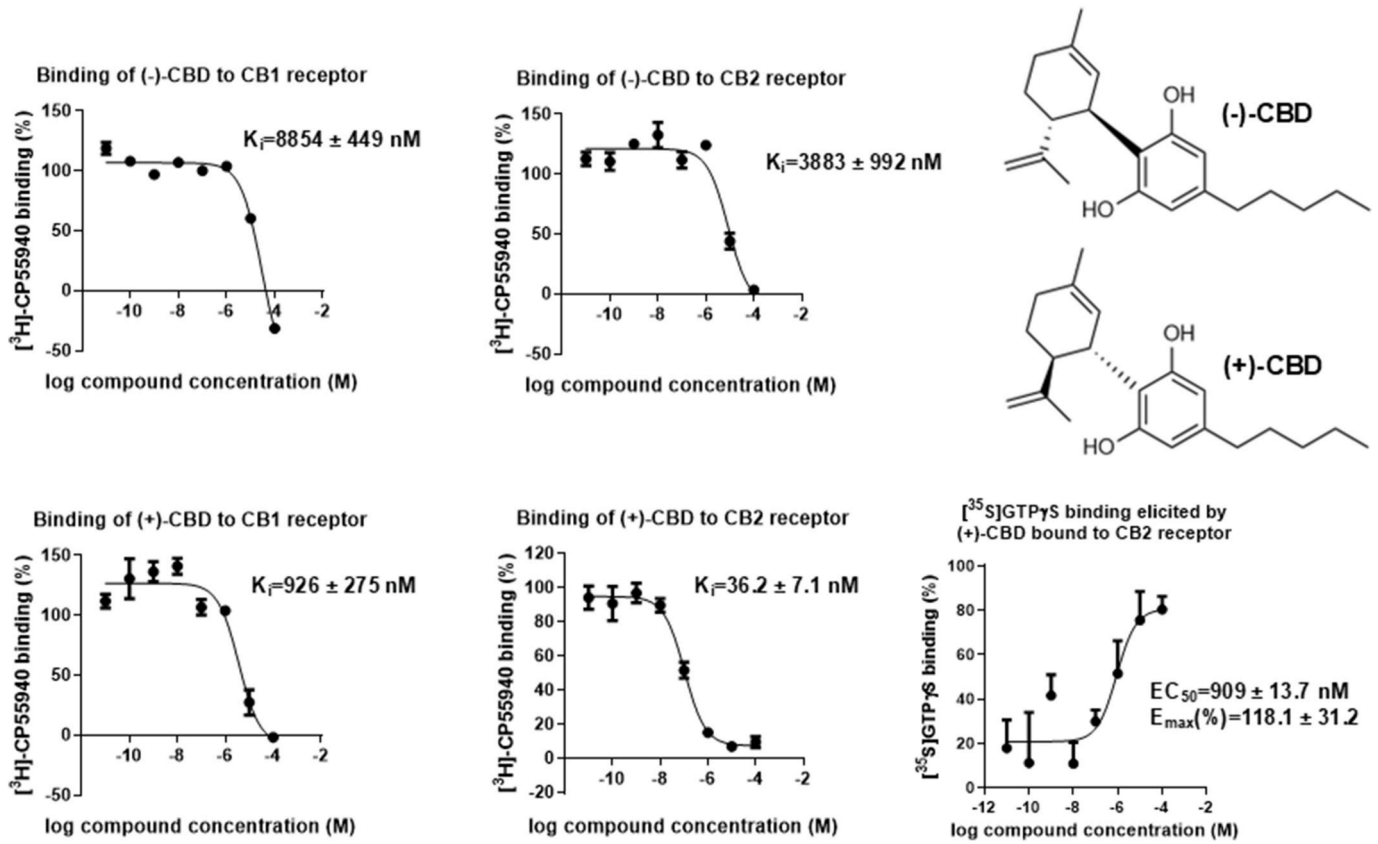


Fig. 2. Representative competition curves for the binding of synthetic (−)- and (+)-CBD at the CB_1 and CB_2 receptors, including the analysis of the intrinsic activity, using the procedure of [^{35}S]GTPγS binding, of those derivatives showing affinity in the low nM range. K_i for receptor binding and EC_{50} and E_{max}(%) values for [^{35}S]GTPγS binding were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

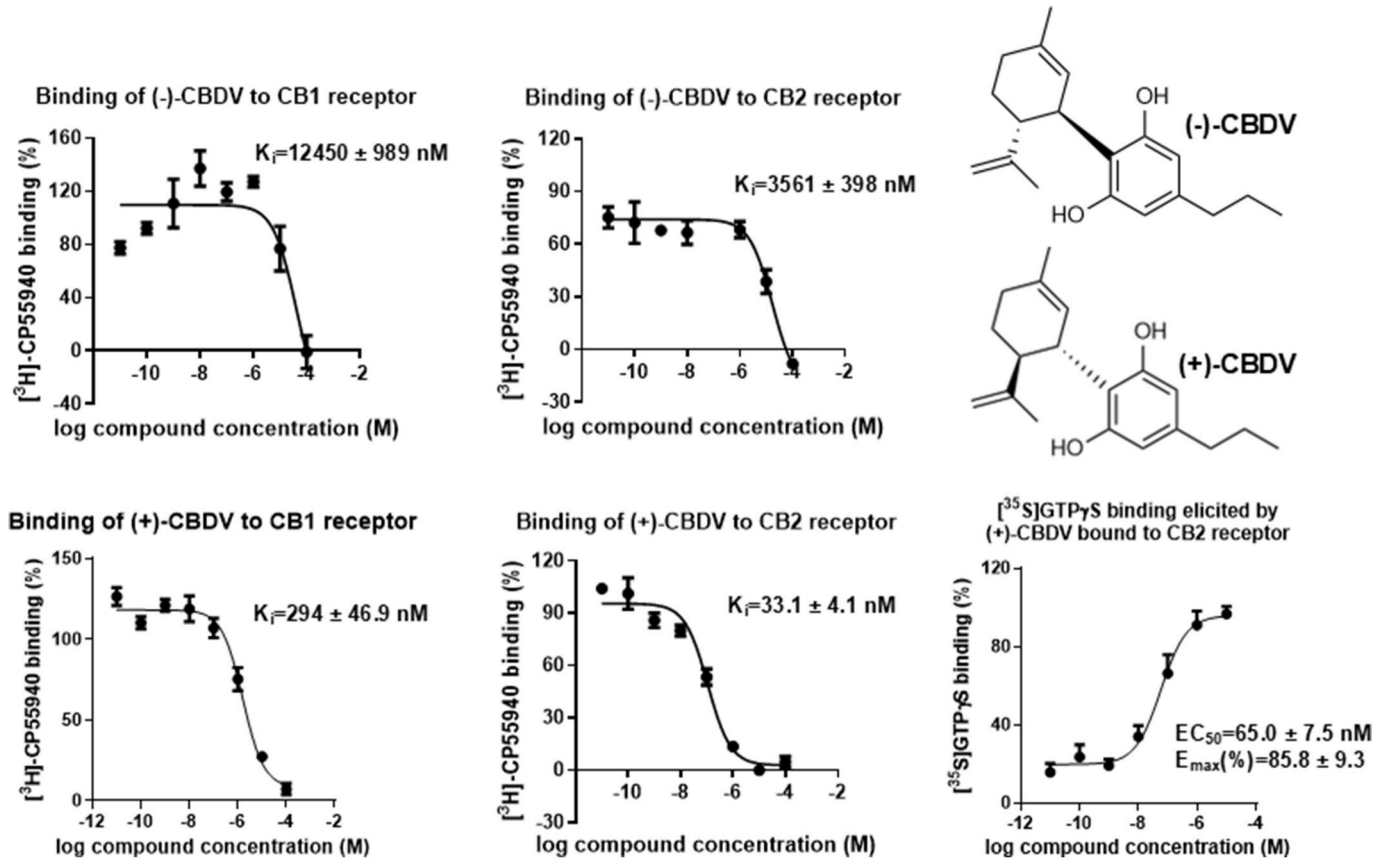


Fig. 3. Representative competition curves for the binding of synthetic (−)- and (+)-CBDV at the CB_1 and CB_2 receptors, including the analysis of the intrinsic activity, using the procedure of [^{35}S]GTPγS binding, of those derivatives showing affinity in the low nM range. K_i for receptor binding and EC_{50} and E_{max}(%) values for [^{35}S]GTPγS binding were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

THC and some analogues, as well as in CBD, enhanced cannabimimetic activity [58–62]. As regards its (−)- and (+)-stereoisomers, our data support their activity at both CB_1 and CB_2 receptors, in contrast to data reviewed in Burstein [2] and Rao et al. [5], who concluded that (−)-DMH-CBD is equally active at both receptors, whereas (+)-DMH-CBD has a lower preference for the CB_2 receptor than for the CB_1 receptor [22,41,74].

3.2. Intrinsic activity of (−)- and (+)-cannabinoids in the [^{35}S]-GTPγS assay

Some of these novel enantiomers, in particular those showing affinities in a relatively low nM range, were furtherly investigated for their intrinsic activity (agonist, antagonist or inverse agonist) using GTPγS binding assays, focusing almost exclusively on the CB_2 receptor, based on the higher preference of all compounds for this receptor (Figs. 2–6, and Table 1). These assays proved that (+)-CBD (Fig. 2), (+)-CBDV (Fig. 3) and (+)-CBDA-Me (Fig. 4) have agonist activity at the CB_2 receptor, with (+)-CBDV having the higher potency (EC_{50} < 100 nM) followed by (+)-CBD (EC_{50} < 1 μM) and (+)-CBDA-Me (EC_{50} < 50 μM) (Figs. 2–4, respectively). On the other hand, (+)-CBDA-Gly was an inverse agonist at this receptor (IC_{50} < 400 nM; Fig. 5), whereas (+)-CBDA-Hyp behaved as agonist at the CB_1 receptor (EC_{50} < 20 nM) but as an inverse agonist at the CB_2 receptor (IC_{50} < 10 nM) (Fig. 6). DMH-CBD was excluded from these analyses as it was already investigated for its intrinsic activity in previous studies [58–62,75], whereas CBDA-Et was not investigated due to compound availability.

3.3. In silico analysis of ADME properties of (+)-CBD and derivatives

Given that previous studies have indicated that different pharmacodynamics of some of these enantiomers could be associated with differences in their pharmacokinetics (e.g., restriction at the brain-blood barrier (BBB) to enter the brain; see Ref. [41,74]), we also wanted to analyze some of the ADME properties of these compounds using *in silico* predictions. This possibility would explain why some (+)-CBD derivatives (including some of the (+)-enantiomers investigated here) bind the CB_1 receptor *in vitro*, but did not reproduce the classical cannabinoid tetrad *in vivo* [74]. This analysis was carried out with five of the (+)-enantiomers (CBD, CBDV, CBDA-Me, CBDA-Gly and CBDA-Hyp) using (−)-CBD as the reference compound. Our data indicated that ADME properties for all compounds, especially for (+)-CBD and (+)-CBDV, were, in general, within the expected ranges (see Supplementary Files 1 and 2). Of particular interest was the case of: (i) QPlogS and CIQPlogS (aqueous solubility) were higher for (+)-CBD and (+)-CBDV than for the three (+)-CBDA derivatives (Supplementary Files 1 and 2); (ii) QPlogHERG (potential cardiotoxicity problems), with some concern for (+)-CBDA-Gly and, in particular, (+)-CBDA-Hyp (Supplementary Files 1 and 2); (iii) QPPCaco, QPlogBB and QPPMDCK (crossing tissue barriers from blood including the BBB), higher for (+)-CBD and (+)-CBDV and lower for (+)-CBDA-Gly (Supplementary Files 1 and 2); and (iv) HumanOralAbsorption (human oral absorption), higher for (+)-CBD and (+)-CBDV, lower for the three (+)-CBDA derivatives. These differences were not found with the equivalent parameter PercentHumanOralAbsorption (Supplementary Files 1 and 2), although this last parameter does not have to correlate with the previous one.

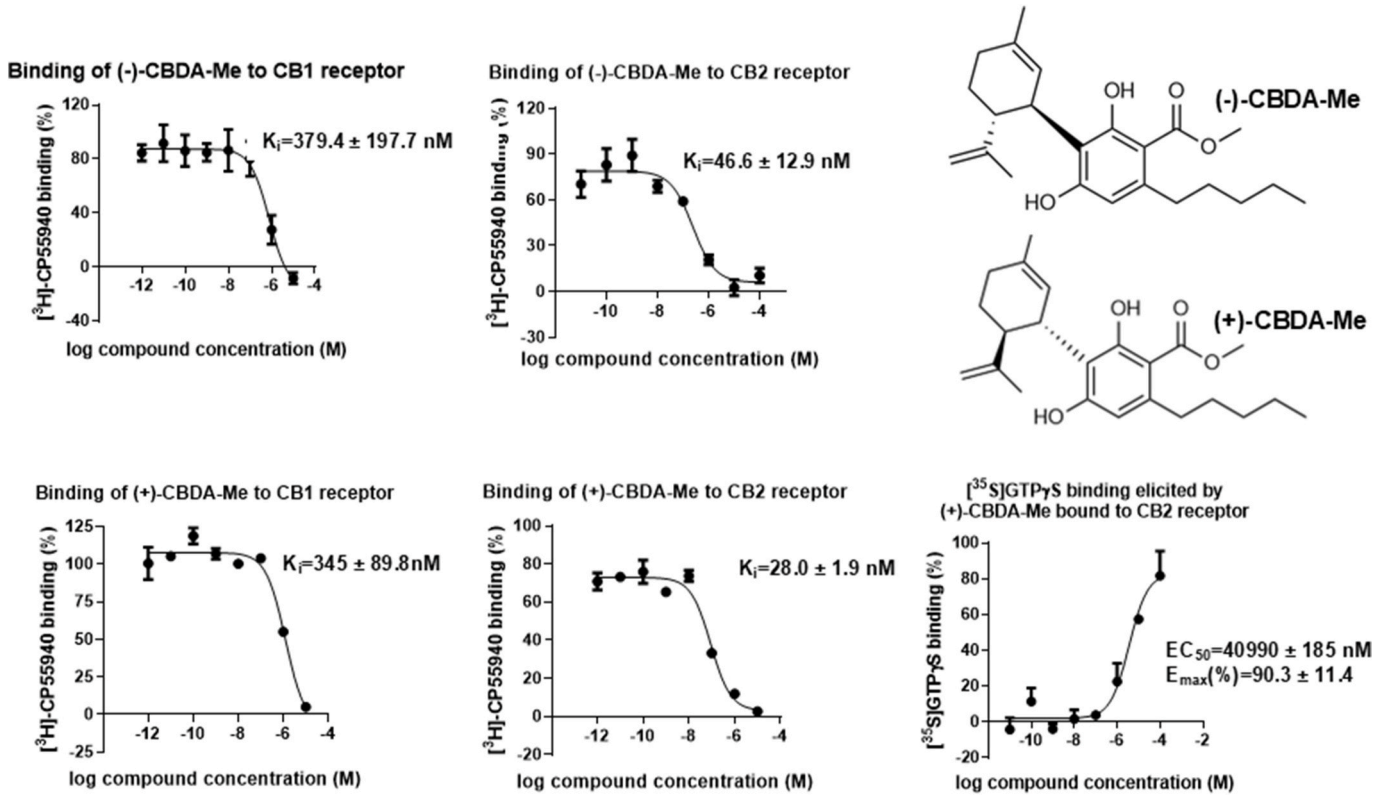


Fig. 4. Representative competition curves for the binding of synthetic (−)- and (+)-CBDA-methyl-ester (CBDA-Me) at the CB_1 and CB_2 receptors, including the analysis of the intrinsic activity, using the procedure of [^{35}S]GTPγS binding, of those derivatives showing affinity in the low nM range. K_i for receptor binding and EC_{50} and E_{max}(%) values for [^{35}S]GTPγS binding were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

3.4. Anti-inflammatory and neuroprotective effects of some (−)- and (+)-enantiomers in cell-based assays

The last experiment in this study was aimed at exploring the ability of (+)-CBD, (+)-CBDV and (+)-CBDA-Me, the three (+)-enantiomers that showed an agonist activity at the CB_2 receptor in [^{35}S]-GTPγS binding assays, to exert anti-inflammatory and neuroprotective properties in some cell-based assays useful for this purpose. Such capability should be an expected consequence of their acquired or improved affinity and agonist activity for this receptor. To this end, we analyzed first the effect of three different concentrations of these three (+)-enantiomers against LPS-induced elevation of gene expression of some relevant proinflammatory markers, IL-1β, TNF-α, COX-2 and iNOS, in cultured iMG cells. Our results confirmed that the addition of LPS to these cells elevated the expression of the four markers analyzed (Fig. 9), whereas the co-incubation with (+)-CBD, (+)-CBDV or (+)-CBDA-Me was able to decrease (or tended to reduce) this elevated expression for all markers (except for (+)-CBDA-Me), with effects more evident for IL-1β ($F(10,37) = 2.15$, $p < 0.05$; Fig. 9), but apparently lower for TNF-α ($F(10,39) = 6.94$, $p < 0.0001$; Fig. 9) and, to a lower extent, COX-2 ($F(10,37) = 2.61$, $p < 0.05$; Fig. 9) and iNOS ($F(10,39) = 1.05$, ns; Fig. 9). In all cases, the highest concentration of 1 μM was the most active and the only being statistically significant with respect to the LPS-treated cells (Fig. 9), with the lower concentrations being inactive or remaining as mere numerical trends (Fig. 9). This is the case of the 0.1 μM which showed values of probability levels respect to the LPS-treated cells close to statistical significance for IL-1β in the case of (+)-CBDA-Me ($p = 0.09$), (+)-CBDV ($p = 0.11$) and (+)-CBD ($p = 0.18$).

In a second experiment, the aim was to demonstrate that the changes elicited by LPS (marked elevation) and by the three (+)-enantiomers (reduction or trends towards a decrease) on the gene expression of these proinflammatory markers in cultured cells would be reflected in the release of these proinflammatory cytokines or other factors in their cultured media as has been described in previous studies [72], so that their addition to cultured neurons may compromise the cell viability. To this end, we generated conditioned media from iMG cells treated with vehicle or LPS in absence or presence of the most active concentration for each (+)-enantiomer in the previous experiment (1 μM), which were added to M-213-2O cells (these cells do not express CB_2 receptors [72]) to induce cell death. Our data confirmed that conditioned media from LPS-exposed iMG cells reduced the cell viability of M-213-2O cells by approximately 40 %, whereas (+)-CBD, (+)-CBDV and (+)-CBDA-Me ($F(10,43) = 9.52$, $p < 0.0001$; Fig. 10) partially reversed the reduction in cell viability (loss of statistical significance respect LPS-treated cells), in particular in the case of (+)-CBDV and (+)-CBDA-Me (Fig. 10). In this experiment, two additional experimental groups of cells were included with the purpose to confirm that the beneficial effects elicited by (+)-enantiomers against LPS-induced cell death were dependent on the activation of CB_2 receptors by the (+)-enantiomer, as well as to discard the contribution of other related receptors (e.g., PPAR-γ). Our data demonstrated that the conditioned media generated by the co-incubation of the (+)-enantiomer and the CB_2 receptor antagonist SR144528 in LPS-exposed iMG cells eliminated the beneficial effect of the (+)-enantiomer alone in the three cases, whereas this did not happen when the co-incubation was with T0070907, an inhibitor of the PPAR-γ (Fig. 10). In fact, in the case of (+)-CBD, but not in the case of the other two (+)-enantiomers, the presence of T0070907 apparently improved the beneficial effect of (+)-CBD on cell viability (Fig. 10). A clarification of the rationale for this improvement will require further research, but *a priori* this could indicate that the activity of (+)-CBD at the CB_2 receptor may be more intense when the enantiomer has a restriction in its capability to act at the PPAR-γ due to the blockade of this receptor. However, the data showed in previous studies using T0070907 or similar covalent antagonists of PPAR-γ, which demonstrated that they

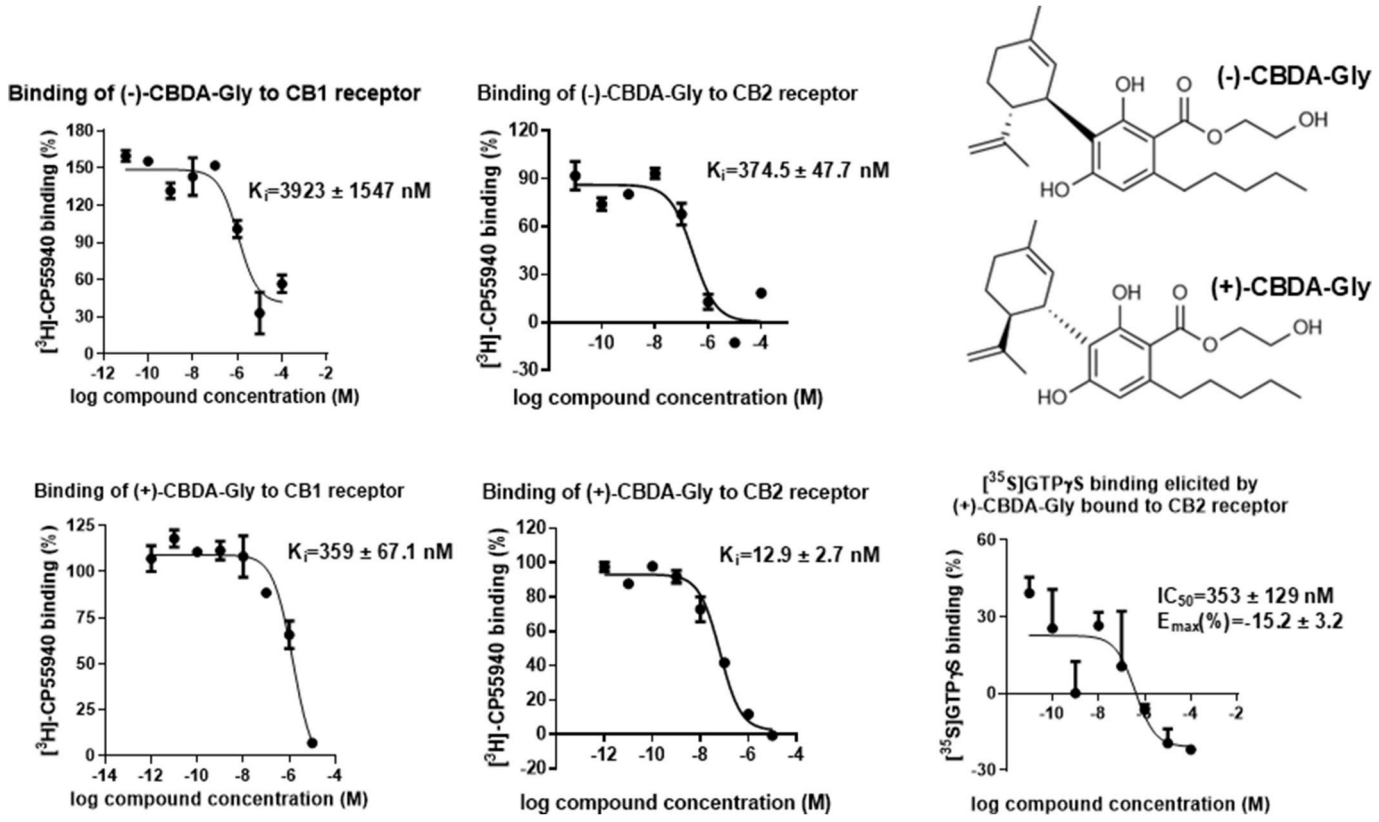


Fig. 5. Representative competition curves for the binding of synthetic (−)- and (+)-CBDA-glycol-ester (CBDA-Gly) at the CB_1 and CB_2 receptors, including the analysis of the intrinsic activity, using the procedure of [^{35}S]GTPγS binding, of those derivatives showing affinity in the low nM range. K_i for receptor binding and IC_{50} and E_{max}(%) values for [^{35}S]GTPγS binding were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

exert only a partial blockade of the orthosteric binding site allowing that certain ligands may still activate PPAR-γ signaling through their binding to so-called omega site in the ligand binding domain [76–78], provide additional explanations based on the activity of (+)-CBD at this omega pocket. Such possibility was also claimed in our previous study carried out with a cannabigerol derivative, so-called VCE-003.2, in an experimental model of Parkinson's disease [79].

The last experiment in this part of the study was aimed at investigating whether (−)-CBD and (−)-CBDV, which, according to our competition studies for the CB_2 receptor are unable to bind and activate this receptor, may, nevertheless, to be also active in reducing the LPS-induced elevation of proinflammatory factors in iMG cells, as their (+)-enantiomers, which are able to bind and activate the CB_2 receptor, did. If this were the case, the mechanism(s) for these effects should be other than the activation of the CB_2 receptor (e.g., activity at the PPAR-γ or other targets), or would imply a possible *in vivo* biotransformation of the (−)-enantiomer into the (+)-isomer. Our data open these two possibilities or others, as we recorded mostly similar patterns in the effects of (−)-enantiomers compared to (+)-enantiomers, in particular for IL-1β ($F(7,31) = 3.36$, $p < 0.01$; Supplementary File 3), COX-2 ($F(7,31) = 9.73$, $p < 0.0001$; Supplementary File 3), iNOS ($F(7,31) = 15.61$, $p < 0.0001$ Supplementary File 3) and, to a lower extent (it happened only for (−)-CBDV), TNF-α ($F(7,31) = 10.78$, $p < 0.0001$; Supplementary File 3), in all cases with the higher concentration (1 μM) being the most active, but also having statistically significant effects (or numerical trends) with the 0.1 μM concentration in some cases (e.g., (−)-CBD and (−)-CBDV for iNOS).

4. Discussion

The aim of this study was to compare the CB_1/CB_2 receptor binding profile and/or intrinsic activity of several synthetic (−)- and (+)-cannabinoids, including CBD, CBDV and some CBDA derivatives, with the aim of demonstrating the existence of important differences between (−)- and (+)-enantiomeric forms of these cannabinoids. However, before addressing this major objective, we wanted to discard the hypothesis that any enantiomer-dependent difference (see below) may be the result of the natural or synthetic origin of the different isomers. The rationale for addressing this question is that most binding data for CBD have been obtained with (−)-CBD from cannabis [58–62] and one recent study claimed that using (−)-CBD generated by chemical synthesis could result in different pharmacodynamics parameters [69]. However, our data confirmed that, independently from the origin: (i) (−)-CBD has a poor affinity at the classic cannabinoid receptors, with K_i values for both CB_1 and CB_2 receptors within the low μM range; (ii) the binding of (−)-CBD to CB_2 receptors was certainly higher (approximately 2-fold) than to CB_1 receptors, although always within the μM range; and (iii) the receptor binding affinity of (−)-CBD was always similar comparing the synthetic (−)-CBD with the plant-derived compound. This result is in agreement with a previous report showing that CBD is a partial agonist for CB_2 receptor at high concentrations [80]. In general, our results confirm previous data showing that (−)-CBD, irrespective of its origin, is a low-affinity ligand for the cannabinoid receptors [81], yet capable to modulate certain intracellular signals that are dependent on the activation of these receptors [82]. Thus, our results may also confirm previous observations on the possible activity of (−)-CBD as a negative allosteric modulator at the CB_1 receptor [69,83]. They also confirm its proposed behavior at the CB_2 receptor, in which claims for an agonist activity have been made in specific experimental conditions [84], despite other studies have also claimed for a similar antagonist/negative allosteric modulator activity [38] that has been proposed for the CB_1 receptor.

Having discarded the possible influence of the natural or synthetic origin of the different compounds under investigation in their receptor

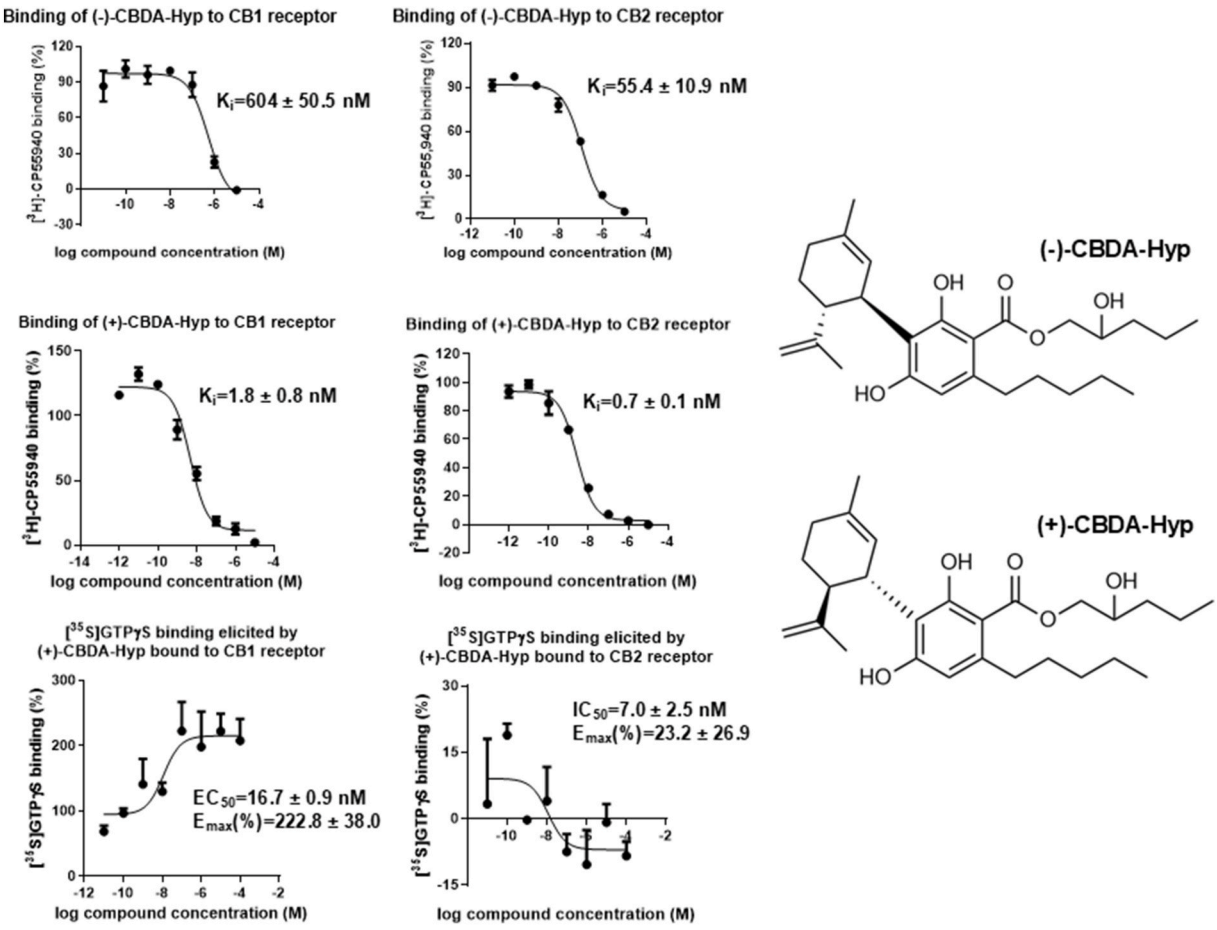


Fig. 6. Representative competition curves for the binding of synthetic (−)- and (+)-CBDA-hydroxypenthyl-ester (CBDA-Hyp) at the CB_1 and CB_2 receptors, including the analysis of the intrinsic activity, using the procedure of [^{35}S]GTPγS binding, of those derivatives showing affinity in the low nM range. K_i for receptor binding and EC_{50} (or IC_{50}) and E_{max}(%) values for [^{35}S]GTPγS binding were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

binding profile, we addressed our major objective, namely evaluating if (−)- and (+)-enantiomeric forms of various chiral cannabinoids showed differences in their receptor affinity for CB_1 and/or CB_2 receptors, as well as in their intrinsic activity at these receptors, following previous results that have been reviewed by Burstein [2] and Rao et al. [5]. Our data fully confirmed this hypothesis, with (+)-enantiomers always showing higher affinities than (−)-enantiomers for both receptors, in particular for the CB_2 receptor, except in the case of CBDA-Me. Our quantification of receptor affinities (K_i) of (+)-enantiomers for the CB_1 receptor yielded to the following order: DMH-CBD = CBDA-Hyp > CBDA-Et > CBDV = CBDA-Me = CBDA-Gly > CBD, with only DMH-CBD and CBDA-Hyp having a K_i in a low nM range. In the case of the CB_2 receptor, the order was CBDA-Hyp > DMH-CBD > CBDA-Et > CBDA-Gly > CBDA-Me = CBDV = CBD, all of them with K_i < 50 nM. These data, however, present differences from those published by Bosquez-Berger et al. [85] who reported that effectively (+)-CBD was more potent than (−)-CBD for some responses related to CB_1 receptor activity (e.g., ~5 fold higher affinity (lower K_i) at the CB_1 receptor; ~10 fold greater potency at inhibiting depolarization-induced suppression of excitation), but lower for others (e.g., CB_1 receptor-mediated suppression of cAMP accumulation). These authors also demonstrated that (+)-CBD, but not (−)-CBD, was active at some non-cannabinoid receptors as sphingosine-1-phosphate receptors [85]. In addition, Golliher and co-workers also described higher affinity to CB_1/CB_2 receptors for (+)-CBD than the natural enantiomer [57], and the same happened with some CBD derivatives as 8,9-dihydro-7-hydroxy-CBD, with the (+)-enantiomer having K_i in the nM range, but the (−)-enantiomer showing very low affinity [86]. A former study also compared several CBD derivatives in both enantiomeric forms and found higher activity of (+)-stereoisomers for the CB_1 receptor in the low nM range, whereas the differences for the CB_2 receptor binding were smaller [41]. These data showed the opposite that we found in our study, although the only common compound with our study was DMH-CBD [41].

In our study, some (+)-enantiomers, selected because their affinities were in the low nM range and also because of their availability and the absence of previous data, were also investigated for their intrinsic activity using GTPγS binding assays. These assays proved that (+)-CBD, (+)-CBDV and (+)-CBDA-Me are agonists at the CB_2 receptor and (+)-CBDA-Gly was an inverse agonist, whereas (+)-CBDA-Hyp behaved as agonist at the CB_1 receptor but as an inverse agonist at the CB_2 receptor. However, it is important to highlight that these intrinsic activities were found in agonist-stimulated GTPγS binding assays, but, in previous studies carried out using a cell-based assay to measure cAMP-mediated signaling, receptor activity for the (+)-CBDA-Hyp proved opposite behaviors with the compound acting as an antagonist at the CB_1 receptor and as an agonist for the CB_2 receptor ([73]; reviewed in Ref. [5]). These differences appear to be related to certain limitations of the agonist-stimulated GTPγS binding assay with respect to similar cell-based assays, which may be based on whether compounds bind orthosteric and/or allosteric sites, the subtype of G protein involved, the possibility of heteromer formation which modifies individual receptor activity, and even possible signaling crosstalks. Further experiments will be necessary to clarify this controversy. Irrespective of this, the fact that some of the (+)-enantiomers investigated in our study (CBD, CBDV and

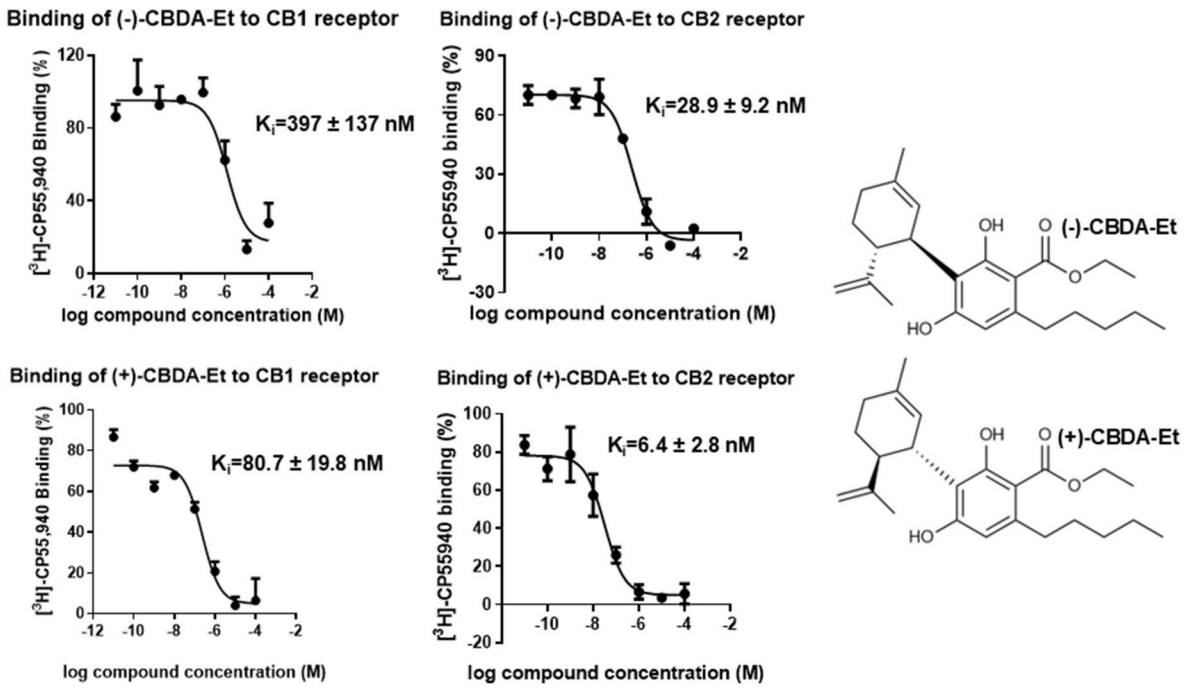


Fig. 7. Representative competition curves for the binding of synthetic (−)- and (+)-CBDA-ethyl-ester (CBDA-Et) at the CB_1 and CB_2 receptors. K_i values were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

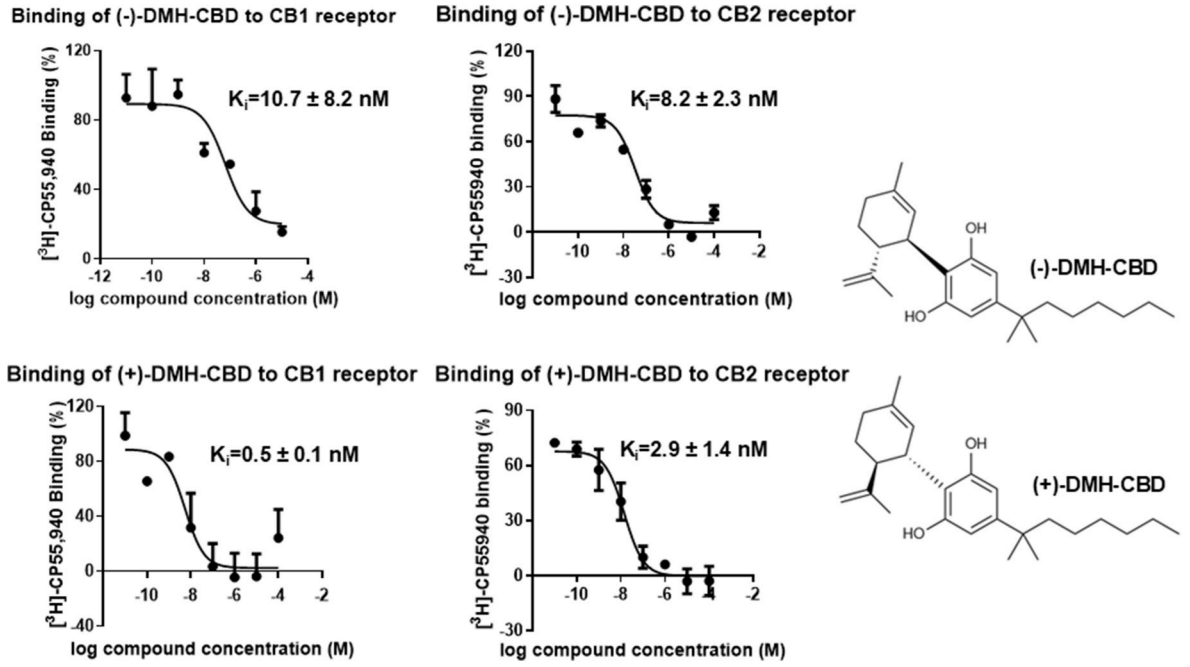


Fig. 8. Representative competition curves for the binding of synthetic (−)- and (+)-dimethyl heptyl-CBD (DMH-CBD) at the CB_1 and CB_2 receptors. K_i values were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

CBDA-Me) are active as agonists of the CB_2 receptors agree with the idea, recently reviewed in Rao et al. [5], that (+)-CBD isomeric derivatives show, in general, a good potential for the treatment of neuroinflammatory disorders. In fact, we investigated here this anti-inflammatory and neuroprotective profile using adequate cell-based assays already used in previous studies [72]. Our data strongly indicated that (+)-CBD, (+)-CBDV and (+)-CBDA-Me were capable of attenuating the effect of LPS exposure in cultured iMG cells on the expression of several proinflammatory cytokines (IL-1β and TNF-α) and enzymes (COX-2 and iNOS), likely reducing the proinflammatory scenario elicited by this inflammatory toxin. As previous studies have demonstrated [72], the use of conditioned media containing several factors generated in this proinflammatory scenario may result toxic for cultured neurons when added in their incubation media. We used this strategy to confirm: (i) the reduced neuronal viability observed with conditioned media derived from iMG cells incubated with LPS; (ii) the reversion of these neurotoxic effects with conditioned media derived from LPS-exposed iMG cells that were treated with the most active

Table 1
Summary of data for the affinity and intrinsic activity at the CB_1 and CB_2 receptors of (−)- and (+)-enantiomers of CBD, CBDV and some derivatives (see details in Figs. 1–8).

Compound	CB_1 receptor		CB_2 receptor	
	Affinity	Intrinsic activity	Affinity	Intrinsic activity
(−)-CBD	Negligible (K_i > 3 μM)	not measured[a]	Negligible (K_i > 3 μM)	not measured[a]
(+)-CBD	Low (K_i > 500 nM)	not measured[a]	High (K_i < 40 nM)	agonist
(−)-CBDV	Negligible (K_i > 3 μM)	not measured[a]	Negligible (K_i > 3 μM)	not measured[a]
(+)-CBDV	Moderate (K_i > 200 nM)	not measured[a]	High (K_i < 40 nM)	agonist
(−)-CBDA-Me	Moderate (K_i > 200 nM)	not measured[a]	Moderate to high (K_i > 40 nM)	not measured[a]
(+)-CBDA-Me	Moderate (K_i > 200 nM)	not measured[a]	High (K_i < 40 nM)	agonist
(−)-CBDA-Gly	Negligible (K_i > 3 μM)	not measured[a]	Moderate (K_i > 200 nM)	not measured[a]
(+)-CBDA-Gly	Moderate (K_i > 200 nM)	not measured[a]	High (K_i < 40 nM)	antagonist
(−)-CBDA-Hyp	Low (K_i > 500 nM)	not measured[a]	Moderate to high (K_i > 40 nM)	not measured[a]
(+)-CBDA-Hyp	Very high (K_i < 5 nM)	agonist[b]	Very high (K_i < 5 nM)	antagonist[b]
(−)-CBDA-Et	Moderate (K_i > 200 nM)	not measured[a]	High (K_i < 40 nM)	not measured
(+)-CBDA-Et	Moderate to high (K_i > 40 nM)	not measured[a]	High (K_i < 40 nM)	not measured
(−)-DMH-CBD	High (K_i < 40 nM)	not measured[c]	High (K_i < 40 nM)	not measured[c]
(+)-DMH-CBD	Very high (K_i < 5 nM)	not measured[c]	Very high (K_i < 5 nM)	not measured[c]

[a] not measured due to the absence of affinity in the low nM range.
[b] opposite results using cell assays instead [^{35}S]GTPγS binding [73].
[c] already described as agonists (see references in the text).

concentration of these (+)-enantiomers; and (iii) the contribution of CB_2 receptors, but not PPAR-γ receptors in the effects of (+)-enantiomers, which agree with the binding profile found for these compounds at the CB_2 receptor compared with their (−)-enantiomers. In addition, in a preliminary experiment addressed to explore an additional objective, we also demonstrated that (−)-CBD and (−)-CBDV mostly mimicked the effects of their (+)-enantiomers despite the (−)-stereoisomers do not bind and activate the CB_2 receptor. Obviously, this observation will require further research to be explained following two major lines of hypothesis: (i) that the molecular mechanism(s) for these effects should be other than the activation of the CB_2 receptor (e.g., activity at the PPAR-γ or other targets); and (ii) the possibility of an *in vivo* biotransformation of the (−)-enantiomer into the (+)-isomer by the activity of cellular racemases that may transform one enantiomer in the other and *vice versa*, something that will represent the follow-up studies generated by the experiments included in the present study. In the case that this racemic transformation may be confirmed, an additional question of high interest would be the relevance that this biotransformation may have in those pathologies in which CBD has been found to be beneficial. An important case for us is Parkinson's disease, in which we and other groups have described numerous neuroprotective effects of CBD in experimental models of this disease ([18,19,87,88]; reviewed in Ref. [89]), which have been frequently assigned to cannabinoid receptor-independent effects of CBD, frequently because the assumption of lack of affinity of (−)-CBD for the two classic cannabinoid receptors (reviewed in Ref. [1]). The present demonstration that (+)-enantiomers of CBD and derivatives are, however, able to more efficiently bind and eventually activate these receptors opens the possibility of alternative mechanisms of action derived from a possible *in vivo* biotransformation of these derivatives, something that will require to be investigated in such follow-up studies.

Finally, we also carried out some *in silico* analyses to predict possible ADME properties of the different (+)-enantiomers (CBD, CBDV, CBDA-Me, CBDA-Gly and CBDA-Hyp) using (−)-CBD as the reference compound. The rationale for this analysis was based on the fact that previous studies have remarked that the differences in pharmacodynamics of (−)- and (+)-enantiomers of CBD and derivatives could be associated with differences in their pharmacokinetics, in particular with a restriction of (+)-enantiomers to enter the brain at the BBB [74]. This fact led authors to propose (+)-CBD and derivatives (e.g., (+)-DMH-CBD) as useful peripherally-restricted CB_1 receptor agonists active against gastrointestinal disorders or peripheral pain and inflammation [74]. Therefore, our *in silico* analysis of ADME properties of (+)-enantiomers was aimed at further exploring this question. Our data indicated positive predicted ADME properties for all compounds, especially for (+)-CBD and (+)-CBDV, including some parameters (QPPCaco, QPlogBB and QPPMDCK), which allow the prediction of the ability of these compounds to cross tissue barriers from blood (including the BBB). The predictions indicated that (+)-CBD and (+)-CBDV may be apparently able to cross these barriers with values similar to (−)-CBD, which were lower for the remaining (+)-CBD derivatives, in particular for the (+)-CBDA-Gly which showed values outside the normal range. These predictions would oppose the idea of (+)-CBD derivatives as peripherally-restricted agonists [74], although they would need to be confirmed in follow-up *in vitro* and *in vivo* studies. In fact, preliminary *in vivo* experiments that are being currently in progress in our laboratory with (+)-CBD and (+)-CBDV in an experimental model of Parkinson's disease appear to indicate benefits against the motor impairment of these compounds which would be presumably caused by their activity at the brain (Rodríguez-Carreiro, unpublished data).

Taken together, the results of our investigation of the CB_1 and CB_2 receptor binding profile and intrinsic activity of CBD and its derivatives show marked differences in the biological profile of (−)- and (+)-enantiomeric forms (higher affinity). A direct consequence of this is the acquired (or enhanced) ability of (+)-enantiomers to act through these receptors, in particular through the CB_2 receptor, a fact that may help to elucidate the mechanisms of action that underlie in some pharmacological effects of these compounds as their anti-inflammatory and neuroprotective effects that have been described here. However, the fact that (−)-CBD and (−)-CBDV mostly mimicked the *in vitro* anti-inflammatory effects found for their (+)-enantiomers, despite the (−)-stereoisomers not binding to and activating the CB_2 receptor, opens the possibility that the activity of these compounds at these receptors in the two enantiomer forms may be influenced by a possible bidirectional biotransformation between these enantiomers in the body, something that will require further research as a highly relevant objective that may impact our understanding on the therapeutic use of these compounds.

CRediT authorship contribution statement

Santiago Rodríguez-Carreiro: Writing – review & editing, Investigation, Formal analysis, Data curation. **María Gómez-Cañas:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Francesca Lubrini:** Writing – review & editing, Formal analysis, Data curation. **Claudia Gonzalo-Consuegra:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Matthias Winkler:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Diego Caprioglio:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Giovanni Appendino:** Writing – review & editing, Supervision, Conceptualization. **Concepción García:** Writing – review & editing, Supervision. **Paula Morales:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Nadine Jagerovic:** Writing – review & editing, Supervision, Conceptualization. **Joerg T. Fischer:** Writing – review & editing,

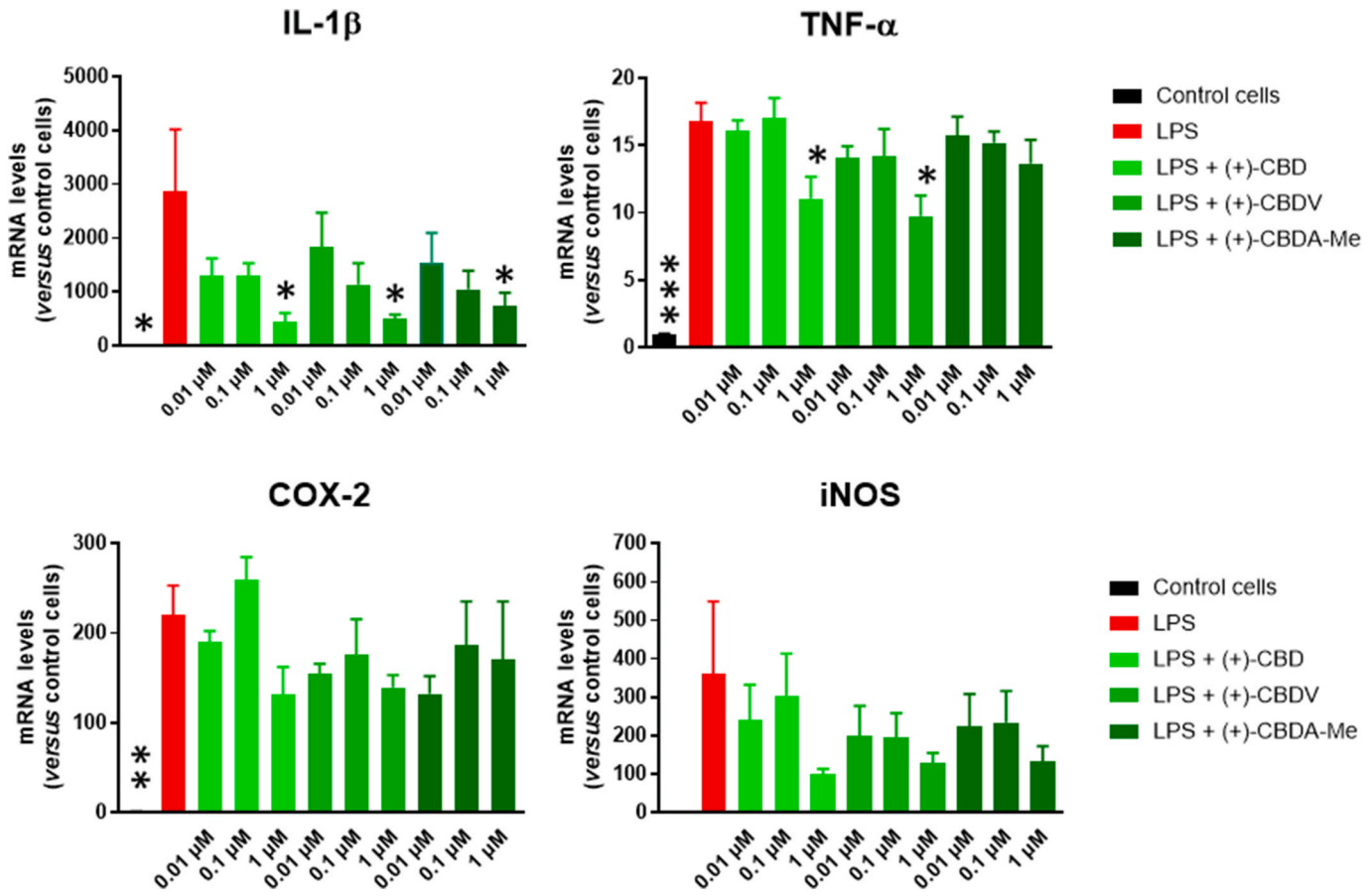


Fig. 9. mRNA levels for IL-1β, TNF-α, COX-2 and iNOS measured by qPCR in iMG cells after the exposure to LPS (100 ng/ml) in absence or presence of three different concentrations of (+)-CBD, (+)-CBDV or (+)-CBDA-Me. Results are means ± SEM of three different experiments carried out in triplicates. Data were assessed by one-way analysis of variance followed by the Dunnett's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ *vs* LPS-treated cells).

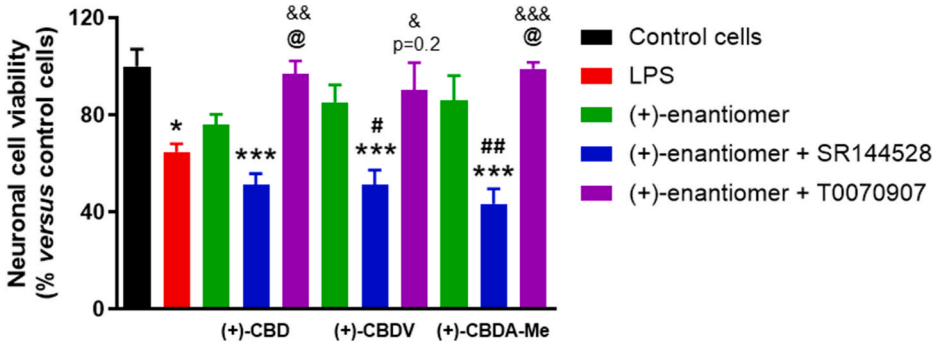


Fig. 10. Cell viability measured by the MTT assay in M-213-2O cells exposed to conditioned media generated from iMG cells after the exposure to LPS (100 ng/ml) in absence or presence of (+)-CBD, (+)-CBDV or (+)-CBDA-Me at the concentration of 1 μM and/or SR144528 (10 μM) or T0070907 (10 μM). Results are means ± SEM of three different experiments carried out in triplicates. Data were assessed by one-way analysis of variance followed by the Tukey's test (*$p < 0.05$, ***$p < 0.005$ *vs* control cells; $^{@}p < 0.05$ *vs* LPS-treated cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* LPS + cannabinoid-treated cells; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.005$ *vs* LPS + cannabinoid + SR144528-treated cells).

Investigation, Conceptualization. **Bernd L. Fiebich:** Writing – review & editing, Investigation, Conceptualization. **Marcus R. Goetz:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Eduardo Muñoz:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Javier Fernández-Ruiz:** Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Data availability statement

The datasets for this manuscript are not publicly available because of the tenure of intellectual property by Symrise AG. Requests to access the datasets should be directed to the corresponding author.

Funding

Authors want to thank the support by grants from MCINN "Programa

Nacional de Biomedicina" (PID2021-128906OB-I00 to JF-R) with co-funding from the European Regional Development Funds (FEDER), "Instituto de Salud Carlos III" (CIBERNED, CB06/05/0089), Symrise AG, Holzminden, Germany, and VivaCell Biotechnology-Spain. These agencies had no further role in study design, the collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: SR-C, MG-C, FL, CG-C, DC, GA, CG, PM, NJ and JF-R declare that they have no conflicts of interest, whereas MW, JTF, BLF, MRG and EM were employees of Symrise AG or VivaCell Biotechnology at the time of study development.

Acknowledgments

SR-C and CG-C were predoctoral fellows supported by the FPU Programme, Ministry of University, Madrid (Spain) and Complutense University, Madrid (Spain). Authors are indebted to Yolanda García-Movellán for administrative assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmcr.2025.100262.

Data availability

Data will be made available on request.

References

[1] J. Fernández-Ruiz, O. Sagredo, M.R. Pazos, C. García, R. Pertwee, R. Mechoulam, J. Martínez-Orgado, Cannabidiol for neurodegenerative disorders: important new clinical applications for this phytocannabinoid? Br. J. Clin. Pharmacol. 75 (2013) 323–333.
[2] S. Burstein, Cannabidiol (CBD) and its analogs: a review of their effects on inflammation, Bioorg. Med. Chem. 23 (2015) 1377–1385.
[3] M. Talebi, M.M. Sadoughi, S.A. Ayatollahi, E. Ainy, R. Kiani, A. Zali, M. Miri, Therapeutic potentials of cannabidiol: focus on the Nrf2 signaling pathway, Biomed. Pharmacother. 168 (2023) 115805.
[4] F.S. Guimarães, Historical perspective on the therapeutic potential of cannabidiol, Int. Rev. Neurobiol. 177 (2024) 1–9.
[5] V.K. Rao, M.M. Lewis-Bakker, E. Wasilewski, H.A. Clarke, L.P. Kotra, Stereoisomers of cannabidiols and their pharmacological activities - a potentially novel direction for cannabinoids, Bioorg. Med. Chem. 117 (2025) 118019.
[6] S. Zaheer, D. Kumar, M.T. Khan, P.R. Giyanwani, F. Kiran, Epilepsy and cannabis: a literature review, Cureus 10 (2018) e3278.
[7] S. Lattanzi, E. Trinka, P. Striano, C. Rocchi, S. Salvemini, M. Silvestrini, F. Brigo, Highly purified cannabidiol for epilepsy treatment: a systematic review of epileptic conditions beyond Dravet syndrome and Lennox-Gastaut syndrome, CNS Drugs 35 (2021) 265–281.
[8] L.A. Parker, E.M. Rock, C.L. Limebeer, Regulation of nausea and vomiting by cannabinoids, Br. J. Pharmacol. 163 (2011) 1411–1422.
[9] T.L. Mortimer, T. Mabin, A.M. Engelbrecht, Cannabinoids: the lows and the highs of chemotherapy-induced nausea and vomiting, Future Oncol. 15 (2019) 1035–1049.
[10] A.R. de Mello Schier, N.P. de Oliveira Ribeiro, D.S. Coutinho, S. Machado, O. Arias-Carrión, J.A. Crippa, A.W. Zuardi, A.E. Nardi, A.C. Silva, Antidepressant-like and anxiolytic-like effects of cannabidiol: a chemical compound of Cannabis sativa, CNS Neurol. Disord.: Drug Targets 13 (2014) 953–960.
[11] K. Han, J.Y. Wang, P.Y. Wang, Y.C. Peng, Therapeutic potential of cannabidiol (CBD) in anxiety disorders: a systematic review and meta-analysis, Psychiatry Res. 339 (2024) 116049.
[12] A.W. Zuardi, J.A. Crippa, J.E. Hallak, S. Bhattacharyya, Z. Atakan, R. Martin-Santos, P.K. McGuire, F.S. Guimarães, A critical review of the antipsychotic effects of cannabidiol: 30 years of a translational investigation, Curr. Pharm. Des. 18 (2012) 5131–5140.
[13] D. Fabris, J.R. Lisboa, F.S. Guimarães, F.V. Gomes, Cannabidiol as an antipsychotic drug, Int. Rev. Neurobiol. 177 (2024) 295–317.
[14] G. Marsicano, B. Moosmann, H. Hermann, B. Lutz, C. Behl, Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1, J. Neurochem. 80 (2002) 448–456.
[15] S. Atalay, I. Jarocka-Karpowicz, E. Skrzydlewska, Antioxidative and anti-Inflammatory properties of cannabidiol, Antioxidants 9 (2019) 21.
[16] P. Nagarkatti, R. Pandey, S.A. Rieder, V.L. Hegde, M. Nagarkatti, Cannabinoids as novel anti-inflammatory drugs, Future Med. Chem. 1 (2009) 1333–1349.
[17] J.P. Hickey, A.E. Collins, M.L. Nelson, H. Chen, B.E. Kalisch, Modulation of oxidative stress and neuroinflammation by cannabidiol (CBD): promising targets for the treatment of Alzheimer's disease, Curr. Issues Mol. Biol. 46 (2024) 4379–4402.
[18] I. Lastres-Becker, F. Molina-Holgado, J.A. Ramos, R. Mechoulam, J. Fernández-Ruiz, Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: relevance to Parkinson's disease, Neurobiol. Dis. 19 (2005) 96–107.
[19] M. García-Arencibia, S. González, E. de Lago, J.A. Ramos, R. Mechoulam, J. Fernández-Ruiz, Evaluation of the neuroprotective effect of cannabinoids in a rat model of Parkinson's disease: importance of antioxidant and cannabinoid receptor-independent properties, Brain Res. 1134 (2007) 162–170.
[20] E. Del-Bel, N. Barros-Pereira, R.P. Moraes, B.A. Mattos, T.A. Alves-Fernandes, L. B. Abreu, G.C. Nascimento, D. Escobar-Espinal, J.F.C. Pedrazzi, G. Jacob, B. A. Milan, G.G. Bálico, L.R. Antonieto, A journey through cannabidiol in Parkinson's disease, Int. Rev. Neurobiol. 177 (2024) 65–93.
[21] N.M. Kogan, R. Mechoulam, Cannabinoids in health and disease, Dialogues Clin. Neurosci. 9 (2007) 413–430.
[22] T. Bisogno, L. Hanus, L. De Petrocellis, S. Tchilibon, D.E. Ponde, I. Brandi, A. S. Moriello, J.B. Davis, R. Mechoulam, V. Di Marzo, Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide, Br. J. Pharmacol. 134 (2001) 845–852.
[23] F.M. Leweke, D. Piomelli, F. Pahlisch, D. Muhl, C.W. Gerth, C. Hoyer, J. Klosterkötter, M. Hellmich, D. Koethe, Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia, Transl. Psychiatry 2 (2012) e94.
[24] R.B. Laprairie, A.M. Bagher, M.E. Kelly, E.M. Denovan-Wright, Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor, Br. J. Pharmacol. 172 (2015) 4790–4805.
[25] E. Martínez-Pinilla, K. Varani, I. Reyes-Resina, E. Angelats, F. Vincenzi, C. Ferreiro-Vera, J. Oyarzabal, E.I. Canela, J.L. Lanciego, X. Nadal, G. Navarro, P.A. Borea, R. Franco, Binding and signaling studies disclose a potential allosteric site for cannabidiol in cannabinoid CB_2 receptors, Front. Pharmacol. 8 (2017) 744.
[26] M. Tham, O. Yilmaz, M. Alaverdashvili, M.E.M. Kelly, E.M. Denovan-Wright, R. B. Laprairie, Allosteric and orthosteric pharmacology of cannabidiol and cannabidiol-dimethylheptyl at the type 1 and type 2 cannabinoid receptors, Br. J. Pharmacol. 176 (2019) 1455–1469.
[27] J. Jakowiecki, R. Abel, U. Orzeł, P. Pasznik, R. Preissner, S. Filipek, Allosteric modulation of the CB1 cannabinoid receptor by cannabidiol-a molecular modeling study of the N-terminal domain and the allosteric-orthosteric coupling, Molecules 26 (2021) 2456.
[28] S. Silvestro, G. Schepici, P. Bramanti, E. Mazzon, Molecular targets of cannabidiol in experimental models of neurological disease, Molecules 25 (2020) 5186.
[29] Y. Miao, F. Zhao, W. Guan, A novel insight into the antidepressant effect of cannabidiol: possible involvement of the 5-HT1A, CB1, GPR55, and PPARγ receptors, Int. J. Neuropsychopharmacol. (2024) in press.
[30] L. Etemad, G. Karimi, M.S. Alavi, A. Roohbakhsh, Pharmacological effects of cannabidiol by transient receptor potential channels, Life Sci. 300 (2022) 120582.
[31] F. Espejo-Porras, J. Fernández-Ruiz, R.G. Pertwee, R. Mechoulam, C. García, Motor effects of the non-psychotropic phytocannabinoid cannabidiol that are mediated by 5-HT1A receptors, Neuropharmacology 75 (2013) 155–163.
[32] M.V. DeVuono, O. La Caprara, G.N. Petrie, C.L. Limebeer, E.M. Rock, M.N. Hill, L. A. Parker, Cannabidiol interferes with establishment of Δ^9-tetrahydrocannabinol-induced nausea through a 5-HT_{1A} mechanism, Cannabis. Cannabinoid. Res 7 (2022) 58–64.
[33] E. Billard, A. Torbey, A. Inserra, E. Grant, A. Bertazzo, D. De Gregorio, S. Comai, D. Chatenet, G. Gobbi, T.E. Hébert, Pharmacological characterization of cannabidiol as a negative allosteric modulator of the 5-HT_{2A} receptor, Cell. Signal. 127 (2025) 111588.
[34] E.J. Carrier, J.A. Auchampach, C.J. Hillard, Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression, Proc. Natl. Acad. Sci. USA. 103 (2006) 7895–7900.
[35] S.E. O'Sullivan, An update on PPAR activation by cannabinoids, Br. J. Pharmacol. 173 (2016) 1899–1910.
[36] J.M. McPartland, M. Duncan, V. Di Marzo, R.G. Pertwee, Are cannabidiol and Δ9-tetrahydrocannabivarin negative modulators of the endocannabinoid system? A systematic review, Br. J. Pharmacol. 172 (2015) 737–753.
[37] G. Navarro, A. Gonzalez, A. Sánchez-Morales, N. Casajuana-Martin, M. Gómez-Ventura, A. Cordomí, F. Busqué, R. Alibés, L. Pardo, R. Franco, Design of negative and positive allosteric modulators of the cannabinoid CB_2 receptor derived from the natural product cannabidiol, J. Med. Chem. 64 (2021) 9354–9364.
[38] P. Morales, P. Goya, N. Jagerovic, L. Hernandez-Folgado, Allosteric modulators of the CB_1 cannabinoid receptor: a structural update review, Cannabis. Cannabinoid. Res 1 (2016) 22–30.
[39] A. Sabatucci, D. Tortolani, E. Dainese, M. Maccarrone, In silico mapping of allosteric ligand binding sites in type-1 cannabinoid receptor, Biotechnol. Appl. Biochem. 65 (2018) 21–28.

[40] H. Li, Y. Liu, D. Tian, L. Tian, X. Ju, L. Qi, Y. Wang, C. Liang, Overview of cannabidiol (CBD) and its analogues: structures, biological activities, and neuroprotective mechanisms in epilepsy and Alzheimer's disease, Eur. J. Med. Chem. 192 (2020) 112163.
[41] L.O. Hanus, S. Tchilibon, D.E. Ponde, A. Breuer, E. Fride, R. Mechoulam, Enantiomeric cannabidiol derivatives: synthesis and binding to cannabinoid receptors, Org. Biomol. Chem. 3 (2005) 1116–1123.
[42] I. Ujváry, L. Hanuš, Human metabolites of cannabidiol: a review on their formation, biological activity, and relevance in therapy, Cannabis. Cannabinoid. Res 1 (2016) 90–101.
[43] A. Breuer, C.G. Haj, M.V. Fogaça, F.V. Gomes, N.R. Silva, J.F. Pedrazzi, E.A. Del Bel, J.C. Hallak, J.A. Crippa, A.W. Zuardi, R. Mechoulam, F.S. Guimarães, Fluorinated cannabidiol derivatives: enhancement of activity in mice models predictive of anxiolytic, antidepressant and antipsychotic effects, PLoS One 11 (2016) e0158779.
[44] C. del Río, C. Navarrete, J.A. Collado, M.L. Bellido, M. Gómez-Cañas, M.R. Pazos, J. Fernández-Ruiz, F. Pollastro, G. Appendino, M.A. Calzado, I. Cantarero, E. Muñoz, The cannabinoid quinol VCE-004.8 alleviates bleomycin-induced scleroderma and exerts potent antifibrotic effects through peroxisome proliferator-activated receptor-γ and CB2 pathways, Sci. Rep. 6 (2016) 21703.
[45] P. Morales, P.H. Reggio, N. Jagerovic, An overview on medicinal chemistry of synthetic and natural derivatives of cannabidiol, Front. Pharmacol. 8 (2017) 422.
[46] A.M. Szczesniak, Y. Maor, H. Robertson, O. Hung, M.E. Kelly, Nonpsychotropic cannabinoids, abnormal cannabidiol and cannabigerol-dimethyl heptyl, act at novel cannabinoid receptors to reduce intraocular pressure, J. Ocul. Pharmacol. Therapeut. 27 (2011) 427–435.
[47] S.E. Turner, C.M. Williams, L. Iversen, B.J. Whalley, Molecular pharmacology of phytocannabinoids, Prog. Chem. Org. Nat. Prod. 103 (2017) 61–101.
[48] P. Alves, C. Amaral, N. Teixeira, G. Correia-da-Silva, Cannabis sativa: much more beyond Δ^9-tetrahydrocannabinol, Pharmacol. Res. 157 (2020) 104822.
[49] A.J. Hill, M.S. Mercier, T.D. Hill, S.E. Glyn, N.A. Jones, Y. Yamasaki, T. Futamura, M. Duncan, C.G. Stott, G.J. Stephens, C.M. Williams, B.J. Whalley, Cannabidivarin is anticonvulsant in mouse and rat, Br. J. Pharmacol. 167 (2012) 1629–1642.
[50] A. Morano, M. Fanella, M. Albini, P. Cifelli, E. Palma, A.T. Giallonardo, C. Di Bonaventura, Cannabinoids in the treatment of epilepsy: current status and future prospects, Neuropsychiatric Dis. Treat. 16 (2020) 381–396.
[51] E. Zamberletti, T. Rubino, D. Parolaro, Therapeutic potential of cannabidivarin for epilepsy and autism spectrum disorder, Pharmacol. Ther. 226 (2021) 107878.
[52] F Wang, T Jin, H Li, H Long, Y Liu, S Jin, Y Lu, Y Peng, C Liu, L Zhao, X Wang, Cannabidivarin alleviates α-synuclein aggregation via DAF-16 in Caenorhabditis elegans, FASEB J. 37 (2) (2023) e22735.
[53] C Aguzzi, L Zeppa, MB Morelli, O Marinelli, M Giangrossi, C Amantini, G Santoni, H Sazzad, M Nabissi, Anticancer effect of minor phytocannabinoids in preclinical models of multiple myeloma, Biofactors 50 (6) (2024) 1208–1219.
[54] D Vigli, L Cosentino, C Raggi, G Laviola, M Woolley-Roberts, B De Filippis, Chronic treatment with the phytocannabinoid Cannabidivarin (CBDV) rescues behavioural alterations and brain atrophy in a mouse model of Rett syndrome, Neuropharmacology 140 (2018) 121–129.
[55] N.L. Stone, A.J. Murphy, T.J. England, S.E. O'Sullivan, A systematic review of minor phytocannabinoids with promising neuroprotective potential, Br. J. Pharmacol. 177 (2020) 4330–4352.
[56] Y. Zhang, H. Li, S. Jin, Y. Lu, Y. Peng, L. Zhao, X. Wang, Cannabidiol protects against Alzheimer's disease in C. elegans via ROS scavenging activity of its phenolic hydroxyl groups, Eur. J. Pharmacol. 919 (2022) 174829.
[57] A.E. Golliher, A.J. Tenorio, N.O. Dimauro, N.R. Mairata, F.O. Holguin, W. Maio, Using (+)-Carvone to access novel derivatives of (+)-*ent*-Cannabidiol: the first asymmetric syntheses of (+)-*ent*-CBDP and (+)-*ent*-CBDV, Tetrahedron Lett. 67 (2021) 152891.
[58] R. Mechoulam, N. Lander, M. Srebnik, A. Breuer, M. Segal, J.J. Feigenbaum, T. U. Jarbe, P. Consroe, Stereochemical requirements for cannabimimetic activity, NIDA Res. Monogr. 79 (1987) 15–30.
[59] R. Mechoulam, A. Breuer, T.U. Järbe, A.J. Hiltunen, R. Glaser, Cannabimimetic activity of novel enantiomeric, benzofuran cannabinoids, J. Med. Chem. 33 (1990) 1037–1043.
[60] A.C. Howlett, T.M. Champion, G.H. Wilken, R. Mechoulam, Stereochemical effects of 11-OH-delta 8-tetrahydrocannabinol-dimethylheptyl to inhibit adenylate cyclase and bind to the cannabinoid receptor, Neuropharmacology 29 (1990) 161–165.
[61] B.R. Martin, D.R. Compton, B.F. Thomas, W.R. Prescott, P.J. Little, R.K. Razdan, M. R. Johnson, L.S. Melvin, R. Mechoulam, S.J. Ward, Behavioral, biochemical, and molecular modeling evaluations of cannabinoid analogs, Pharmacol. Biochem. Behav. 40 (1991) 471–478.
[62] R.G. Pertwee, L.A. Stevenson, D.B. Elrick, R. Mechoulam, A.D. Corbett, Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of Guinea-pig small intestine, Br. J. Pharmacol. 105 (1992) 980–984.
[63] P.J. Little, D.R. Compton, M.R. Johnson, L.S. Melvin, B.R. Martin, Pharmacology and stereoselectivity of structurally novel cannabinoids in mice, J. Pharmacol. Exp. Therapeut. 247 (1988) 1046–1051.
[64] M.A. Schafroth, G. Mazzoccanti, I. Reynoso-Moreno, R. Erni, F. Pollastro, D. Caprioglio, B. Botta, G. Allegrone, G. Grassi, A. Chicca, F. Gasparrini, J. Gertsch, E.M. Carreira, G. Appendino, Δ^9-*cis*-Tetrahydrocannabinol: natural occurrence, chirality, and pharmacology, J. Nat. Prod. 84 (2021) 2502–2510.
[65] P.J. Little, D.R. Compton, R. Mechoulam, B.R. Martin, Stereochemical effects of 11-OH-delta 8-THC-dimethylheptyl in mice and dogs, Pharmacol. Biochem. Behav. 32 (1989) 661–666.
[66] T.U. Järbe, A.J. Hiltunen, R. Mechoulam, Stereospecificity of the discriminative stimulus functions of the dimethylheptyl homologs of 11-hydroxy-delta 8-tetrahydrocannabinol in rats and pigeons, J. Pharmacol. Exp. Therapeut. 250 (1989) 1000–1005.
[67] A. Ottani, D. Giuliani, HU-210: a potent tool for investigations of the cannabinoid system, CNS Drug Rev. 7 (2001) 131–145.
[68] E. Pop, Dexanabinol pharmos, Curr. Opin. Invest. Drugs 1 (2000) 494–503.
[69] N. Bruni, C. Della Pepa, S. Oliaro-Bosso, E. Pessione, D. Gastaldi, F. Dosio, Cannabinoid delivery systems for pain and inflammation treatment, Molecules 23 (2018) 2478.
[70] B. Fiebich, M. Winkler, M.R. Götz, O. Koch, Synthesis of (+)-cannabinoids and their therapeutic effects, Patent US 2021/0253598 A1 (2020).
[71] D. Mattoteia, A. Schiano Moriello, O. Taglialatela-Scafati, P. Amodeo, L. De Petrocellis, G. Appendino, R.M. Vitale, D. Caprioglio, The combined effect of branching and elongation on the bioactivity profile of phytocannabinoids. Part I: Thermo-TRPs, Biomedicines 9 (2022) 1070.
[72] M. Gómez-Cañas, P. Morales, L. García-Toscano, C. Navarrete, E. Muñoz, N. Jagerovic, J. Fernández-Ruiz, M. García-Arencibia, M.R. Pazos, Biological characterization of PM226, a chromenoisoxazole, as a selective CB2 receptor agonist with neuroprotective profile, Pharmacol. Res. 110 (2016) 205–215.
[73] I González-Mariscal, B Carmona-Hidalgo, M Winkler, JD Unciti-Broceta, A Escamilla, M Gómez-Cañas, J Fernández-Ruiz, BL Fiebich, SY Romero-Zerbo, FJ Bermúdez-Silva, JA Collado, E Muñoz, (+)-trans-Cannabidiol-2-hydroxy pentyl is a dual CB_1R antagonist/CB_2R agonist that prevents diabetic nephropathy in mice, Pharmacol. Res. 169 (2021) 105492.
[74] E. Fride, C. Feigin, D.E. Ponde, A. Breuer, L. Hanus, N. Arshavsky, R. Mechoulam, (+)-Cannabidiol analogues which bind cannabinoid receptors but exert peripheral activity only, Eur. J. Pharmacol. 506 (2004) 179–188.
[75] J. Bouma, J.D. Broekhuis, C. van der Horst, P. Kumar, A. Ligresti, M. van der Stelt, L.H. Heitman, Dual allosteric and orthosteric pharmacology of synthetic analog cannabidiol-dimethylheptyl, but not cannabidiol, on the cannabinoid CB_2 receptor, Biochem. Pharmacol. 218 (2023) 115924.
[76] T.S. Hughes, P.K. Giri, I.M. de Vera, D.P. Marciano, D.S. Kuruvilla, Y. Shin, A. L. Blayo, T.M. Kamenecka, T.P. Burris, P.R. Griffin, D.J. Kojetin, An alternate binding site for PPARγ ligands, Nat. Commun. 5 (2014) 3571.
[77] Å. Kaupang, T.V. Hansen, The PPAR Ω pocket: renewed opportunities for drug development, PPAR Res. 2020 (2020) 9657380.
[78] J. Shang, D.J. Kojetin, Unanticipated mechanisms of covalent inhibitor and synthetic ligand cobinding to PPARγ, Elife 13 (2024) RP99782.
[79] C. García, M. Gómez-Cañas, S. Burgaz, B. Palomares, Y. Gómez-Gálvez, C. Palomo-Garo, S. Campo, J. Ferrer-Hernández, C. Pavicic, C. Navarrete, M. Luz Bellido, M. García-Arencibia, M. Ruth Pazos, E. Muñoz, J. Fernández-Ruiz, Benefits of VCE-003.2, a cannabigerol quinone derivative, against inflammation-driven neuronal deterioration in experimental Parkinson's disease: possible involvement of different binding sites at the PPARγ receptor, J. Neuroinflammation 15 (2018) 19.
[80] R. Mechoulam, M. Peters, E. Murillo-Rodriguez, L.O. Hanus, Cannabidiol–recent advances, Chem. Biodivers. 4 (2007) 1678–1692.
[81] S. Zou, U. Kumar, Cannabinoid receptors and the endocannabinoid system: signaling and function in the Central Nervous System, Int. J. Mol. Sci. 19 (2018) 833.
[82] C. Muller, P. Morales, P.H. Reggio, Cannabinoid ligands targeting TRP channels, Front. Mol. Neurosci. 11 (2019) 487.
[83] J.M. McPartland, M. Glass, R.G. Pertwee, Meta-analysis of cannabinoid ligand binding affinity and receptor distribution: interspecies differences, Br. J. Pharmacol. 152 (2007) 583–593.
[84] A. Dhopeshwarkar, K. Mackie, CB2 Cannabinoid receptors as a therapeutic target-what does the future hold? Mol. Pharmacol. 86 (2014) 430–437.
[85] T. Bosquez-Berger, S. Wilson, C. Iliopoulos-Tsoutsouvas, S. Jiang, J. Wager-Miller, S.P. Nikas, K.P. Mackie, A. Makriyannis, A. Straiker, Differential enantiomer-specific signaling of cannabidiol at CB_1 receptors, Mol. Pharmacol. 102 (2022) 259–268.
[86] E. Kozela, C. Haj, L. Hanuš, M. Chourasia, A. Shurki, A. Juknat, N. Kaushansky, R. Mechoulam, Z. Vogel, HU-446 and HU-465, derivatives of the non-psychoactive cannabinoid cannabidiol, decrease the activation of encephalitogenic T cells, Chem. Biol. Drug Des. 87 (2016) 143–153.
[87] C. García, C. Palomo-Garo, M. García-Arencibia, J. Ramos, R. Pertwee, J. Fernández-Ruiz, Symptom-relieving and neuroprotective effects of the phytocannabinoid Δ^9-THCV in animal models of Parkinson's disease, Br. J. Pharmacol. 163 (2011) 1495–1506.
[88] Y. Gómez-Gálvez, C. Palomo-Garo, J. Fernández-Ruiz, C. García, Potential of the cannabinoid CB2 receptor as a pharmacological target against inflammation in Parkinson's disease, Prog. Neuropsychopharmacol. Biol. Psychiatry 64 (2016) 200–208.
[89] A.D.F. Alves, F.C.R. Dias, P.G. Cadena, V.A. Silva-Jr, Use of phytocanabinoids in animal models of Parkinson's disease: systematic review, Neurotoxicology 105 (2024) 34–44.

brain sciences

MDPI

Article

The Cannabigerol Derivative VCE-003.2 Exerts Therapeutic Effects in 6-Hydroxydopamine-Lesioned Mice: Comparison with The Classic Dopaminergic Replacement Therapy

Santiago Rodríguez-Carreiro [1,2,3], Elisa Navarro [1,2,3], Eduardo Muñoz [4,5,6] and Javier Fernández-Ruiz [1,2,3,*]

1 Instituto Universitario de Investigación en Neuroquímica, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain; santirod@ucm.es (S.R.-C.); elisnava@ucm.es (E.N.)
2 Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), 28029 Madrid, Spain
3 Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), 28034 Madrid, Spain
4 Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), 14004 Córdoba, Spain; fi1muble@uco.es
5 Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, 14004 Córdoba, Spain
6 Hospital Universitario Reina Sofía, 14004 Córdoba, Spain
* Correspondence: jjfr@med.ucm.es; Tel.: +34-913941450

Abstract: (1) Background: A cannabigerol aminoquinone derivative, so-called VCE-003.2, has been found to behave as a neuroprotective agent (administered both i.p. and orally) in different experimental models of Parkinson's disease (PD) in mice. These effects were exerted through mechanisms that involved the activation of a regulatory site within the peroxisome proliferator-activated receptor-γ (PPAR-γ). (2) Methods: We are now interested in comparing such neuroprotective potential of VCE-003.2, orally administered, with the effect of the classic dopaminergic replacement therapy with L-DOPA/benserazide in similar conditions, using 6-hydroxydopamine-lesioned mice. (3) Results: The oral administration of VCE-003.2 during 14 days at the dose of 20 mg/kg improved, as expected, the neurological status (measured in motor tests) in these mice. This correlated with a preservation of TH-labelled neurons in the substantia nigra. By contrast, the treatment with L-DOPA/benserazide (during 7 days at 2 mg/kg) was significantly less active in these experimental conditions, in concordance with their profile as a mere symptom-alleviating agent. (4) Conclusions: Our results confirmed again the therapeutic profile of VCE-003.2 in experimental PD and revealed a different and more relevant effect, as a disease modifier, compared to the classic symptom-alleviating L-DOPA treatment. This reinforces the interest in VCE-003.2 for a future clinical development in this disease.

Keywords: VCE-003.2; L-DOPA/benserazide; Parkinson's disease; 6-OHDA-lesioned mice; cannabinoids; PPAR-γ

check for updates

Citation: Rodríguez-Carreiro, S.; Navarro, E.; Muñoz, E.; Fernández-Ruiz, J. The Cannabigerol Derivative VCE-003.2 Exerts Therapeutic Effects in 6-Hydroxydopamine-Lesioned Mice: Comparison with The Classic Dopaminergic Replacement Therapy. *Brain Sci.* **2023**, *13*, 1272. https://doi.org/10.3390/brainsci13091272

Academic Editor: Mohammed Emdadul Haque

Received: 23 July 2023
Revised: 18 August 2023
Accepted: 24 August 2023
Published: 31 August 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Cannabinoids are pleiotropic compounds that, acting through multiple pharmacological targets within the endocannabinoid system, as well as in other signaling systems, have demonstrated to be promising neuroprotective agents [1]. Such neuroprotective potential has been preclinically investigated in different neurological conditions, such as accidental brain damage (e.g., stroke and brain trauma) and chronic progressive disorders (e.g., Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease (HD)) [2]. One of the disorders that has been more investigated to date is Parkinson's disease (PD). In this disease, cannabinoid-based therapies may serve to delay disease progression, but also to alleviate specific parkinsonian symptoms [3,4]. Thus, preclinical studies have demonstrated that modulating the cannabinoid receptor type 1 (CB_1) may serve for reducing parkinsonian signs such as bradykinesia and immobility [5], tremor [6]

and/or L-DOPA-induced dyskinesia [7], This can occur even combined in combination with other agents, such adenosine receptor ligands [8,9] such as these receptors may form heteromers with the CB_1 receptor resulting in possible synergic effects at a pharmacological level [10,11]. Such a combination between CB_1 and adenosine receptor ligands has been already investigated in pathological conditions other than PD [12,13].

Modulating the CB_1 receptor may also serve to afford neuroprotective effects in PD [14,15]. However, most cannabinoids proposed as neuroprotectant agents in this disease target the cannabinoid receptor type 2 (CB_2), whose activation entails anti-inflammatory effects in different PD models [16–19]. A similar effect has been found with cannabinoids that activate the peroxisome proliferator-activated receptor (PPAR)-γ [20,21] and even that target GPR55, an orphan receptor that has been recently associated with the endocannabinoid system [22,23]. Lastly, the neuroprotective effects in PD of some specific phytocannabinoids (e.g., cannabidiol) have been assigned to receptor-independent antioxidant effects [24] or even associated to activity as modulators of the antioxidant transcription factor Nrf2 [25]. Therefore, these studies have placed several cannabinoid compounds in a promising position for serving to generate a cannabinoid-based therapy for specific symptoms and, in particular, for disease progression in patients affected by PD.

An interesting cannabinoid compound in PD is the non-thiophilic CBG quinone derivative VCE-003.2, whose chemical structure, mechanism of action and other characteristics (in comparison with CBG, its original naturally-occurring phytocannabinoid) have been previously published [20,26,27]. VCE-003.2 behaves as a PPAR-γ activator with no activity at the CB_1/CB_2 receptors [26]. This study investigated the effect of VCE-003.2 in murine models of HD, confirming its neuroprotectant profile exerted by activating PPAR-γ and its ability to cross the blood–brain barrier after systemic administration. Moreover, VCE-003.2, given orally, was found to also be neuroprotective and to induce neurogenesis in experimental HD [28]. As regards to experimental models of PD, VCE-003.2, given i.p. or orally, has been found to be active as an anti-inflammatory and neuroprotective agent against inflammation-driven neuronal deterioration in LPS-lesioned mice [20,21]. It was also active in 6-hydroxydopamine (6-OHDA)-lesioned mice [29], a model characterized by mitochondrial dysfunction and oxidative stress. These effects were found to be mediated by its binding at a functional alternative site (different from the canonical binding site used by glitazones) in the PPAR-γ receptor, as revealed in in vitro studies carried out in cell-based assays [20,29].

We wanted now is to further explore the neuroprotective potential of VCE-003.2, using an oral formulation, comparison with the reference therapy in PD using L-DOPA/benserazide [30]. Such a comparison is a necessary step in the development of any new antiparkinsonian agent, in this case VCE-003.2, towards its clinical exploitation in PD. However, it is important to remark that such dopaminergic replacement therapy with L-DOPA/benserazide was approved to alleviate specific parkinsonian symptoms (e.g., akinesia and rigidity) in PD patients [30]. By contrast, it is generally accepted that L-DOPA/benserazide has no effect on disease progression, despite a few studies that reported certain neuroprotective effects in experimental models [31,32] pending further confirmation. To this end, our experimental design was aimed at evaluating the neuroprotective effect of a chronic administration of VCE-003.2 given orally to 6-OHDA-lesioned mice for 14 days, which was compared with the effect of a chronic administration of L-DOPA/benserazide given i.p. for 7 days (to use a shorter treatment was necessary to diminish the occurrence of dyskinesia [33]). The efficacy of both treatments was determined with motor tests and immunostaining for tyrosine hydroxylase (TH) and glial markers (GFAP and CD68) in the substantia nigra. It is important to remark that all analyses, but this is particularly important in the case of behavioral tests, were carried out at 24 h from the last injection. This means that the possible behavioral effects found should be related more to neuroprotection rather than to symptom alleviation, which would be visible only at shorter times (1–2 h).

2. Materials and Methods

2.1. Animal and Surgical Lesions

Male C57BL/6 mice were housed in our animal facilities (CAI-Animalario, Faculty of Medicine, Complutense University, ref. ES280790000086) under controlled photoperiod (08:00–20:00 light) and temperature (22 ± 1 °C), and with free access to standard diet and water. They were used at adult age (6–8 month-old; 24–30 g weight) for experimental purposes. All experiments were conducted according to national and European guidelines (directive 2010/63/EU), as well as conforming to ARRIVE guidelines, and were approved by the "Comité de Experimentación Animal" of our university (PROEX: 201.8/22). Mice were anaesthetized (ketamine 40 mg/kg + xylazine 4 mg/kg, i.p. purchased from Sigma-Aldrich, Madrid, Spain). This was followed by pretreatment with desipramine (25 mg/kg, i.p., purchased from Sigma-Aldrich, Madrid, Spain) just 30 min before mice received 6-OHDA free base (2 µL at a concentration of 2 µg/µL saline in 0.02% ascorbate to avoid oxidation; both purchased from Sigma-Aldrich, Madrid, Spain) or saline (for control mice) injected stereotaxically into the right striatum at a rate of 0.5 µL/min. To this end, we used the following coordinates: +0.4 mm AP, ±1.8 mm ML and −3.5 mm DV, as described in Alvarez-Fischer et al. [34]. Once injected, the needle was left in place for 5 min before being slowly withdrawn. This avoided generating reflux and a rapid increase in intracranial pressure. Control animals were sham-operated and injected with 2 µL of saline using the same coordinates. After the application of 6-OHDA or saline, mice were subjected to pharmacological treatments as described in the following section. The lesions were generated using unilateral injection with the contralateral structures serving as controls for the different analyses.

2.2. Pharmacological Treatments and Sampling

After the application of 6-OHDA, animals were treated with the following compounds: (i) VCE-003.2 (provided by Emerald Health Pharmaceuticals, San Diego, CA, USA) given orally at a dose of 20 mg/kg according to previous studies [29], initiating the treatment at 24 h after the lesion and daily repeating during 14 days; (ii) L-DOPA (Sigma-Aldrich Chem., Madrid, Spain) and benserazide (Sigma-Aldrich Chem., Madrid, Spain), given i.p. at the dose of 2 mg/kg in both cases according to previous studies [33], initiating the treatment 7 days after the lesion and daily repeating during 7 additional days (as indicated before, the use of a shorter treatment was necessary to diminish the occurrence of dyskinesia [33]); or (iii) the vehicle for VCE-003.2 (sesame oil) given orally during 14 days (50% of mice in this group) and the vehicle for L-DOPA/benserazide (0.9% saline) given i.p. during 7 days (remaining 50% of animals). At the end of the treatment (24 h after the last injection), mice were analysed in different behavioural tests just before being killed by rapid and careful decapitation. Their brains were rapidly removed and fixed for one day at 4 °C in fresh 4% paraformaldehyde (Sigma-Aldrich, Madrid, Spain) prepared in 0.1 M PBS, pH 7.4. Samples were cryoprotected by immersion in a 30% sucrose (Sigma-Aldrich, Madrid, Spain) solution for 48 h, and finally stored at −80 °C for immunohistochemical analysis in the substantia nigra.

2.3. Behavioural Tests

Cylinder rearing test (CRT). Given that the lesions were unilateral, this test attempts to quantify the degree of forepaw (ipsilateral, contralateral, or both) preference for wall contacts after placing the mouse in a methacrylate transparent cylinder (diameter: 15.5 cm; height: 12.7 cm; [35]). Each score was made from a 3 min trial with a minimum of 4 wall contacts.

Pole test. Mice were placed head upward on the top of a vertical rough-surfaced pole (diameter 8 mm; height 55 cm) and the time until animals descended to the floor was recorded with a maximum duration of 90 s. When the mouse was not able to turn downward and instead dropped from the pole, the time was taken as 90 s (default value).

2.4. Immunohistochemical Procedures

Brains were sliced in coronal sections (30 μm thick; containing the substantia nigra) in a cryostat (Leica Biosystems, Wetzlar, Germany) and collected on antifreeze solution (glycerol/ethylene glycol/PBS; 2:3:5) and stored at −20 °C until used. Brain sections were mounted on gelatin-coated slides, and once adhered, washed in 0.1 M potassium PBS (KPBS) at pH 7.4. Endogenous peroxidase was blocked by 30 min incubation at room temperature in peroxidase blocking solution (Dako Cytomation, Glostrup, Denmark). After several washes with KPBS, sections were incubated overnight at room temperature with the following polyclonal antibodies: (i) rabbit anti-mouse TH (Chemicon-Millipore, Temecula, CA, USA) used at 1:200; (ii) rat anti-mouse CD68 antibody (AbD Serotec, Oxford, UK) used at 1:200; or (iii) rabbit anti-mouse GFAP antibody (Dako Cytomation, Glostrup, Denmark) used at 1:200. Dilutions were carried out in KPBS containing 2% bovine serum albumin and 0.1% Triton X-100 (both purchased in Sigma-Aldrich, Madrid, Spain). After incubation, sections were washed in KPBS, followed by incubation with the corresponding biotinylated secondary antibody (1:200) (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine substrate–chromogen system (Dako Cytomation, Glostrup, Denmark) were used to obtain a visible reaction product. Negative control sections were obtained using the same protocol with omission of the primary antibody. A Leica DMRB microscope and a DFC300FX camera (both from Leica Biosystems, Wetzlar, Germany) were used for the observation and photography of the slides, respectively. For quantification of TH, CD68, or GFAP immunostaining in the substantia nigra, we used the NIH Image Processing and Analysis software (ImageJ; NIH, Bethesda, MD, USA) using 4–5 sections, separated approximately by 200 μm, and observed with a 10× objective. In all sections, the same areas of the substantia nigra were analyzed. The analyses were always conducted by experimenters who were blinded to all animal characteristics. Data were expressed as percentage of immunostaining intensity in the ipsilateral (lesioned) side over the contralateral (non-lesioned) side.

2.5. Statistics

Data were assessed using one-way ANOVA, followed by the Tukey test using GraphPad Prism, version 8.00 for Windows (GraphPad Software, San Diego, CA, USA). A p value lower than 0.05 was used as the limit for statistical significance. The sample sizes in the different experimental groups for both behavioral and histopathological analyses were always $\geq$5 (exact sample sizes are visible in the scatter plots presented in the figures). In the case of the groups treated with vehicle, there was not any significant difference between the values in the two subgroups (sesame oil given orally during 14 days and saline given i.p. during 7 days) for both behavioral and histopathological data, so they were combined for statistical analysis and presentation.

3. Results

In this study, we first pursued to confirm whether an oral formulation of VCE-003.2 in sesame oil at 20 mg/kg (two weeks of daily treatment) is active as a neuroprotectant in 6-OHDA-lesioned mice, as previously found in this [29] and other experimental models [20,21] of PD. Thus, our data indicated the occurrence of motor (deteriorated animal performance in the pole test and the CRT) and histopathological (loss of TH-positive neurons in the substantia nigra) abnormalities in 6-OHDA-lesioned mice to an extent similar to the data described in previous studies using this model [29]. These motor and histopathological abnormalities were again attenuated after the oral administration of VCE-003.2, to an extent similar to the previous data obtained in this model [29] and also in additional models [20,21]. These beneficial effects were evident, in particular, in the animal response in the CRT, as the elevation in the score of 6-OHDA-lesioned mice that reflects hemiparesis was significantly reversed by the treatment with oral VCE-003.2 ($F(3,29) = 7.42$, $p < 0.001$; Figure 1). Similar changes were seen in the pole test, with a total reversion of the elevated

time to descend the pole after the treatment with oral VCE-003.2 (F(3,28) = 6.01, $p < 0.005$) (Figure 1).

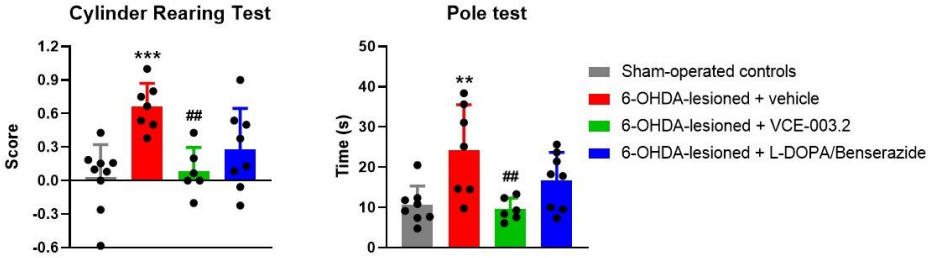


Figure 1. Response in the cylinder rearing and pole tests of 6-OHDA-lesioned mice treated with VCE-003.2 (at an oral dose of 20 mg/kg), L-DOPA/benserazide (at an i.p. dose of 2 mg/kg) or vehicle (sesame oil or 0.9% saline, respectively), and the corresponding controls. Data corresponded to 24 h after the last dose of VCE-003.2 (2 weeks of daily treatment) or L-DOPA (1 week of daily treatment). Values are mean ± SD and were analyzed by one-way ANOVA followed by the Tukey test (** $p < 0.01$, *** $p < 0.005$ versus vehicle-treated sham mice; ## $p < 0.01$ versus vehicle-treated 6-OHDA-lesioned mice).

These neurological benefits elicited by VCE-003.2 were associated with an apparent preservation of nigrostriatal neurons as detected using TH immunostaining in the substantia nigra (Figure 2). Thus, TH immunoreactivity levels in 6-OHDA-lesioned mice were strongly reduced compared to the control mice, but this reduction was significantly reversed by the chronic treatment with oral VCE-003.2 (F(3,29) = 11.31, $p < 0.0001$; Figure 2). It is true that our analysis only detected immunoreactivity levels for TH and that this is not necessarily a confirmation of more TH-positive cells. However, the morphological analysis of TH-immunostained sections in the different experimental groups appears to confirm the existence of more TH-immunolabelled cells in VCE-003.2-treated mice.

As indicated in the Introduction, we also pursued to compare the efficacy of VCE-003.2 as an antiparkinsonian agent with the effects of L-DOPA/benserazide (2 mg/kg, i.p., one week of daily treatment following the method described in Lundblad et al. [33]). This is the reference dopaminergic replacement therapy approved for PD patients, but this therapy is, in general, not active as a disease modifier being exclusively addressed to alleviate certain parkinsonian signs [30]. Thus, our data confirmed that the treatment with L-DOPA/benserazide only caused very modest effects (without reaching statistical significance compared to vehicle-treated 6-OHDA-lesioned mice) in the CRT and the pole test (Figure 1), as well as in TH immunostaining (Figure 2), in concordance with the notion that it only serves as a symptom-alleviating agent. Such an observation discards the data published previously in a few studies that suggest a certain neuroprotective activity for L-DOPA/benserazide too [31,32]. In addition, it demonstrates that the expected benefits of L-DOPA/benserazide against parkinsonian symptoms (without preservation of TH-positive neurons) were not visible here, as the response in the motor tests was analyzed at 24 h after the last injection, and not at shorter times (1–2 h) when they should be much more visible.

Lastly, our study also included immunohistochemical analysis of glial reactivity. However, according to the data described in our previous study [29], the elevation of immunoreactivity levels for the astrocyte marker GFAP (F(3,29) = 0.62, ns) and for the microglial marker CD68 (F(3,29) = 1.88, ns) was modest (or did not exist) with this 6-OHDA lesion, counteracting the determination of whether VCE-003.2 or L-DOPA/benserazide may be active at this level (Figures 3 and 4).

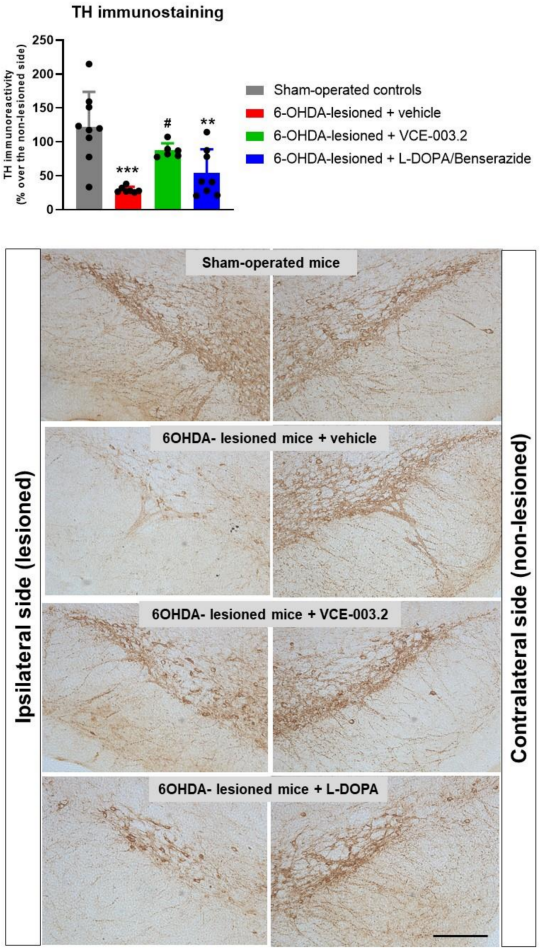


Figure 2. TH immunoreactivity levels (expressed as % over the contralateral non-lesioned side) in the substantia nigra of 6-OHDA-lesioned mice treated with VCE-003.2 (at an oral dose of 20 mg/kg), L-DOPA/benserazide (at an i.p. dose of 2 mg/kg) or vehicle (sesame oil or 0.9% saline, respectively), and the corresponding controls. The figure also includes representative microphotographs of ipsilateral lesioned and contralateral non-lesioned sides for each experimental group (scale bar = 200 µm). Data corresponded to 24 h after the last dose of VCE-003.2 (2 weeks of daily treatment) or L-DOPA (1 week of daily treatment). Values are mean ± SD, and were analyzed by one-way ANOVA followed by the Tukey test (** $p < 0.01$, *** $p < 0.005$ versus vehicle-treated sham mice; # $p < 0.05$ versus vehicle-treated 6-OHDA-lesioned mice).

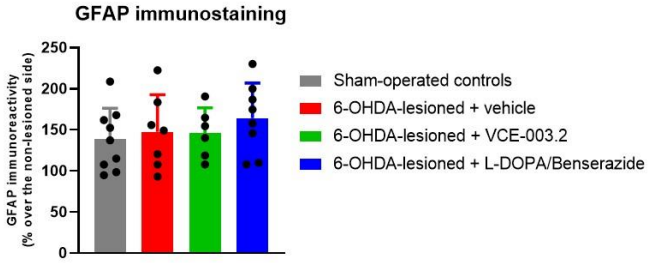


Figure 3. GFAP immunoreactivity levels (expressed as % over the contralateral non-lesioned side) in the substantia nigra of 6-OHDA-lesioned mice treated with VCE-003.2 (at an oral dose of 20 mg/kg), L-DOPA/benserazide (at an i.p. dose of 2 mg/kg) or vehicle (sesame oil or 0.9% saline, respectively), and the corresponding controls. Data corresponded to 24 h after the last dose of VCE-003.2 (2 weeks of daily treatment) or L-DOPA (1 week of daily treatment). Values are mean ± SD and were analyzed by one-way ANOVA followed by the Tukey test.

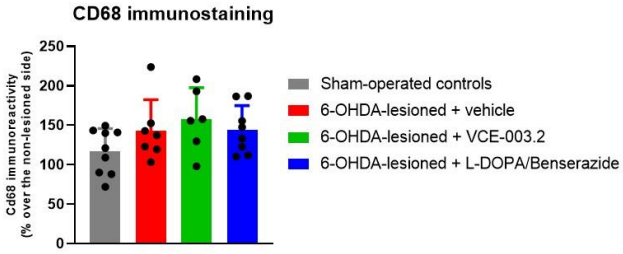


Figure 4. Cd68 immunoreactivity levels (expressed as % over the contralateral non-lesioned side) in the substantia nigra of 6-OHDA-lesioned mice treated with VCE-003.2 (at an oral dose of 20 mg/kg), L-DOPA/benserazide (at an i.p. dose of 2 mg/kg) or vehicle (sesame oil or 0.9% saline, respectively), and the corresponding controls. Data corresponded to 24 h after the last dose of VCE-003.2 (2 weeks of daily treatment) or L-DOPA (1 week of daily treatment). Values are mean ± SD and were analyzed by one-way ANOVA followed by the Tukey test.

4. Discussion

Our study is a new step in the process to move the phytocannabinoid derivative VCE-003.2 towards the clinical scenario as a potential neuroprotective therapy for PD patients. The first step in this process was to demonstrate the efficacy of VCE-003.2 as neuroprotective agent in different in vivo models of PD that recapitulate the different pathogenic events in this disease. Thus, we demonstrated that VCE-003.2 was active against inflammatory events eliciting neuronal injury (LPS model) [20,21], as well as against mitochondrial dysfunction and oxidative stress (6-OHDA model) [20,29] and could also be effective against α-synuclein dysregulation and aggregation (work in progress). This was followed, in a second step, by the confirmation that VCE-003.2 was also active not only after i.p. administration, but also when given orally (LPS model) [21], which facilitates its formulation for administration in patients.

Now, a necessary new step is to compare the efficacy of VCE-003.2 with the effect in the same conditions of the classic dopaminergic replacement therapy with L-DOPA/benserazide licensed for PD patients. It is important to remark, as indicated in the Introduction, that L-DOPA/benserazide treatment is addressed to attenuate specific parkinsonian symptoms (e.g., akinesia, rigidity, and postural instability), not to slow disease progression. However, this comparison is necessary given that this dopaminergic replacement treatment is the reference therapy approved in PD patients [28]. In this sense, any new antiparkinsonian agent needs to confirm that it offers advantages, for example, more potent effects, less side effects, or activity at areas not covered by the approved therapy (the latter is the option investigated here). It was also necessary to confirm, or not, the data provided by a few studies that reported certain neuroprotective activity in experimental PD models with this dopaminergic replacement therapy [31,32]. Thus, according to the data obtained in the study presented here, we can confirm that VCE-003.2 was also active given orally in 6-OHDA-lesioned mice and presented a neuroprotective effect that was not found with L-DOPA/benserazide. This was seen in the animal response in the two motor tests analyzed here, in which the effects of VCE-003.2 almost completely reversed the motor deficiencies. Nevertheless, L-DOPA/benserazide only showed very small trends towards a reversion that were not statistically significant compared to lesioned animals treated with the vehicle. It is likely that these modest effects only reflect the symptom-alleviating effect of L-DOPA/benserazide. In addition, its small magnitude may be caused because the motor tests were carried out at 24 h after the last administration, and not during the acute period (e.g., 1–2 h) where this combination should be more active as a symptomatic treatment. By contrast, VCE-003.2 showed a complete reversion seen at the behavioral level, which should be caused by its capability to preserve nigral TH-positive neurons. In the case of L-DOPA/benserazide, such capability was not evident at the statistical level, as already indicated in previous studies [31,32]. This different response is possibly associated with the molecular mechanisms that are activated by each of these two treatments. In the

case of L-DOPA/benserazide, the mechanism would be the recovery of dopamine levels enabling only symptom-alleviating effects [30]. In the case of VCE-003.2, the mechanism would be the activation of PPAR-γ signaling, which would result in the preservation of TH-positive neurons, as demonstrated in previous studies [20,21]. It is also important to remark that PPAR-γ receptors have been already found to serve as a neuroprotective target in PD when activated by non-cannabinoid compounds (e.g., glitazones) [36]. Therefore, this is possibly the major difference between the two treatments investigated here, which situates PPAR-γ as a relevant protein that may work as a promising target to develop a cannabinoid-based neuroprotective therapy.

Another aspect of our study that requires some discussion is the lack of any changes in astroglial and microglial reactivity with this 6-OHDA lesion. This avoids to see whether the treatment with VCE-003.2 was effective against this pathogenic event as found in PD models in which inflammation drives neuronal deterioration [20,21]. Our observation here is likely associated with the fact that glial reactivity in the 6-OHDA model is relatively residual and always secondary to neuronal injury, as described in previous studies [18,29].

5. Conclusions

Our data confirmed that 6-OHDA-lesioned mice exhibited an altered response in the cylinder rearing and pole tests, recapitulating some PD-like neurological signs. These responses were provoked by an intense loss of nigral dopaminergic neurons. The treatment with an oral dose of VCE-003.2 (20 mg/kg) avoids the deterioration of animal performance in the two motor tests, and such a benefit was associated with a parallel preservation of nigral TH-containing neurons. All these beneficial effects were not seen (or were resulted to be mere trends) after the treatment with L-DOPA/benserazide, then revealing the advantages of VCE-003.2 against the standard L-DOPA/benserazide therapy in PD. This finding will require further confirmation in future studies aimed at exploring VCE-003.2 administered with: (i) L-DOPA/benserazide to optimize the combination of disease modifying and symptom-alleviating effects; (ii) other cannabinoids active at CB_1, CB_2, and/or GPR55 receptors to obtain more intense neuroprotective effects; and (iii) with non-cannabinoid ligands (e.g., adenosine-active ligands) to sum additional therapeutic properties.

Author Contributions: Funding acquisition, E.M. and J.F.-R.; study design, coordination, and supervision, E.N., E.M. and J.F.-R.; studies in 6-OHDA-lesioned mice: design and methodology, S.R.-C. and E.N.; statistical analysis of the data, S.R.-C. and J.F.-R.; and manuscript preparation, J.F.-R., with the revision and approval of all authors. All authors have read and agreed to the published version of the manuscript.

Funding: This work has been supported by grants from CIBERNED (CB06/05/0089), MINECO Retos-Colaboración (RTC-2014-1877-1) and MICINN-Biomedicina (RTI2018-098885-B-100 and PID2021-128906OB-I00) with FEDER funds. Part of the study was also funded by Emerald Health Pharmaceuticals (San Diego, CA, USA).

Institutional Review Board Statement: All experiments were conducted according to European guidelines (directive 2010/63/EU), as well as conformed to ARRIVE guidelines, and approved by the "Comité de Experimentación Animal" of our university (ref. PROEX 201.8/22).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data supporting reported results may be supplied upon request to the authors.

Acknowledgments: Santiago Rodríguez-Carreiro is a predoctoral fellow supported by the FPU Program (Ministry of Education and Universities). Authors are indebted to Yolanda García-Movellán for administrative assistance.

Conflicts of Interest: The authors declare that they have no conflict of interest in relation to this study. The funders had no role in study design, the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

References

1. Fernández-Ruiz, J. The biomedical challenge of neurodegenerative disorders: An opportunity for cannabinoid-based therapies to improve on the poor current therapeutic outcomes. *Br. J. Pharmacol.* **2019**, *176*, 1370–1383. [CrossRef]
2. Fernández-Ruiz, J.; Moro, M.A.; Martínez-Orgado, J. Cannabinoids in neurodegenerative disorders and stroke/brain trauma: From preclinical models to clinical applications. *Neurotherapeutics* **2015**, *12*, 793–806. [CrossRef] [PubMed]
3. Antonazzo, M.; Botta, M.; Bengoetxea, H.; Ruiz-Ortega, J.A.; Morera-Herreras, T. Therapeutic potential of cannabinoids as neuroprotective agents for damaged cells conducing to movement disorders. *Int. Rev. Neurobiol.* **2019**, *146*, 229–257. [PubMed]
4. Baul, H.S.; Manikandan, C.; Sen, D. Cannabinoid receptor as a potential therapeutic target for Parkinson's Disease. *Brain Res. Bull.* **2019**, *146*, 244–252. [CrossRef]
5. Kelsey, J.E.; Harris, O.; Cassin, J. The CB1 antagonist rimonabant is adjunctively therapeutic as well as monotherapeutic in an animal model of Parkinson's disease. *Behav. Brain Res.* **2009**, *203*, 304–307. [CrossRef] [PubMed]
6. Sañudo-Peña, M.C.; Patrick, S.L.; Khen, S.; Patrick, R.L.; Tsou, K.; Walker, J.M. Cannabinoid effects in basal ganglia in a rat model of Parkinson's disease. *Neurosci. Lett.* **1998**, *248*, 171–174. [CrossRef] [PubMed]
7. Espadas, I.; Keifman, E.; Palomo-Garo, C.; Burgaz, S.; García, C.; Fernández-Ruiz, J.; Moratalla, R. Beneficial effects of the phytocannabinoid Δ9-THCV in L-DOPA-induced dyskinesia in Parkinson's disease. *Neurobiol. Dis.* **2020**, *141*, 104892. [CrossRef] [PubMed]
8. Zhao, Y.; Liu, X.; Yang, G. Adenosinergic pathway in Parkinson's disease: Recent advances and therapeutic perspective. *Mol. Neurobiol.* **2023**, *60*, 3054–3070. [CrossRef]
9. Pinna, A.; Parekh, P.; Morelli, M. Serotonin 5-HT_{1A} receptors and their interactions with adenosine A_{2A} receptors in Parkinson's disease and dyskinesia. *Neuropharmacology* **2023**, *226*, 109411. [CrossRef]
10. Ferré, S.; Sarasola, L.I.; Quiroz, C.; Ciruela, F. Presynaptic adenosine receptor heteromers as key modulators of glutamatergic and dopaminergic neurotransmission in the striatum. *Neuropharmacology* **2023**, *223*, 109329. [CrossRef]
11. Pinna, A.; Bonaventura, J.; Farré, D.; Sánchez, M.; Simola, N.; Mallol, J.; Lluís, C.; Costa, G.; Baqi, Y.; Müller, C.E.; et al. L-DOPA disrupts adenosine A(2A)-cannabinoid CB(1)-dopamine D(2) receptor heteromer cross-talk in the striatum of hemiparkinsonian rats: Biochemical and behavioral studies. *Exp. Neurol.* **2014**, *253*, 180–191. [CrossRef]
12. Haddad, M.; Alsalem, M.; Aldossary, S.A.; Kalbouneh, H.; Jaffal, S.M.; Alshawabkeh, Q.; Al Hayek, S.; Abdelhai, O.; Barakat, N.A.; El-Salem, K. The role of adenosine receptor ligands on inflammatory pain: Possible modulation of TRPV1 receptor function. *Inflammopharmacology* **2023**, *31*, 337–347. [CrossRef] [PubMed]
13. Bassani, D.; Pavan, M.; Federico, S.; Spalluto, G.; Sturlese, M.; Moro, S. The multifaceted role of GPCRs in amyotrophic lateral sclerosis: A new therapeutic perspective? *Int. J. Mol. Sci.* **2022**, *23*, 4504. [CrossRef]
14. Chung, Y.C.; Bok, E.; Huh, S.H.; Park, J.Y.; Yoon, S.H.; Kim, S.R.; Kim, Y.S.; Maeng, S.; Park, S.H.; Jin, B.K. Cannabinoid receptor type 1 protects nigrostriatal dopaminergic neurons against MPTP neurotoxicity by inhibiting microglial activation. *J. Immunol.* **2011**, *187*, 6508–6517. [CrossRef] [PubMed]
15. Pérez-Rial, S.; García-Gutiérrez, M.S.; Molina, J.A.; Pérez-Nievas, B.G.; Ledent, C.; Leiva, C.; Leza, J.C.; Manzanares, J. Increased vulnerability to 6-hydroxydopamine lesion and reduced development of dyskinesias in mice lacking CB1 cannabinoid receptors. *Neurobiol. Aging* **2011**, *32*, 631–645. [CrossRef]
16. García, C.; Palomo-Garo, C.; García-Arencibia, M.; Ramos, J.A.; Pertwee, R.G.; Fernández-Ruiz, J. Symptom-relieving and neuroprotective effects of the phytocannabinoid Δ^9-THCV in animal models of Parkinson's disease. *Br. J. Pharmacol.* **2011**, *163*, 1495–1506. [CrossRef]
17. Javed, H.; Azimullah, S.; Haque, M.E.; Ojha, S.K. Cannabinoid type 2 (CB2) receptors activation protects against oxidative stress and neuroinflammation associated dopaminergic neurodegeneration in rotenone model of Parkinson's disease. *Front. Neurosci.* **2016**, *10*, 321. [CrossRef] [PubMed]
18. Gómez-Gálvez, Y.; Palomo-Garo, C.; Fernández-Ruiz, J.; García, C. Potential of the cannabinoid CB2 receptor as a pharmacological target against inflammation in Parkinson's disease. *Prog. Neuropsychopharm. Biol. Psychiatry* **2016**, *64*, 200–208. [CrossRef]
19. Wang, G.; Ma, W.; Du, J. β-Caryophyllene (BCP) ameliorates MPP+ induced cytotoxicity. *Biomed. Pharmacother.* **2018**, *103*, 1086–1091. [CrossRef] [PubMed]
20. García, C.; Gómez-Cañas, M.; Burgaz, S.; Palomares, B.; Gómez-Gálvez, Y.; Palomo-Garo, C.; Campo, S.; Ferrer-Hernández, J.; Pavicic, C.; Navarrete, C.; et al. Benefits of VCE-003.2, a cannabigerol quinone derivative, against inflammation-driven neuronal deterioration in experimental Parkinson's disease: Possible involvement of different binding sites at the PPARγ receptor. *J. Neuroinflamm.* **2018**, *15*, 19. [CrossRef]
21. Burgaz, S.; García, C.; Gómez-Cañas, M.; Muñoz, E.; Fernández-Ruiz, J. Development of an oral treatment with the PPAR-γ-acting cannabinoid VCE-003.2 against the inflammation-driven neuronal deterioration in experimental Parkinson's disease. *Molecules* **2019**, *24*, 2702. [CrossRef] [PubMed]
22. Celorrio, M.; Rojo-Bustamante, E.; Fernández-Suárez, D.; Sáez, E.; Estella-Hermoso de Mendoza, A.; Müller, C.E.; Ramírez, M.J.; Oyarzábal, J.; Franco, R.; Aymerich, M.S. GPR55: A therapeutic target for Parkinson's disease? *Neuropharmacology* **2017**, *125*, 319–332. [CrossRef]
23. Burgaz, S.; García, C.; Gonzalo-Consuegra, C.; Gómez-Almería, M.; Ruiz-Pino, F.; Unciti, J.D.; Gómez-Cañas, M.; Alcalde, J.; Morales, P.; Jagerovic, N.; et al. Preclinical investigation in neuroprotective effects of the GPR55 ligand VCE-006.1 in experimental models of Parkinson's disease and amyotrophic lateral sclerosis. *Molecules* **2021**, *26*, 7643. [CrossRef]

24. Lastres-Becker, I.; Molina-Holgado, F.; Ramos, J.A.; Mechoulam, R.; Fernández-Ruiz, J. Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: Relevance to Parkinson's disease. *Neurobiol. Dis.* **2005**, *19*, 96–107. [CrossRef] [PubMed]
25. Gugliandolo, A.; Pollastro, F.; Grassi, G.; Bramanti, P.; Mazzon, E. In vitro model of neuroinflammation: Efficacy of cannabigerol, a non-psychoactive cannabinoid. *Int. J. Mol. Sci.* **2018**, *19*, 1992. [CrossRef]
26. Díaz-Alonso, J.; Paraíso-Luna, J.; Navarrete, C.; Del Río, C.; Cantarero, I.; Palomares, B.; Aguareles, J.; Fernández-Ruiz, J.; Bellido, M.L.; Pollastro, F.; et al. VCE-003.2, a novel cannabigerol derivative, enhances neuronal progenitor cell survival and alleviates symptomatology in murine models of Huntington's disease. *Sci. Rep.* **2016**, *6*, 29789. [CrossRef]
27. Granja, A.G.; Carrillo-Salinas, F.; Pagani, A.; Gómez-Cañas, M.; Negri, R.; Navarrete, C.; Mecha, M.; Mestre, L.; Fiebich, B.L.; Cantarero, I.; et al. A cannabigerol quinone alleviates neuroinflammation in a chronic model of multiple sclerosis. *J. Neuroimmune. Pharmacol.* **2012**, *7*, 1002–1016. [CrossRef] [PubMed]
28. Aguareles, J.; Paraíso-Luna, J.; Palomares, B.; Bajo-Grañeras, R.; Navarrete, C.; Ruiz-Calvo, A.; García-Rincón, D.; García-Taboada, E.; Guzmán, M.; Muñoz, E.; et al. Oral administration of the cannabigerol derivative VCE-003.2 promotes subventricular zone neurogenesis and protects against mutant huntingtin-induced neurodegeneration. *Transl. Neurodegener.* **2019**, *8*, 9. [CrossRef]
29. Burgaz, S.; García, C.; Gómez-Cañas, M.; Navarrete, C.; García-Martín, A.; Rolland, A.; Del Río, C.; Casarejos, M.J.; Muñoz, E.; Gonzalo-Consuegra, C.; et al. Neuroprotection with the cannabigerol quinone derivative VCE-003.2 and its analogs CBGA-Q and CBGA-Q-Salt in Parkinson's disease using 6-hydroxydopamine-lesioned mice. *Mol. Cell. Neurosci.* **2021**, *110*, 103583. [CrossRef]
30. Cenci, M.A. Transcription factors involved in the pathogenesis of L-DOPA-induced dyskinesia in a rat model of Parkinson's disease. *Amino Acids* **2002**, *23*, 105–109. [CrossRef]
31. Lu, D.S.; Chen, C.; Zheng, Y.X.; Li, D.D.; Wang, G.Q.; Liu, J.; Shi, J.; Zhang, F. Combination treatment of icariin and L-DOPA against 6-OHDA-lesioned dopamine neurotoxicity. *Front. Mol. Neurosci.* **2018**, *11*, 155. [CrossRef] [PubMed]
32. Kondo, T. Levodopa therapy from the neuroprotection viewpoint. From a clinical outlook. *J. Neurol.* **2005**, *252* (Suppl. 4), IV32–IV36. [CrossRef] [PubMed]
33. Lundblad, M.; Picconi, B.; Lindgren, H.; Cenci, M.A. A model of L-DOPA-induced dyskinesia in 6-hydroxydopamine lesioned mice: Relation to motor and cellular parameters of nigrostriatal function. *Neurobiol. Dis.* **2004**, *16*, 110–123. [CrossRef] [PubMed]
34. Alvarez-Fischer, D.; Henze, C.; Strenzke, C.; Westrich, J.; Ferger, B.; Höglinger, G.U.; Oertel, W.H.; Hartmann, A. Characterization of the striatal 6-OHDA model of Parkinson's disease in wild type and alpha-synuclein-deleted mice. *Exp. Neurol.* **2008**, *210*, 182–193. [CrossRef]
35. Fleming, S.M.; Ekhator, O.R.; Ghisays, V. Assessment of sensorimotor function in mouse models of Parkinson's disease. *J. Vis. Exp.* **2013**, *76*, 50303.
36. Prabitha, P.; Justin, A.; Ananda Kumar, T.D.; Chinaswamy, M.; Kumar, B.R.P. Glitazones activate PGC-1α signaling via PPAR-γ: A promising strategy for antiparkinsonism therapeutics. *ACS Chem. Neurosci.* **2021**, *12*, 2261–2272.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Role of non-neuronal cells in neurorepair: a focus on proteinopathy and neurodegeneration

Santiago Rodríguez-Carreiro[1, 2, 3, 4], Maria del Carmen Nogales[1], David Jiménez-Galán[1], Sara Carmona-Lorenzo[1], Alicia Caro-Martín[1], Elisa Navarro[1, 2, 3, 4, *, #], Noemí Esteras[1, 2, 3, 5, 6, *, #]

1 Department of Biochemistry and Molecular Biology, School of Medicine, Complutense University of Madrid, Madrid, Spain
2 Neurochemistry Research Institute, Complutense University of Madrid, Madrid, Spain
3 CIBERNED, Network Center for Biomedical Research in Neurodegenerative Diseases, Madrid, Spain
4 Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain
5 Group of Neurodegenerative Diseases, Hospital Universitario 12 de Octubre Research Institute (imas12), Madrid, Spain.
6 Department of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology, London, UK

#Both authors contributed equally to this work.
*Correspondence to: Elisa Navarro, PhD, elisnava@ucm.es; Noemí Esteras, PhD, nesteras@ucm.es.
orcid: 0000-0002-4056-7146 (Elisa Navarro); 0000-0002-7938-6131 (Noemí Esteras)

Abstract

Despite advances in biomedicine, therapies that prevent neuronal loss have not been effective. Several strategies targeting the defining markers of the main neurodegenerative diseases, such as amyloid beta or tau, which are pivotal in Alzheimer's disease or frontotemporal dementia, or α-synuclein, the leading actor in Parkinson's disease, have shown limited success. Nevertheless, these proteins do not act only in neurons but also are part of a network involving non-neuronal brain cells, such as astrocytes, microglia and oligodendrocytes, as well as the peripheral immune system. In this review, we aim to cover the role of non-neuronal central and peripheral cells in proteinopathies associated with α-synuclein, amyloid-β and tau, with the objective of identifying new options for regenerative medicine aimed at neuroprotection and neurorepair.

Key words: amyloid-β; astrocytes; biomedical engineering; microglia; neurodegeneration; oligodendrocytes; peripheral cells; regenerative medicine; regenerative therapy; synuclein; tau

doi: 10.4103/REGENMED.REGENMED-D-24-00005
Funding: This work was supported by grants from the Spanish Ministry of Science, Innovation and Universities to EN (PID2022-139936OA-I00) and NE (PID2022-137011OA-I00/AEI/10.13039/501100011033/FEDER, EU) and a Ramon y Cajal Fellowship to NE (RYC2021-034267-I) funded by the Spanish Ministry of Science and Innovation MCIN/AEI/10.13039/501100011033 and the European Union "NextGenerationEU"/PRTR. SRC is a predoctoral fellow supported by the FPU Program (Ministry of Education and Universities).
How to cite this article: Rodríguez-Carreiro S, Nogales MDC, Jiménez-Galán D, Carmona-Lorenzo S, Caro-Martín A, Navarro E, Esteras N. Role of non-neuronal cells in neurorepair: a focus on proteinopathy and neurodegeneration. Regen Med Rep. 2024;1(1):31-51.

Introduction

The deposition of protein aggregates in specific brain regions is a common feature of many neurodegenerative conditions. In particular, the accumulation of misfolded amyloid-β (Aβ) and tau in Alzheimer's disease (AD) and of α-synuclein in Parkinson's disease (PD) is closely associated with neurodegeneration. Traditionally, research has focused predominantly on neurons, as neuronal loss is a hallmark of these disorders. However, despite extensive efforts, these diseases remain incurable, and existing treatments offer only temporary relief without addressing the underlying neurodegeneration, underscoring the need for a broader perspective. Emerging evidence highlights the significant role of non-neuronal cells, including central cells — astrocytes, microglia, and oligodendrocytes — and peripheral cells, such as lymphocytes, in modulating disease processes and contributing to neurorepair. These insights have opened new avenues for therapeutic interventions in regenerative medicine. In this review, we examine recent advances in understanding the role of non-neuronal cells in the pathogenesis of proteinopathies. We begin by providing an overview of the physiology of

these cells, followed by a discussion of their contributions to proteinopathies induced by α-synuclein, Aβ and tau, focusing on their roles in protein aggregation, clearance and spread. Modulating these non-neuronal functions in regenerative medicine has promising potential for developing therapies aimed at promoting neurorepair in patients with proteinopathies.

Search Strategy

We searched PubMed for journal articles using different terms such as "astrocytes," "microglia," "oligodendrocytes," "peripheral immune cells," "tau," "amyloid beta," "alpha synuclein," "Alzheimer's disease," "Parkinson's disease," "frontotemporal dementia," "proteinopathy," "uptake," "spreading," and "removal." We included those papers judged to be more relevant for this review, prioritizing papers published in the last 5 years. We also included seminal papers with groundbreaking findings.

Physiological Role of Non-neuronal Cells

Non-neuronal cells in the brain: microglia, astrocytes and oligodendrocytes

When discovered, glial cells were considered mere backup players, while neurons the main character within the central nervous system (CNS) (**Figure 1**). This misconception was initiated when, in 1846, Rudolf Virchow described glia as connective tissue that served as support for nervous elements in the brain, the spinal cord and higher sensory nerves. Thus, he named this supportive structure "neuroglia" or "Nervenkitt," a German word meaning "nerve glue." Throughout the 19th and 20th centuries, the development of several staining techniques led to a more refined characterization of glia, distinguishing different types of glial cells. Additionally, by this time, the scarce functions attributed by Virchow had already been questioned by Golgi and Ramón y Cajal. Currently, the scope of glial cells has a wide variety of functions beyond being a connective tissue and will be briefly reviewed below.

Astrocytes

Michael von Lenhossek was the first to introduce the term "astrocyte" to describe these star-shaped cells, although it was popularized by Ramón y Cajal, who developed an astroglia-specific gold-sublimated staining technique that labels glial fibrillary acidic protein (GFAP). Astrocytes, as well as neurons and oligodendrocytes, are derived from neuroepithelium radial glia through a process involving extracellular and intracellular signals that regulate the neural or glial fate of progenitors.[1]

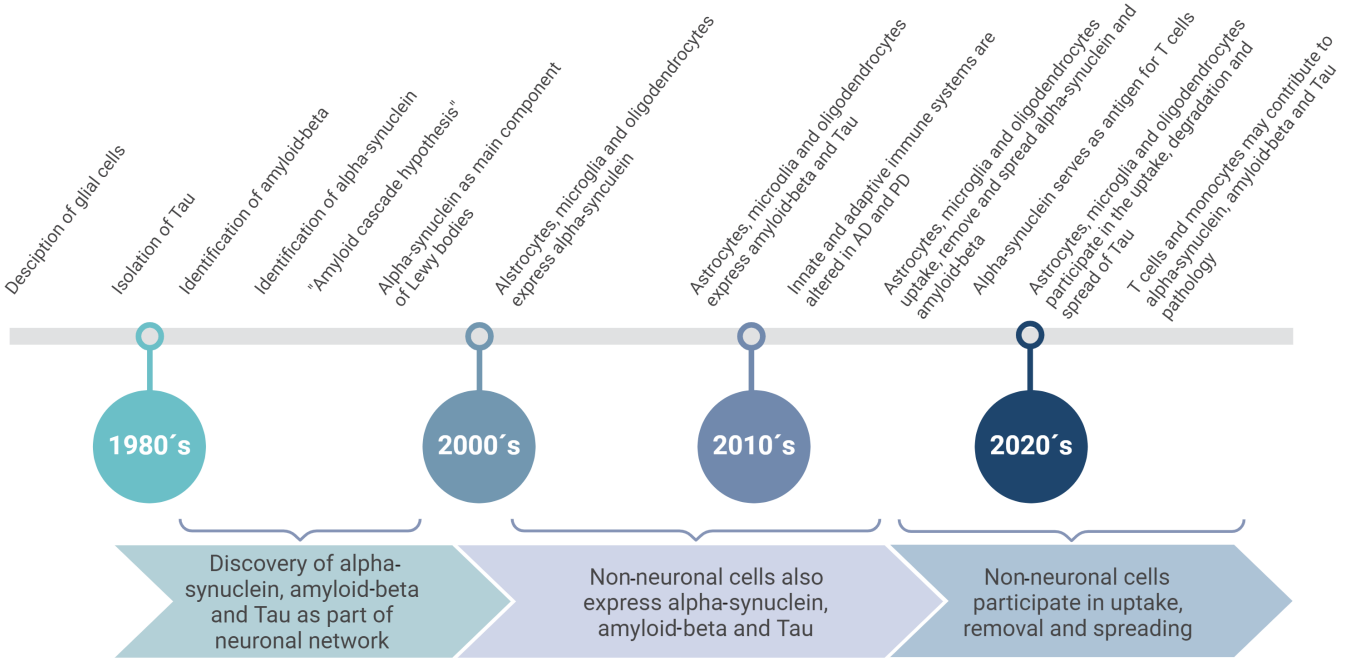


Figure 1: Timeline of research on non-neuronal cells and alpha-synuclein, amyloid-beta and tau proteinopathies.
Note: Created with BioRender.com. AD: Alzheimer's disease; PD: Parkinson's disease.

These GFAP-positive cells are essential for maintaining CNS homeostasis and carry out pivotal functions both in development and in the adult brain. During development, astrocytes shape synapses and induce synapse maturation, whereas in the adult brain, they participate in neurotransmitter recycling, maintenance of the blood–brain barrier (BBB), ionic buffering, energy homeostasis and neural synapsis.[2, 3] Astrocytes respond extensively to CNS injury. Although in the past, there were binary divisions of "good" *vs*. "bad" or "A1" *vs.* "A2," the explosion of "omic" tools has expanded our knowledge of molecular alterations. Although there has been some confusion and controversy in the field, the accepted term underlying these changes during damage or neurodegeneration is "reactive astrogliosis."[4] These reactive astrocytes are astrocytes that undergo profound and multifactorial functional, molecular and morphological changes in response to brain damage.[4]

Among the alterations during neurodegeneration, astrocytes display calcium dyshomeostasis, altered glutamate uptake or mitochondrial dysfunction,[3] contributing to synapse loss and inflammation. The outcomes of manipulating reactive astrocytes in different neurodegenerative diseases suggest complex and context-dependent roles. Reactive astrocytes can both lose beneficial functions and gain harmful functions, impacting synaptic function, neuroinflammation and neuronal survival, highlighting their potential for protective and detrimental effects.[3]

Microglia

The term "microglia" was first used by the Spanish researcher Pio del Río-Hortega, who proposed a mesodermal origin for these cells. This proposal took hold over the years with several studies identifying typical macrophage markers within microglial cells.[5] This supposed a change of paradigm, as macrophages derive from the hematopoietic system, which differentiates from mesoderm. Thus, the current hypothesis is that macrophage progenitors, which originate in the yolk sac during primitive hematopoiesis, migrate throughout the embryo to the CNS to form microglia.[6]

The microglial depletion paradigm and ablation of microglial genes have led to the discovery of a variety of functions controlled by microglia. Because they are immune cells, they oversee immunosurveillance through surface receptors that act as immune pattern recognition receptors, leading to signaling pathways that mediate neuroinflammation. Microglia contribute to CNS homeostasis, accomplishing a variety of functions, such as regulating brain development through the modulation of synapses via a process called "synaptic pruning."[7] Moreover, microglia adapt the CNS structure by controlling neurogenesis, phagocytizing apoptotic neurons during development, guiding vasculogenesis or contributing to myelinization.[8]

Traditionally, microglia were classified into two main states: a "bad" state, associated with disease, in which microglia are reactive, change to an ameboid morphology and are proinflammatory (also M1), and a "good" state, associated with a homeostatic situation, in which microglia are resting and have a ramified morphology (also M2). This dichotomy is no longer accepted, as it has been described that microglia are heterogeneous and plastic cells in constant adjustment depending on their local environment stimulus: even in a "homeostatic" state, microglia can change depending on intrinsic cues such as age, sex or circadian rhythms.[8, 9] According to this theory, many microglia coexist in a variety of context-dependent microglial states depending on environmental perturbations and can actively switch among these phenotypes.[8]

Interestingly, specific phenotypes have been described to appear in neurodegenerative diseases, with the first phenotype being disease-associated microglia in a model of AD.[10] These neurodegenerative phenotypes present a signature morphology and gene expression that varies throughout the progression of the disease. However, whether these profiles are detrimental is still elusive.

Oligodendrocytes

We also owe the introduction of the term "oligodendrocytes" to Pío del Río-Hortega, which was the first to stain and precisely describe these cells. Like neurons and astrocytes, oligodendrocytes originate from neuroepithelial cells in the neural tube. Thus, radial glia serve as common progenitors for these three types of cells, in the case of oligodendrocytes, through the generation of oligodendrocyte precursor cells (OPCs).[1]

Owing to their similarities to Schwann cells, which are in charge of myelinating the peripheric neural system, the hallmark roles of oligodendrocytes are myelinization and myelin regeneration within the developing and injured CNS,[11] which are essential for reducing energy consumption and increasing the velocity of electrical transmission along neurons.[12] Apart from maintaining neuronal function through enhancing neuronal action potentials, they also support neurons metabolically by accumulating metabolites and shuttling them to the neurons in accordance with their metabolic necessity.[13, 14] Moreover, OPCs are involved in innate immunity by communicating with microglia to modulate

neuroinflammation.[15]

Emerging evidence points to oligodendrocytes, the cells that have received less attention, as the most vulnerable cell types in response to brain changes that occur during neurodegenerative diseases.[16] Their functionality is extensively compromised during neurodegeneration, including disruption of metabolic and trophic support to neurons as well as aggravation of oxidative stress. Different oligodendrocyte subsets are described in the context of aging and neurodegeneration,[17] although more research is needed to fully understand their role in disease.

Peripheral cells: crosstalk between the central nervous system and the peripheral immune system

In addition to the non-neuronal cells previously reviewed, brain homeostasis and damage are also shaped by the collaboration of peripheral cells. Although the CNS has been regarded as immune-privileged for many years, there is evidence supporting the idea of central–peripheral immune crosstalk, especially in pathological conditions.[18] In this context, cytokines released in the periphery can cross the BBB and directly cause neurotoxicity, as well as activate microglia and other glial cells.[18] Additionally, immune cells from the periphery can enter the brain and worsen neuroinflammation, especially after BBB disruption, highlighting the relevance of the peripheral immune system (both innate and adaptive) in neuropathological events, including proteinopathy and neurodegeneration.[19] The innate immune system serves as the body's initial line of defense, which is predominantly nonspecific and driven by monocytes, macrophages, neutrophils, dendritic cells, mast cells and natural killers. In contrast, adaptive immunity offers highly specific responses, confers long-lasting protection against pathogens[20] and is composed of B cells and T cells. In the context of neuropathological processes, lymphocytes and monocytes/macrophages seem to be the main players[19]; therefore, we will focus on them.

Lymphocytes: responsible for adaptive immunity

Lymphocytes constitute the principal cellular components of lymphoid tissue present in the blood, and on the basis of their receptors and functionality, lymphocytes are classified into two main populations. One type, thymus-derived T cells, is involved in mediating cellular immunity, whereas the other type, bone marrow or bursa-derived B cells, is responsible for synthesizing and secreting antibodies, thereby mediating the humoral immune response.[20]

T lymphocytes are characterized by the expression of the T-cell receptor, which simultaneously recognizes two distinct molecules, an antigen and a molecule encoded by the genes of the major histocompatibility complex (MHC), and they can be classified as $CD8^+$ or $CD4^+$.[20] Under normal conditions, lymphocyte trafficking into the CNS is significantly lower than that into other organs because of the tight junctions in the cerebral vasculature and the lower binding affinity to cultured cerebral and retinal endothelium than to nonspecialized endothelia.[21] However, activated T cells express adhesion molecules (such as the intercellular (ICAM-1) and vascular (VCAM) adhesion molecules), chemokine receptors and integrins that allow them to traverse the BBB to carry out immune surveillance of the CNS.[22] Although naïve T cells, which do not express crucial proteolytic enzymes and adhesion ligands, are believed to circulate only between the blood, lymph, and secondary lymphoid organs, they can also be present in the healthy, noninflamed CNS.[23]

B lymphocytes originate and differentiate in the bone marrow. Unlike T lymphocytes, the B-cell receptor on B cells is an immunoglobulin that recognizes only one molecule, the antigen, leading to B-cell activation.[24] Upon activation, naïve B cells undergo proliferation and differentiation, resulting in the production of humoral responses through antibody production by terminally differentiated plasma cells and thereby the establishment of long-term immunity.[24] Additionally, B cells play crucial roles in neutrophil recruitment and the activation of other immune cells, such as T cells.[24] Like T lymphocytes, the infiltration of B lymphocytes into the CNS and their contribution to neurodegenerative diseases have been demonstrated.[25]

Monocytes and macrophages: part of the peripheral innate immune system

Myeloid cells are a group of hematopoietic-derived cells that include neutrophils, monocytes, macrophages and dendritic cells and play essential roles in inflammation; antimicrobial defense; and tissue repair and remodeling. Specifically, monocytes and macrophages are central to tissue homeostasis and immune responses and are essential components of the mononuclear phagocyte system.[26] Monocytes are present in the blood and spleen, but their largest reservoir under homeostatic conditions is the bone marrow. Monocytes leave the bone marrow and move to inflamed tissues through the peripheral blood system. They are attracted to and mobilized from the bloodstream by adhering to the endothelium, followed by transendothelial migration and movement

to the target tissue in response to immunologic or inflammatory stimuli. In tissues, monocytes differentiate and mature into macrophages, which are the active cells of their lineage.[26] Macrophages are highly plastic cells whose phenotype and transcriptional program are adjusted on the basis of the microenvironmental stimuli they encounter. As a result, macrophages exhibit a diverse range of activation states.[27] In addition, they colonize every organ in the body and have specific names depending on their location, such as Kupffer cells in the liver, microglia in the brain, and osteoclasts in the bone.[26]

Like lymphocytes, monocytes can infiltrate the CNS.[28, 29] Regarding neuropathological processes, several studies have indicated that monocyte infiltration into the brain is correlated with various forms of injury and disorders, suggesting that monocyte infiltration is involved in neurodegenerative diseases, not only in the context of inflammatory diseases.[30]

Role of Non-neuronal Cells in Proteinopathy, Neurodegeneration, and Regenerative Medicine

AD, PD and frontotemporal dementia (FTD) are neurodegenerative disorders characterized by progressive cognitive and/or motor impairments. A common factor among these diseases is the abnormal accumulation of specific proteins in the brain, a condition known as proteinopathy. This pathological build-up disrupts normal neuronal function and leads to the deterioration of brain tissue, ultimately resulting in the clinical manifestations of each disease.[31] In the following sections, we focus on proteinopathies derived from non-neuronal α-synuclein, Aβ and tau dysregulation, which are leading factors of PD, AD and FTD. Moreover, we review the contribution of non-neuronal cells to the pathology derived from the dysregulation of these proteins. The role of non-neuronal cells in proteinopathy is summarized in **Figure 2**.

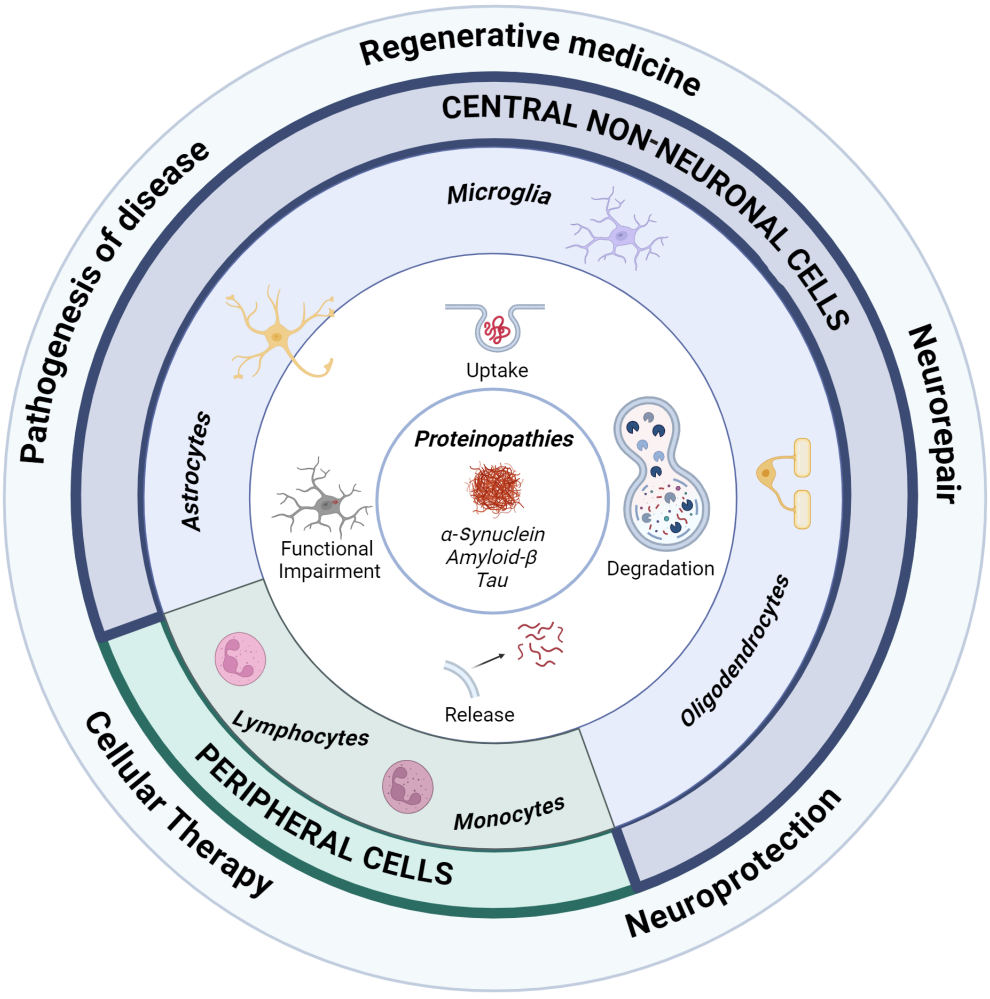


Figure 2: Schematic illustration of the role of non-neuronal cells in proteinopathy.
Note: Created with BioRender.com.

α-Synuclein

α-Synuclein was first identified in 1988 as a neuron-specific protein localized to presynaptic terminals and the nucleus[32]; hence, it was named synuclein, with "syn" referring to synaptic vesicles and "nuclein" to the nuclear envelope. This small protein (14.5 kDa) consists of a 140 amino acid sequence characterized by three distinct regions: an amphipathic *N*-terminal lipid-binding domain, which facilitates interaction with membranes and vesicles; a hydrophobic nonamyloid component prone to clustering; and an intrinsically disordered acidic *C*-terminal region, enabling interactions with metals and other proteins. This structural organization allows α-synuclein to adopt a dual conformation, transitioning between a soluble unfolded monomer[33] and membrane-bound multimers with helical structures.[34] While the normal cellular role of α-synuclein has not been fully elucidated, its ability to bind to phospholipid membranes and its presynaptic distribution suggest functions in vesicle trafficking,[35] neurotransmitter release[36] and synaptic plasticity.[37]

Since the discovery of α-synuclein as the main component in Lewy bodies, this protein has been the focus of numerous investigations because of its prominent role in several neurodegenerative disorders characterized by the accumulation of α-synuclein aggregates in the brain.[38, 39] These disorders are collectively known as synucleinopathies and include PD, dementia with Lewy bodies and multiple system atrophy (MSA). In this type of disorders, α-synuclein appears to acquire neurotoxic effects upon its misfolding and fibrillation, which ultimately leads to neuron degeneration.[40] Moreover, α-synuclein is thought to propagate in a cell-to-cell manner,[41] spreading the synucleinopathy to different brain areas. Much of the research on α-synuclein pathology has focused on neurons, but the involvement of other non-neuronal cell populations, such as glial cells, in these disorders should not be underestimated.

α-Synuclein expression in central non-neuronal cells

α-Synuclein is most highly expressed in neurons, where it primarily aggregates, resulting in the formation of Lewy bodies. In addition to neuronal aggregation, glial α-synuclein inclusions are also a well-described feature of synucleinopathies. Aggregates of this protein have been observed in astrocytes in PD and dementia with Lewy bodies,[42] as well as in oligodendrocytes in MSA.[43] The origin of protein inclusions in glial cells is debated, but it is generally agreed that neuron-to-glia transfer may contribute to the propagation of synucleinopathy.[44, 45] Endogenous expression of α-synuclein has also been identified in glial populations (see www.brainrnaseq.org), which points to the possibility of *de novo* induction of α-synuclein pathology in glia. Indeed, the detection of α-synuclein-positive astrocytes in the human brain, which usually do not display Lewy bodies,[46] might support this idea. However, the level of α-synuclein expression in these glial cells is notably lower than what would be expectedly required to initiate *de novo*-templated aggregation.[47] Therefore, there is still controversy regarding this theory.

Tanji et al.[48] were the first to detect α-synuclein mRNA and protein in human astrocytic cell lines, confirming the presence of this protein via an immunofluorescence assay. *In vitro* evidence has also shown that, under conditions of inflammation or oxidative stress, the expression of α-synuclein in astrocytes increases,[48, 49] indicating that the increase in α-synuclein levels is related to the activation status of astrocytes. Since then, numerous transcriptomic studies conducted in astrocytes have confirmed the expression of α-synuclein in this brain cell type,[50-53] even though its expression is much lower than that in neurons. This consistent evidence of α-synuclein expression by astrocytes may indicate a role of this protein in normal astrocytic function. In fact, α-synuclein in astrocytes has been implicated in fatty acid metabolism,[54] glutamate uptake, BBB function and water transport.[55]

Like in other immune cells, detectable expression levels of α-synuclein have also been identified in microglia,[56] but to a much lesser extent than in astrocytes.[57, 58] Through these studies, it has been possible to establish a main role of α-synuclein during microglial differentiation, as higher levels of expression were found in microglial precursors than in mature cells.[59] Both overexpression and deficiency of α-synuclein lead to impaired cytokine release and phagocytosis, ultimately affecting the overall reactive phenotype.[60, 61] One speculation about how α-synuclein can affect these processes is that, as occurs in neurons, it may also contribute to SNARE complex assembly in microglia. This step is crucial for the formation of vesicles that participate in autophagic clearance and the release of cytokines.[62]

Different α-synuclein conformations can induce activation in astrocytes and microglia, upregulate the expression of proinflammatory cytokines,[63, 64] promote the generation of reactive oxygen and nitrogen species and induce morphological changes.[64, 65] Extracellular α-synuclein can induce this activated glial state through its interaction with different cell surface receptors, such

as toll-like receptors (TLRs),[66] and others.

Finally, glial cytoplasmic inclusions, consisting of inclusions of α-synuclein residing in oligodendrocytes, are a major hallmark of MSA.[67] Although increasing evidence supports the neuronal origin of the α-synuclein that composes these inclusions,[45, 68] the aggregation of endogenous oligodendroglial α-synuclein should not be dismissed. Among brain cells, oligodendrocytes and OPCs exhibit the highest levels of α-synuclein expression, after neurons[57](see www.brainrnaseq.org). Most studies have confirmed the expression and presence of α-synuclein in oligodendrocyte lineage cells *in vitro*,[69] revealing a developmental pattern in the expression of this protein. Djelloul et al.[70] conducted one of the most comprehensive studies on the expression of α-synuclein in oligodendroglial lineage cells of rodent and human origin, both *in vitro* and *in vivo*. Using quantitative PCR, Western blotting and immunocytochemistry, they reported a significant decrease in α-synuclein during oligodendrocyte maturation,[70] similar to what was found in microglia. This compelling evidence for α-synuclein expression during oligodendrocyte lineage cell development substantiates a functional role for α-synuclein, even though this role has not been adequately clarified. When synucleinopathy reaches oligodendrocytes, as occurs in MSA, it leads to oligodendroglial dysfunction, demyelination and neuroinflammation.[71]

α-Synuclein uptake and spreading

The low endogenous expression of α-synuclein by astrocytes, microglia, and oligodendrocytes suggests that additional mechanisms are involved in the spread of synucleinopathy. An increasing body of evidence shows that α-synuclein can propagate through neurons in the CNS[72, 73] in a widely accepted prion-like manner, with misfolded α-synuclein serving as a template for monomeric α-synuclein.[74] α-Synuclein can be transferred to surrounding neurons either through passive release into the extracellular space from dead neurons[75] or through active secretion from diseased neurons overloaded with α-synuclein as part of the cellular quality control system to eliminate damaged and harmful proteins. These mechanisms include the formation of exosomes[76] and tunneling nanotubes (TNTs).[77]

Importantly, α-synuclein is taken up not only by neighboring neurons but also by glia in a more efficient way.[44, 78, 79] Thus, glial cells can be initially activated in response to extracellular α-synuclein, promoting a resolutive inflammatory profile, phagocytosis and clearance. However, the eventual accumulation of damage associated with protein overload drives a harmful, disease-accelerating phenotype,[79, 80] which alters glial homeostasis and favors the spread of the synucleinopathy.

In addition to receptor-mediated endocytosis,[44, 81] other pathways synergistically contribute to the internalization of neuronal α-synuclein into glial cells. Exosomes, which are small membrane-enclosed vesicles in the extracellular space, allow more efficient uptake of α-synuclein in neurons and microglia.[76, 82] Although the uptake mechanism responsible for exosome incorporation has not yet been established, phagocytosis seems to involve professional phagocytes such as microglia.[83] Clathrin-mediated, lipid raft-mediated and actin network-dependent endocytosis have also been reported as potential means for exosome internalization.[84]

With respect to astrocytes, little evidence has proven the ability of this cell type to take up exosomes, which are very rare compared with those of neurons and microglia.[85] The other means by which neurons transfer α-synuclein to glial cells is based on networks of TNTs, which are actin-based membranous extensions 50–200 nm in diameter that allow communication and exchange between neighboring cells.[86] These structures commonly connect neurons to astrocytes as well as astrocytes with each other.[77] This astrocyte-to-astrocyte communication appears to act as a double-edged sword in synucleinopathy, given that overloaded astrocytes can recruit functional mitochondria from healthy astrocytes as a rescue strategy, whereas aggregated α-synuclein is also transferred from affected to unaffected astrocytes, initiating α-synuclein pathology and propagating proteinopathy.[87] Although a similar behavior has been described among microglia,[88] the presence of functional TNTs between neuronal and microglial cell lines that allow the movement of mitochondrial and α-synuclein aggregates uni- and bidirectionally has been reported recently.[89]

Astrocytes and microglia can act as "spreaders" when they eventually become overloaded by synucleinopathy. The means by which these glial cells can transmit α-synuclein to glia and neurons are primary exosomes and TNTs. Exosomes are released by microglia and astrocytes[90, 91] and are able to selectively target neurons and induce further aggregation in those that assimilate them. In the case of TNTs, aggregated α-synuclein increases the formation of nanotubes in microglia,[88] which permits the accumulation of proteins through an intercellular network. As mentioned previously, astrocytes can also display TNTs for α-synuclein

transmission.[77] These connections do not appear to be formed indistinctively among brain cells, but each cell type preferentially connects to specific cell populations. For example, while microglia tend to communicate among themselves for putative collaborative clearance,[88] astrocytes can connect with neurons,[55] other astrocytes[92] and microglia[87] to potentially recruit help for removing α-synuclein, playing a pivotal role as major spreading agents. Less is known about the role of oligodendrocytes, given that more studies are necessary to explore their ability to spread α-synuclein, in which new models of propagation from peripheral tissues to white matter within the CNS are used.[93]

The accumulation of intracellular α-synuclein in oligodendrocytes may have a more complex origin than that in astrocytes and microglia. Although α-synuclein transfer from neurons must occur,[94] it is believed that increased expression[70] and decreased degradation[95] might contribute to α-synuclein aggregation in oligodendrocytes. Nevertheless, several studies have shown that oligodendrocytes and OPCs have the ability to take up neuronal secreted α-synuclein monomers and oligomers both *in vitro*[45, 69] and *in vivo*.[45] These studies suggest that endocytic mechanisms are involved in the internalization of α-synuclein in oligodendrocytes. This process seems to be mediated by cell surface heparan sulfate proteoglycans, at least for α-synuclein fibrils.[96] Moreover, the release of neuronal α-synuclein through vesicles is believed to also be a crucial mechanism, considering the role of exosomes in communication between neurons and oligodendrocytes.[97]

α-Synuclein removal and clearance

Once α-synuclein is internalized by cells, it is degraded, mainly through two pathways: the ubiquitin–proteasome system[98] and lysosomal-mediated pathways (phagocytosis and autophagy).[99] In microglia, a specific α-synuclein degradation pathway termed "synucleinphagy" has been identified.[100] Synucleinphagy is regulated by TLR4/nuclear factor-κB signaling, through which the transcription of the autophagy receptor p62/SQSTM1 is upregulated. p62 binding to ubiquitin-linked α-synuclein is likely to mediate the sequestration of the protein into autophagosomes for lysosomal degradation. These findings correlate with previous studies involving microglial TLR4 in the clearance of extracellular α-synuclein.[66] Ingested α-synuclein aggregates are also thought to be efficiently degraded by astrocytes following proteasomal[101] and lysosomal digestion,[81] even more efficiently than neurons.[78] Thus, when α-synuclein is recognized by specific receptor(s) on the astrocytic surface, it is enclosed in phagosomes, which finally fuse with lysosomes.[44, 79] Multiple studies have also reported several forms of autophagy as degradative processes for α-synuclein within affected oligodendroglia in MSA, including macroautophagy and chaperone-mediated autophagy,[69, 102] as well as the ubiquitin–proteasome system.[103]

In addition to being cleared via intracellular degradative pathways, various forms of α-synuclein in the extracellular space have been shown to be cleared directly by extracellular proteases, including plasmin, neurosin or matrix metalloproteases (MMPs).[104-106] Some species of these proteolytic enzymes are secreted by glial cells, such as microglial insulin-degrading enzymes or MMP1, MMP3, MMP9 and MMP13[107] or the oligodendroglial serine protease KLK6.[108] However, when cleavage occurs at specific sites, it can contribute to pathology though the generation of truncated species that favor nucleation and aggregation.[109]

Overall, the ability of glial cells to clear α-synuclein is suggested to be initially beneficial, as these cells act as neuroprotective scavengers that do not contribute to the propagation of the disease.[78, 87] However, upon extensive uptake, the lysosomal machinery may become overloaded, resulting in incomplete degradation of α-synuclein. Indeed, α-synuclein has been shown to inhibit autophagic flux in microglia,[110, 111] astrocytes,[101] and oligodendrocytes.[103] This situation of autophagy blockage leads to the formation of intracellular deposits in astrocytes.[79] Astrocytic deposits exhibit distinctive characteristics from those of neuronal inclusions, such as truncation *of the N*- and *C*-termini as well as phosphorylation at tyrosine 39.[42] These unique structural properties can alter the dynamics of α-synuclein in terms of aggregation and prion-like transmission.[112] Therefore, owing to excessive burden, a threshold is eventually reached whereby glial impairment occurs, affecting several specific cellular processes, such as mitochondrial damage,[79] aberrant calcium signaling[113] and the establishment of an inflammatory microenvironment that leads to sustained neuronal damage.[44, 64]

Implication of peripheral cells in α-synuclein pathology

Among peripheral players, T cells, which have been found in the brains of PD mice,[114] particularly in the substantia nigra, are known to be triggered by α-synuclein expression.[115] Notably, microglia and astrocytes can function as antigen-presenting cells. Partial digestion-derived α-synuclein peptides may be displayed by MHC class II molecules on microglial and

astrocytic surfaces and act as epitopes for $CD4^+$ T-cell activation,[116] leading to an adaptative immune response in which α-synuclein is targeted.

The activation of T cells by α-synuclein has been shown to kill dopaminergic neurons, contributing to pathology.[114, 117] This response elicited by T cells to α-synuclein presentation seems to be dependent on MHCII.[118] Furthermore, it was recently shown by Sulzer et al.[119] that peptides derived from α-synuclein act as antigenic epitopes displayed by MHC alleles, driving the T-cell response in PD patients. The specificity of MHCII alleles in the T-cell response draws a bridge between the known genetic associations of MHC alleles and PD observed through genome-wide association studies.[120] Similarly, T-cell depletion has been demonstrated to prevent reverse behavioral deficits and most histological alterations in PD model mice[114] and reduce CNS myeloid activation associated with α-synuclein expression.[115]

Conversely, the role of B cells in PD is less well understood and is still under investigation.[121] A recent study explored the associations between B-cell immunological traits and more than 480,000 individuals from genome-wide association studies via Mendelian randomization.[122] Although no significant associations were observed, several B-cell traits were observed to be either detrimental or protective at a nominal p value.

Other peripheral immune cells also seem to be involved in α-synuclein pathology. In this context, natural killer cells have been proven to internalize and degrade α-synuclein aggregates via the endosomal/lysosomal pathway, playing a beneficial role in modulating synuclein pathology and motor symptoms in a preclinical mouse model of PD.[123] In contrast, a variety of studies have shown how monocytes and macrophages can respond to α-synuclein and in a murine model of α-synuclein fibril expression, where monocytes have been shown to be recruited to the striatum and can express MHCII.[124] This peripheral immune recruitment seems to occur prior to neurodegeneration and might also be implicated in neuronal loss.

Amyloid-β

Aβ was identified in the 1980s as the main component of neuritic plaques, which are extracellular plaques that deposit in the brains of patients with AD.[125] Since then, its deposition has long been considered the central event in the pathogenesis of the disease, leading to the so-called "amyloid cascade hypothesis."[126] Many efforts have been made in recent years to pharmacologically target this protein in the development of therapies for AD, leading to repeated failures but also promising results.[127] In recent years, a more complex hypothesis in which AD is explained as a multifactorial disease in which glial cells and the immune system also interact with Aβ during disease progression has been formulated.[128]

Aβ is a peptide of approximately 40 amnoacids formed by proteolytic cleavage of the amyloid precursor protein (APP). APP is a transmembrane protein found mainly in the axonal and somatodendritic compartments of neurons and is thought to play a role in many aspects of neuronal homeostasis, including neuronal development, signaling and intracellular transport.[129] It undergoes sequential proteolytic processing, which occurs via two alternate pathways, leading to the extracellular release of different peptides: processing by β- and γ-secretase complexes is involved in the amyloidogenic processing of APP, which generates Aβ, whereas α- and γ-secretases are involved in the nonamyloidogenic pathway, which generates soluble fragments not linked to disease.[129] Under physiological conditions, APP is processed predominantly through the nonamyloidogenic pathway. However, some mutations in APP and/or in the γ-secretases, which cause familial AD, as well as certain age-related risk factors, increase the processing of APP through the amyloidogenic pathway, leading to an increase in the relative production of Aβ.[130] Aβ monomers can rapidly aggregate to form oligomers and later fibrils, which are deposited into neuritic plaques. While fibrils are larger and insoluble, Aβ oligomers are soluble and are thought to play a key role in the pathogenic process and the spread of the protein through the brain.[130]

Amyloid-β expression in non-neuronal cells

Traditionally, excitatory neurons have been considered the main source of Aβ in AD.[131] However, there is evidence that glial cells also contribute to the overall Aβ load in the brain.[131, 132]

Although to a much lesser extent than neurons, reactive astrocytes recruited to amyloid plaques positively regulate APP,[133] β-secretase[134] and γ-secretase in the brain.[132] This increased expression is mediated by cellular stress and proinflammatory cytokines,[135] although the influence of inflammation on γ-secretase expression has not yet been demonstrated. Aβ itself from nearby neurons induces Aβ production in astrocytes,[136, 137] thus generating a vicious cycle in which astrocytes produce Aβ in a self-sustaining manner. During this cycle, exposure of astrocytes to Aβ triggers reactive astrogliosis, including increased GFAP, hypertrophy, and the release of proinflammatory

cytokines such as tumor necrosis factor α, interleukin (IL)-6, IL-1β, and IL-1α.[138] Aβ also leads to dysregulation of astrocytic calcium levels, leading to disruption of gliotransmission and, consequently, to neuronal hyperactivity.[139] Synaptic dysregulation is also linked to an Aβ-induced decrease in astrocytic uptake of glutamate[140] and GABA[141] and an increase in the release of both neurotransmitters by these cells.[141, 142] Finally, astrocytes exposed to Aβ exhibit decreased glycolytic metabolism, leading to reduced lactate production for neuronal consumption[143] and mitochondrial dysfunction, which further worsens pathology.[144]

There is not much evidence about the involvement of microglia in Aβ synthesis, although microglia have been shown to possess the cellular machinery to cleave APP[145] and generate Aβ.[131] When microglia are exposed to Ab, they release profinlammatory cytokines and reactive oxygen and nitrogen species through the activation of several receptors in the surface of microglia, including TLRs or the receptor for advanced glycation end products.[146] Similarly, Aβ stimulates the microglial NLRP3 inflammasome, leading to IL-1β release, Aβ aggregation and phagocytic dysfunction.[147]

High levels of APP mRNA and amyloidogenic processing enzymes have been detected in oligodendrocytes, although they seem to be less efficient in the production of Aβ than neurons.[148] Interestingly, myelin dysfunction affects APP metabolism and alters microglial responses, which fail to clear Aβ deposits in a mouse model of AD.[149]

In summary, there is evidence that glial cells possess the Aβ synthesis machinery and can produce Aβ, especially under neuroinflammatory conditions such as those characteristic of AD. However, it is important to develop studies that provide quantitative data to determine to what extent astrocytes, microglia and oligodendrocytes contribute to the Aβ burden in the AD brain. Importantly, soluble oligomeric Aβ and amyloid plaque-deposited Aβ interact not only with neurons but also with microglia, astroglia, and oligodendrocytes, triggering a range of harmful cellular responses that eventually result in neuronal dysfunction and death.

Amyloid-β uptake and spreading

Aβ is known to spread through the AD brain via different mechanisms, including the seeding of soluble forms of Aβ[150] and neuron–neuron transfer.[151] Importantly, glial cells also play an indirect role in Aβ diffusion in AD.

Microglia are the key players in the uptake and removal of Aβ. During pathology, microglia lose their ability to clear Aβ, as they reduce the expression of Aβ-binding receptors and exhibit deleterious phagocytosis.[152, 153] While soluble Aβ forms are commonly internalized by microglia through pinocytosis,[154] fibrillar forms of Aβ are recognized primarily through the activation of innate immunity receptors, most notably TLRs.[154, 155] Another key receptor involved in this process is triggering receptor expressed on myeloid cells 2 (TREM2), which is expressed on the surface of microglia and is involved in Aβ phagocytosis.[156] However, the R47H mutation of this receptor, a major risk factor for sporadic AD, impairs the ability of TREM2 to recognize Aβ.[157] Another recently described receptor is PIEZO1, a mechanosensory ion channel expressed in microglia, which translates the stiffness elicited by Aβ plaques into a cell signaling cascade that promotes phagocytosis.[158] This channel is altered in specific subpopulations of microglia in AD in humans and in mouse models of AD. Activation of these receptors by Aβ stimulates microglial phagocytosis,[154, 155] although the proinflammatory state characteristic of AD leads to inhibition of phagocytosis,[159] contributing to amyloid accumulation. When microglia are overwhelmed in their ability to phagocytose and degrade Aβ, they release soluble neurotoxic Aβ species within microvesicles, thus contributing to the spread of Aβ to other brain regions.[160, 161] The release of these microvesicles is induced by ATP released by damaged neurons and reactive astrocytes.[162] Moreover, microglia increase the production of proinflammatory cytokines that further reduce the expression of the Aβ degradation machinery[153] and, as previously mentioned, induce its synthesis in neurons and glial cells.[135]

In addition to microglia, astrocytes can also efficiently internalize large amounts of soluble Aβ aggregates. Astrocytes internalize Aβ monomers and oligomers via phagocytosis and subsequent lysosomal degradation.[163] Oligomeric and fibrillar Aβ can be internalized via receptor-mediated endocytosis. Low-density lipoprotein receptor-related protein 1 (LRP1) takes up Aβ either directly or indirectly through its association with apolipoprotein E (ApoE).[164] ApoE is expressed and secreted mainly by astrocytes under basal conditions, although in the context of neurodegeneration, it can also be secreted by activated microglia.[165] In addition to mediating Aβ clearance through LRP1 via its Aβ-binding site, ApoE promotes Aβ degradation by proteases[164] and mediates the chemotaxis of microglia toward Aβ plaques.[166] There are three polymorphic alleles of *APOE* (ε2, ε3 and ε4),[167] which have different effects on Aβ clearance and aggregation and, therefore, may contribute to Aβ deposition. While ApoE2 is a protective factor for AD, ApoE4 dose-dependently increases the probability

of AD and reduces the age of onset.[167] Compared with ApoE2 and ApoE3, ApoE4 forms fewer stable complexes with Aβ and confers greater stability to Aβ oligomers.[168] The different ApoE isoforms also affect microglial uptake of different substrates.[169] Other receptors that mediate Aβ internalization in astrocytes include receptor for advanced glycation end products, some TLRs, some scavenger receptors, and Fc receptors.[163] Using induced pluripotent-derived astrocytes, it has been demonstrated that after uptake, astrocytes can truncate and pack Aβ with the subsequent release of toxic forms, affecting neighboring cells.[170]

As mentioned above, oligodendrocytes also produce Aβ and contribute to amyloid plaque formation in AD, even in the primary setting of pathology. According to a recent preprint, a threshold of Aβ accumulation is required for amyloid plaque to occur, and it appears that this threshold cannot be reached without the contribution of excitatory neurons; thus, oligodendroglia Aβ monomers can only aggregate if there is previous deposition.[171] These findings suggest that oligodendroglial Aβ assembles in the secondary nucleation phase of amyloid aggregate production. However, further studies on the aggregation kinetics of Aβ peptides from this cellular source are needed.

Amyloid-β removal and clearance

Some authors note that while in early-onset familial AD, there is increased production of Aβ or species that are more prone to aggregation, in late-onset sporadic AD, there is defective clearance of Aβ.[172] Therefore, efficient clearance of Aβ appears to be essential for preventing its accumulation in the brain.

Astrocytes use several mechanisms to degrade and eliminate Aβ from the brain. First, they express different enzymes that cleave Aβ into smaller, less toxic peptides.[173] Metalloendopeptidases and MMPs, such as MMP-2 and MMP-9 expressed by these cells, can degrade both Aβ monomers and fibrils.[163, 174] Nevertheless, AD is associated with dysregulation of the levels of these enzymes, which could contribute to pathology.[175] Lysosomal degradation may also be altered in astrocytes, as LC3 expression in astrocytes surrounding amyloid plaques in AD mice is decreased, possibly due to senescence-associated autophagic decline.[176] Moreover, astrocytes can scavenge Aβ through the BBB owing to their projections around blood vessels.[163] Maintenance of the BBB is also compromised in AD,[177] causing an imbalance in the clearance of Aβ through the BBB, thereby promoting its deposition in the brain.

Aβ monomers can also be degraded by microglia via proteolytic enzymes or LRP1-mediated endocytosis, but these are not the main pathways of Aβ degradation in these cells.[154] Microglia mediate Aβ degradation mainly through phagocytosis and subsequent lysosomal degradation. In AD, lysosomal acidification and the autophagic flux of microglia are impaired, leading to increased secretion of proinflammatory cytokines, recruitment and activation of immune cells, and induction of astrocyte reactivity.[178] Furthermore, there is a bidirectional relationship between lysosomal dysfunction in microglia and Aβ accumulation, which perpetuates neuroinflammation and exacerbates neurodegeneration.[178] One study detected colocalization of Iba1 (a microglial marker), ubiquitin and p62 in amyloid plaque-associated microglia. In contrast to what was observed in astrocytes, the presence of p62 indicates that autophagic flux could underlie Aβ phagocytic dysfunction[178] and could be compensated for by the ubiquitin–proteasome system.[179]

Implication of peripheral cells in amyloid-β pathology

Regarding the peripheral alterations driven by the Aβ burden, early in pathology, Aβ accumulation is associated with abnormalities in peripheral cells, especially T cells,[180] although its contribution to AD is still under study. General knowledge has pointed to a detrimental role of T cells in AD**,** mainly due to a more proinflammatory status,[181] although a recent investigation identified a subset of $CD8^+$ T cells that seem to have a suppressive role in Aβ pathology.[182] They demonstrated that $CD8^+$ T cells that express CXCR6 communicate with microglia and localize in close proximity to plaque-associated microglia. Interestingly, ablation of this subset of T cells seems to be detrimental, increasing Aβ deposition.

The contribution of B cells to Aβ pathology has been less studied, with conflicting results. While B-cell depletion has been shown to retard the Aβ burden and reduce the damage-associated microglia;[183] other authors pointed to interleukin 35 release by B cells to be protective, as neutralization of this cytokine led to an increased burden of Aβ.[184]

Peripheral monocytes are also capable of taking up Aβ, and phagocytosis is compromised in AD patients.[185, 186] These abnormalities may be derived from the reduced expression of TREM2 and CD33, which are involved in Aβ internalization and phagocytosis.[187, 188] Additionally, *in vivo* murine animal models have shown the presence of monocytes associated with vascular Aβ, whereas selective depletion of monocyte subsets

contributes to Aβ deposition in the APP/PS1 model.[189] The extent of infiltrating myeloid cells *vs.* resident-derived cells is still elusive, as is their beneficial or detrimental role.[190]

Tau

Tau was first isolated in 1975 from the porcine brain as a protein present in association with tubulin, which is essential for microtubule assembly.[191] Its main known physiological role is the stabilization of the cytoskeleton, whereas in neurons, where it is highly expressed, it has an essential role in the maintenance of neuronal integrity and axoplasmic transport.[192] In addition, it participates in the protection of genomic architecture, the regulation of myelination, synaptic plasticity, iron homeostasis and neurogenesis.[193] The tau protein is encoded by the *MAPT* gene, which is located on chromosome 17q21.3. It contains 16 exons, and alternative splicing of exons 2, 3 and 10 of the pre-mRNA results in 6 isoforms between 352 and 441 aminoacids, which are classified into two main groups depending on the number of microtubule-binding domains (encoded in exons 9–12) that they express: 3R (exon 10 exclusion) and 4R (exon 10 inclusion).[193]

Under normal conditions, tau is soluble and unfolded, but different pathological modifications, such as hyperphosphorylation, lead to its polymerization and insolubilization. Abnormal processing of tau results in its deposition in the brain in the form of intraneuronal neurofibrillary tangles and glial inclusions. These are common hallmarks of neurodegenerative disorders, such as AD, FTD and other disorders, which are collectively termed tauopathies and have very different clinical and neuropathological manifestations.[194] The alteration of tau homeostasis is thought to lead to the impairment of several physiological functions as well as apoptosis and neuronal loss, processes that are reflected as cognitive impairment and even death in the late stages of the disease.[195]

Tau expression in non-neuronal cells

Tau protein is expressed mainly in neurons, but non-neuronal cells also play a role in tau homeostasis. In fact, although neurofibrillary tangles have been more widely studied in the context of neurodegeneration, tau aggregates are also found in astrocytes in different tauopathies. Interestingly, different tauopathies are associated with different tau–astroglial morphologies, and the presence of predominant specific astrocytic tau immunoreactivity is a defining hallmark in some tauopathies. For example, the neuropathological diagnosis of progressive supranuclear palsy includes the presence of "tufted" astrocytes, which accumulate 4R tau aggregates,[196] whereas astrocytic plaques are found in corticobasal degeneration and ramified astrocytes in Pick´s disease.[197] These specific tau immunoreactivities in astrocytes present in the so-called primary tauopathies listed above differ from the astrocytic inclusions found in age-related tau astrogliopathy, which comprises a group of astroglial tau pathologies detected in the elderly population. In these pathologies, tau deposition induces the formation of thorn-shaped astrocytes and granular/fuzzy astrocytes[197, 198] in the absence of neuronal tau accumulation.

The presence of tau aggregates in glial cells has been interpreted in the verse of glial cells internalizing tau released from neurons, assuming that glial cells do not express enough amounts of tau to construct fibrillar inclusions independently.[199] Although this is the general view, the presence of tau has been observed in oligodendrocytes, suggesting that this protein has a role in myelinization.[200] Moreover, other studies using human and cultured cells have shown that oligodendrocytes contain all six tau isoforms and that tau mRNA and protein expressions are developmentally regulated.[201, 202] More recently, RNA-seq studies have shown that the *MAPT* gene is expressed mainly in neurons but also at lower levels in fetal and mature astrocytes as well as in oligodendrocytes and microglia (see www.brainrnaseq.org), all of which were isolated from the human brain.[51] Recent studies have confirmed that, in addition to neurons, *MAPT* mRNA is consistently expressed in oligodendrocytes and astrocytes, indicating that cellular *MAPT* expression varies between and within cell types even within the same anatomical area.[199] The authors suggest that this *MAPT* expression provides a pool for local protein production that can accentuate the pathological process by, for example, being phosphorylated and aggregated.[199] Interestingly, studies in induced pluripotent stem cells-derived cells with the FTD-associated mutation 10+16 revealed a different regulation of tau production when these cells were derived to either neurons or astrocytes.[203] This intronic mutation increases the 4R:3R ratio of the protein, which is otherwise balanced in the adult brain. When derived to astrocytes, these cells presented a much greater increase in the 4R:3R-tau ratio than induced pluripotent stem cells-derived neurons did, implicating glial cells in the pathogenic process and supporting the existence of cell type-specific regulation.[203] In addition to its pathological role, the fact that astrocytes and oligodendrocytes express tau under

physiological conditions indicates that this protein may play a role in the correct functioning of these cell types.

The accumulation of tau in non-neuronal cells, either by expression or uptake, can have detrimental effects on glial function. For example, in some studies, tau pathology induced an astrocyte signature involving the repression of bioenergetic and translational processes as well as the induction of inflammation pathways and protein degradation/proteostasis genes such as the inflammatory mediator Spi1 and stress-activated cytoprotective Nrf2.[204] Tau-induced impairment of glial function has also been shown to affect neighboring neurons. Uptake and accumulation of extracellular tau oligomers in astrocytes impaired ATP release, disrupting synaptic transmission in neighboring neurons.[205] Similar effects were found in a transgenic tau mouse model, in which early tau pathology affected the neurosupportive capacity of astrocytes, leading to neuron loss,[206] whereas transplantation of control astrocytes in the same transgenic mouse model rescued neuronal death.[207] Conversely, intraneuronal aggregation of tau specifically induces oxidative stress and activation of the integrated stress response in astrocytes.[208]

Together with astrocytes, tau pathology is associated with microglial activation and polarization to an inflammatory phenotype. Specifically, microglia has been reported to sense extrinsic tau 3R/4R proteins by direct interaction and trigger an innate immune response promoting brain inflammation and cognitive impairment in mice.[209] Furthermore, studies have shown that Aβ and tau pathologies act synergistically to induce disease stage-specific microglial subtypes, such as early-stage AD-associated microglia and late-stage AD-associated microglia, with distinct magnitudes and compositions of gene signatures.[210]

Tau uptake and spreading

As part of its cellular pathways, tau can be released into the extracellular space, both physiologically and by dying neurons. Released tau can then be taken up by surrounding neurons and spread, inducing the progression of the disease through synaptically connected regions of the brain but also in neurons that are not connected by synapses.[211] In the case of AD, tau protein spreads from the entorhinal cortex to the hippocampal region early in the disease.[212] Tau has been shown to colocalize with microglia in the postmortem brain tissue of AD patients and healthy controls, suggesting a role for this cell type in this process.[213] Asai et al.[214] showed that microglia promote the dissemination of tau in two independent models of tauopathy: transgenic PS19 mice and an adeno-associated virus-based mouse model, in which neuronal tau rapidly propagates from the entorhinal cortex to the dentate gyrus. Depletion of microglia in these models suppressed the propagation of tau via a mechanism involving tau phagocytosis by microglia and secretion via exosomes. Further studies confirmed that microglia can take up both soluble and insoluble tau.[213] The molecular mechanisms linking microglial activation and tau uptake, release and pathology are still unknown. A recent study has shown that tau-dependent activation of microglial nuclear factor-κB signaling is a key transcription factor in tau seeding and spreading in PS19 mice.[215] In this work, the authors showed that genetic activation of microglial nuclear factor-κB promoted tau spreading, whereas its inactivation rescued learning and memory deficits.[215]

Astrocytes can also internalize extracellular tau[216] and contribute to tau spread via multiple mechanisms.[196] Some works have investigated how astrocytic and microglial-associated genetic factors affect the spread of tau in AD. This is the case for ApoE. It is known that ApoE4 carriers present exacerbated tau pathology, but the mechanisms are not well understood. Wang et al.[217] recently showed that the selective deletion of ApoE4 in astrocytes of P301S tau transgenic mice reduced tau pathology and synaptic loss, and decreased synaptic phagocytosis by microglia, connecting both processes. Importantly, the astrocyte-secreted protein GPC-4 has been described as an inducer of ApoE4-dependent tau accumulation and propagation by promoting its endocytosis.[218] In addition to ApoE4, TREM2, a pattern recognition receptor highly expressed in microglia, represents another genetic risk factor for AD. Employing an AAV-based tau spread model, Zhu et al.[219] reported that TREM2 KO mice presented exacerbated propagation of tau into the dentate gyrus via exosomes, leading to cognitive decline and synaptic loss. Nevertheless, the role of TREM2 in tau pathology remains controversial, with different models of tauopathy presenting variable effects of TREM2 on tau pathology.[220] Indeed, sustained activation of TREM2 appears to exacerbate amyloid-induced tau pathology.[221]

Tau removal and clearance

Intrinsically connected with tau uptake, the maintenance of controlled levels of tau is essential for cellular function and health. Thus, alterations in tau clearance mechanisms are pivotal contributors to tauopathies. The two main cellular mechanisms that drive tau degradation are the proteasome and

the autophagy–lysosome pathway,[222] with astrocytes, particularly microglia, playing a key role at the cellular level. Some studies have proposed that astrocyte–microglia crosstalk may contribute to tau clearance. In humans and mice, IL-3 produced by astrocytes causes transcriptional, morphological, and functional reprogramming of microglia to clear aggregates of Aβ and tau.[223] The role of microglia is particularly important in the process of clearance and removal and has a dual and opposite role: while they are able to engulf tau seeds and process them, alterations in microglial function, or the inability to degrade the seeds might lead to further microglial dysfunction and the spread of tau.[224] Interestingly, a recent cross-sectional postmortem study with 40 different human brains examined the effects of tau oligomers on synapse loss.[225] The authors reported that tau-containing synapses were preferentially engulfed by microglia and astrocytes in the brains of patients with dementia, suggesting a cycle of glial-mediated elimination of tau-containing synapses, followed by glial dysfunction and subsequent release and propagation of oligomers, which could explain the progression of pathology.[225]

In recent years, although still causing some controversy, a new mechanism for tau (as with other proteins that accumulate in neurodegenerative disorders) removal and clearance from the brain has emerged. This system is the glymphatic system, akin to the lymphatic system but in the CNS, which is claimed to be crucial for directing the movement of interstitial fluid and clearing waste.[226] This system appears to facilitate the flux of cerebrospinal fluid into the brain and the clearance of metabolic waste, sparking growing interest in its role in the pathogenesis of proteinopathies.[227] Astrocytes are believed to be the main regulators of glymphatic fluid movement, with aquaporin-4, a water channel situated in the endfeet of astrocyte membranes, considered a primary driver of the clearance system. Recent studies have shown that aquaporin-4 facilitates the elimination of extracellular tau from the brain to the cerebrospinal fluid and then to deep cervical lymph nodes.[228] Deletion of aquaporin-4 led to increased tau levels in the cerebrospinal fluid and worsened phosphorylated tau deposition and related neurodegeneration in transgenic mice expressing the P301S MAPT mutant tau.[228] These data highlight the essential role of astroglia in tau clearance and removal.

Implication of peripheral cells in tau pathology

Tau pathology has also been recently described to be modulated by adaptive immune cells. A greater proportion of T cells associated with tauopathies have been widely described, but its involvement in pathogenesis is not clearly understood. Recently, Chen and collaborators demonstrated a greater proportion of T cells associated with tau-related neurodegeneration[229] than in models associated with amyloid burden. Authors show how T cells directly interact with specific subsets of disease-associated microglia, while either microglia or T-cell depletion prevents immune activation and tau-related neurodegeneration.[229] The specific antigens implicated in the mechanism are still elusive; either myelin debris, forms of tau or other proteins are candidates, although further analyses are needed.

Limitations

Despite the comprehensive nature of this review on the role of non-neuronal cells in proteinopathies, several limitations should be acknowledged. First, the review primarily synthesizes findings from various animal models and *in vitro* studies, which may not fully capture the complexity of human disease pathology. The heterogeneity of non-neuronal cell types and their diverse functions further complicates the integration of results across different studies, potentially leading to generalized conclusions that may not apply universally. Additionally, the rapid pace of research in this field means that new discoveries could quickly shift the current understanding, which may impact the relevance of some reviewed studies. Although we have tried our best to include all the most recent advances in the field, it is not possible to include all of them, so we might have failed at identifying all the relevant literature. Finally, the review may be limited by publication bias, as studies with positive or novel findings are more likely to be published, potentially overlooking negative or inconclusive results.

Conclusions

Synuclein, amyloid or tau proteins, among others, play multifaceted roles in both neuronal and non-neuronal cells, contributing to the maintenance of cellular integrity and function. However, pathological modifications of these proteins lead to their accumulation and the formation of aggregates, which are central to the development of proteinopathies such as PD, AD disease or FTD. Importantly, the cross-collaboration of non-neuronal cells, such as microglia, astrocytes and oligodendrocytes, in these pathologies highlights the complex interplay between different cell types in the brain (**Figure 3**). Non-neuronal cells participate in protein homeostasis through, among

other processes, the uptake, clearance, and degradation of these proteins, which is crucial for preventing neurodegeneration and promoting neurorepair. However, protein overload often leads to impaired protein function, which can contribute to the spread and exacerbation of pathology. Additionally, the emerging role of peripheral immune cells in modulating pathology underscores the intricate immune responses involved in proteinopathy-related diseases. Understanding these diverse mechanisms is essential for advancing regenerative medicine, as it offers potential for targeted therapies aimed at mitigating synuclein, amyloid and tau pathology and promoting neuronal regeneration to counteract associated neurodegenerative processes.

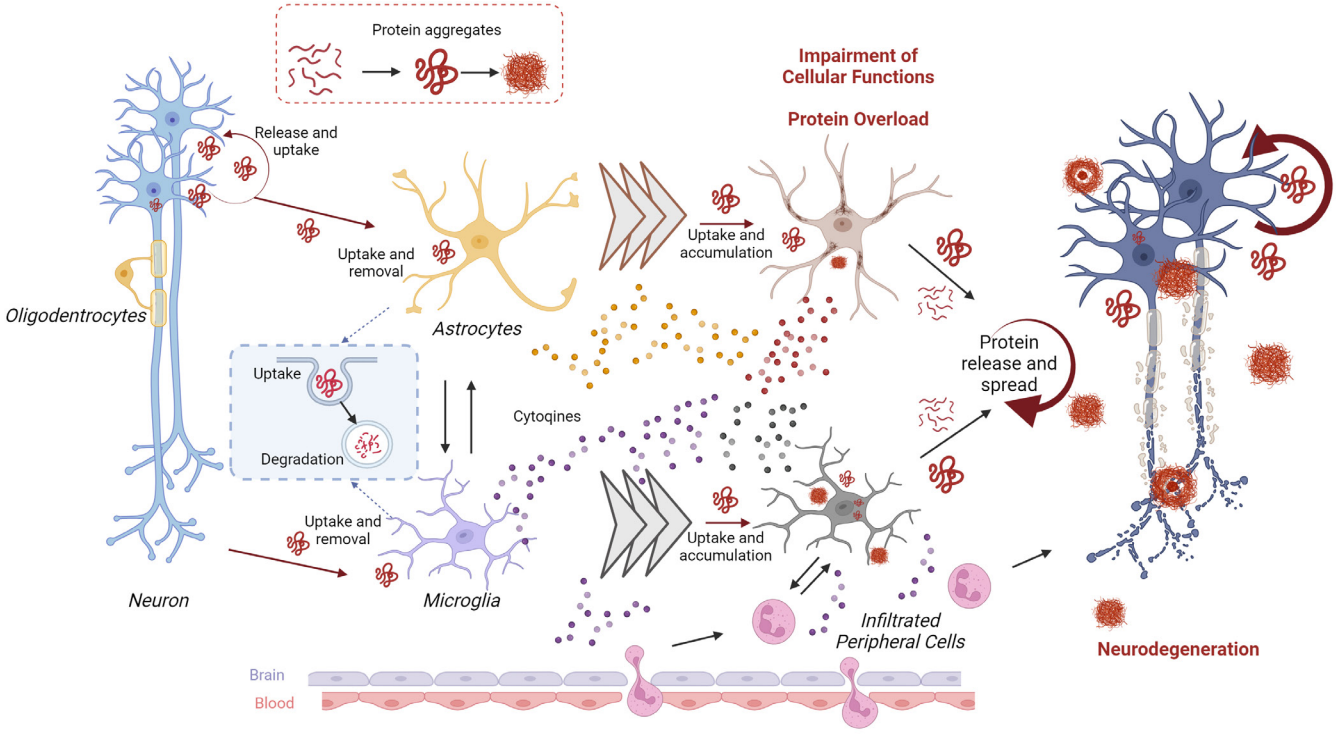


Figure 3: The role of non-neuronal cells in proteinopathy and neurodegeneration.
Note: The aggregation of specific proteins, such as synuclein, amyloid, and tau, is a hallmark of various neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease, and frontotemporal dementia. The interaction and collaboration between non-neuronal cells, such as microglia, astrocytes, and oligodendrocytes, play a significant role in this process. These cells initially contribute to the uptake and removal of these proteins through proteasomal or autophagy pathways. As the disease progresses, protein overload disrupts the primary functions of both neuronal and non-neuronal cells, leading to further protein release and spread, creating a vicious cycle that exacerbates the pathology. Additionally, peripheral cells such as lymphocytes may infiltrate the brain and contribute to neurodegeneration. Created with BioRender.com.

Author contributions
All the authors read and approved the manuscript, participated in writing and editing and believed that the manuscript represents honest work.

Conflicts of interest
The authors declare that they have no conflicts of interest.

Data availability statement
Not applicable.

Open access statement
This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

REFERENCES

1. Jovanovic VM, Weber C, Slamecka J, et al. A defined roadmap of radial glia and astrocyte differentiation from human pluripotent stem cells. *Stem Cell Reports*. 2023;18:1701-1720.
2. Barres BA. New roles for glia. *J Neurosci*. 1991;11:3685-3694.
3. Brandebura AN, Paumier A, Onur TS, Allen NJ. Astrocyte contribution to dysfunction, risk and progression in neurodegenerative disorders. *Nat Rev Neurosci*. 2023;24:23-39.
4. Escartin C, Galea E, Lakatos A, et al. Reactive astrocyte nomenclature, definitions, and future directions. *Nat Neurosci*. 2021;24:312-325.
5. Ginhoux F, Greter M, Leboeuf M, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science*. 2010;330:841-845.

6. Stremmel C, Schuchert R, Wagner F, et al. Yolk sac macrophage progenitors traffic to the embryo during defined stages of development. *Nat Commun*. 2018;9:75.
7. Wilton DK, Dissing-Olesen L, Stevens B. Neuron-glia signaling in synapse elimination. *Annu Rev Neurosci*. 2019;42:107-127.
8. Paolicelli RC, Sierra A, Stevens B, et al. Microglia states and nomenclature: A field at its crossroads. *Neuron*. 2022;110:3458-3483.
9. Ransohoff RM. A polarizing question: do M1 and M2 microglia exist? *Nat Neurosci*. 2016;19:987-991.
10. Keren-Shaul H, Spinrad A, Weiner A, et al. A unique microglia type associated with restricting development of Alzheimer's disease. *Cell*. 2017;169:1276-1290.e17.
11. Kuhn S, Gritti L, Crooks D, Dombrowski Y. Oligodendrocytes in development, myelin generation and beyond. *Cells*. 2019;8:1424.
12. Nave KA. Myelination and support of axonal integrity by glia. *Nature*. 2010;468:244-252.
13. Fünfschilling U, Supplie LM, Mahad D, et al. Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature*. 2012;485:517-521.
14. Lee Y, Morrison BM, Li Y, et al. Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature*. 2012;487:443-448.
15. Zhang SZ, Wang QQ, Yang QQ, et al. NG2 glia regulate brain innate immunity via TGF-β2/TGFBR2 axis. *BMC Med*. 2019;17:204.
16. Han S, Gim Y, Jang EH, Hur EM. Functions and dysfunctions of oligodendrocytes in neurodegenerative diseases. *Front Cell Neurosci*. 2022;16:1083159.
17. Kaya T, Mattugini N, Liu L, et al. CD8(+) T cells induce interferon-responsive oligodendrocytes and microglia in white matter aging. *Nat Neurosci*. 2022;25:1446-1457.
18. Passaro AP, Lebos AL, Yao Y, Stice SL. Immune response in neurological pathology: emerging role of central and peripheral immune crosstalk. *Front Immunol*. 2021;12:676621.
19. Berriat F, Lobsiger CS, Boillée S. The contribution of the peripheral immune system to neurodegeneration. *Nat Neurosci*. 2023;26:942-954.
20. DeMaio A, Mehrotra S, Sambamurti K, Husain S. The role of the adaptive immune system and T cell dysfunction in neurodegenerative diseases. *J Neuroinflammation*. 2022;19:251.
21. Pryce G, Male D, Campbell I, Greenwood J. Factors controlling T-cell migration across rat cerebral endothelium in vitro. *J Neuroimmunol*. 1997;75:84-94.
22. Herz J, Paterka M, Niesner RA, et al. In vivo imaging of lymphocytes in the CNS reveals different behaviour of naïve T cells in health and autoimmunity. *J Neuroinflammation*. 2011;8:131.
23. Cose S, Brammer C, Khanna KM, Masopust D, Lefrançois L. Evidence that a significant number of naive T cells enter non-lymphoid organs as part of a normal migratory pathway. *Eur J Immunol*. 2006;36:1423-1433.
24. Hoffman W, Lakkis FG, Chalasani G. B cells, antibodies, and more. *Clin J Am Soc Nephrol*. 2016;11:137-154.
25. Schafflick D, Xu CA, Hartlehnert M, et al. Integrated single cell analysis of blood and cerebrospinal fluid leukocytes in multiple sclerosis. *Nat Commun*. 2020;11:247.
26. Richards DM, Hettinger J, Feuerer M. Monocytes and macrophages in cancer: development and functions. *Cancer Microenviron*. 2013;6:179-191.
27. Nielsen MC, Andersen MN, Møller HJ. Monocyte isolation techniques significantly impact the phenotype of both isolated monocytes and derived macrophages in vitro. *Immunology*. 2020;159:63-74.
28. Niu F, Liao K, Hu G, et al. Cocaine-induced release of CXCL10 from pericytes regulates monocyte transmigration into the CNS. *J Cell Biol*. 2019;218:700-721.
29. Hohsfield LA, Najafi AR, Ghorbanian Y, et al. Effects of long-term and brain-wide colonization of peripheral bone marrow-derived myeloid cells in the CNS. *J Neuroinflammation*. 2020;17:279.
30. Muñoz-Castro C, Mejias-Ortega M, Sanchez-Mejias E, et al. Monocyte-derived cells invade brain parenchyma and amyloid plaques in human Alzheimer's disease hippocampus. *Acta Neuropathol Commun*. 2023;11:31.
31. Wilson DM, 3rd, Cookson MR, Van Den Bosch L, Zetterberg H, Holtzman DM, Dewachter I. Hallmarks of neurodegenerative diseases. *Cell*. 2023;186:693-714.
32. Maroteaux L, Campanelli JT, Scheller RH. Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. *J Neurosci*. 1988;8(8):2804-2815.
33. Weinreb PH, Zhen W, Poon AW, Conway KA, Lansbury PT, Jr. NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. *Biochemistry*. 1996;35:13709-13715.
34. Davidson WS, Jonas A, Clayton DF, George JM. Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. *J Biol Chem*. 1998;273:9443-9449.
35. Scott D, Roy S. α-Synuclein inhibits intersynaptic vesicle mobility and maintains recycling-pool homeostasis. *J Neurosci*. 2012;32:10129-10135.
36. Burré J, Sharma M, Tsetsenis T, Buchman V, Etherton MR, Südhof TC. Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. *Science*. 2010;329:1663-1667.
37. George JM, Jin H, Woods WS, Clayton DF. Characterization of a novel protein regulated during the critical period for song learning in the zebra finch. *Neuron*. 1995;15:361-372.
38. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. *Nature*. 1997;388:839-840.
39. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*. 1997;276:2045-2047.
40. Calabresi P, Mechelli A, Natale G, Volpicelli-Daley L, Di Lazzaro G, Ghiglieri V. Alpha-synuclein in Parkinson's disease and other synucleinopathies: from overt neurodegeneration back to early synaptic dysfunction. *Cell Death Dis*. 2023;14:176.
41. Desplats P, Lee HJ, Bae EJ, et al. Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. *Proc Natl Acad Sci U S A*. 2009;106:13010-13015.
42. Altay MF, Liu AKL, Holton JL, Parkkinen L, Lashuel HA. Prominent astrocytic alpha-synuclein pathology with unique post-translational modification signatures unveiled across Lewy body disorders. *Acta Neuropathol Commun*. 2022;10:163.
43. Ubhi K, Low P, Masliah E. Multiple system atrophy: a clinical and neuropathological perspective. *Trends Neurosci*. 2011;34:581-590.

44. Lee HJ, Suk JE, Patrick C, et al. Direct transfer of alpha-synuclein from neuron to astroglia causes inflammatory responses in synucleinopathies. *J Biol Chem*. 2010;285:9262-9272.
45. Reyes JF, Rey NL, Bousset L, Melki R, Brundin P, Angot E. Alpha-synuclein transfers from neurons to oligodendrocytes. *Glia*. 2014;62:387-398.
46. Braak H, Sastre M, Del Tredici K. Development of alpha-synuclein immunoreactive astrocytes in the forebrain parallels stages of intraneuronal pathology in sporadic Parkinson's disease. *Acta Neuropathol*. 2007;114:231-241.
47. Booth HDE, Hirst WD, Wade-Martins R. The role of astrocyte dysfunction in Parkinson's disease pathogenesis. *Trends Neurosci*. 2017;40:358-370.
48. Tanji K, Imaizumi T, Yoshida H, et al. Expression of alpha-synuclein in a human glioma cell line and its up-regulation by interleukin-1beta. *Neuroreport*. 2001;12:1909-1912.
49. Cheng SY, Trombetta LD. The induction of amyloid precursor protein and alpha-synuclein in rat hippocampal astrocytes by diethyldithiocarbamate and copper with or without glutathione. *Toxicol Lett*. 2004;146:139-149.
50. Srinivasan R, Lu TY, Chai H, et al. New transgenic mouse lines for selectively targeting astrocytes and studying calcium signals in astrocyte processes in situ and in vivo. *Neuron*. 2016;92:1181-1195.
51. Zhang Y, Sloan SA, Clarke LE, et al. Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. *Neuron*. 2016;89:37-53.
52. Yu X, Nagai J, Marti-Solano M, et al. Context-specific striatal astrocyte molecular responses are phenotypically exploitable. *Neuron*. 2020;108:1146-1162.e10.
53. Chai H, Diaz-Castro B, Shigetomi E, et al. Neural circuit-specialized astrocytes: transcriptomic, proteomic, morphological, and functional evidence. *Neuron*. 2017;95:531-549.e9.
54. Castagnet PI, Golovko MY, Barceló-Coblijn GC, Nussbaum RL, Murphy EJ. Fatty acid incorporation is decreased in astrocytes cultured from alpha-synuclein gene-ablated mice. *J Neurochem*. 2005;94:839-849.
55. Gu XL, Long CX, Sun L, Xie C, Lin X, Cai H. Astrocytic expression of Parkinson's disease-related A53T alpha-synuclein causes neurodegeneration in mice. *Mol Brain*. 2010;3:12.
56. Shin EC, Cho SE, Lee DK, et al. Expression patterns of alpha-synuclein in human hematopoietic cells and in Drosophila at different developmental stages. *Mol Cells*. 2000;10:65-70.
57. Zhang Y, Chen K, Sloan SA, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci*. 2014;34:11929-11947.
58. Li Q, Cheng Z, Zhou L, et al. Developmental heterogeneity of microglia and brain myeloid cells revealed by deep single-cell RNA sequencing. *Neuron*. 2019;101:207-223.e10.
59. Svoboda DS, Barrasa MI, Shu J, et al. Human iPSC-derived microglia assume a primary microglia-like state after transplantation into the neonatal mouse brain. *Proc Natl Acad Sci U S A*. 2019;116:25293-25303.
60. Austin SA, Rojanathammanee L, Golovko MY, Murphy EJ, Combs CK. Lack of alpha-synuclein modulates microglial phenotype in vitro. *Neurochem Res*. 2011;36:994-1004.
61. Haenseler W, Zambon F, Lee H, et al. Excess α-synuclein compromises phagocytosis in iPSC-derived macrophages. *Sci Rep*. 2017;7:9003.
62. Booms A, Coetzee GA. Functions of intracellular alpha-synuclein in microglia: implications for Parkinson's disease risk. *Front Cell Neurosci*. 2021;15:759571.
63. Hoffmann A, Ettle B, Bruno A, et al. Alpha-synuclein activates BV2 microglia dependent on its aggregation state. *Biochem Biophys Res Commun*. 2016;479:881-886.
64. Chavarría C, Rodríguez-Bottero S, Quijano C, Cassina P, Souza JM. Impact of monomeric, oligomeric and fibrillar alpha-synuclein on astrocyte reactivity and toxicity to neurons. *Biochem J*. 2018;475:3153-3169.
65. Hughes CD, Choi ML, Ryten M, et al. Picomolar concentrations of oligomeric alpha-synuclein sensitizes TLR4 to play an initiating role in Parkinson's disease pathogenesis. *Acta Neuropathol*. 2019;137:103-120.
66. Stefanova N, Fellner L, Reindl M, Masliah E, Poewe W, Wenning GK. Toll-like receptor 4 promotes α-synuclein clearance and survival of nigral dopaminergic neurons. *Am J Pathol*. 2011;179:954-963.
67. Gai WP, Power JH, Blumbergs PC, Culvenor JG, Jensen PH. Alpha-synuclein immunoisolation of glial inclusions from multiple system atrophy brain tissue reveals multiprotein components. *J Neurochem*. 1999;73:2093-2100.
68. Frühbeis C, Fröhlich D, Kuo WP, et al. Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte-neuron communication. *PLoS Biol*. 2013;11:e1001604.
69. Kaji S, Maki T, Kinoshita H, et al. Pathological endogenous α-synuclein accumulation in oligodendrocyte precursor cells potentially induces inclusions in multiple system atrophy. *Stem Cell Reports*. 2018;10:356-365.
70. Djelloul M, Holmqvist S, Boza-Serrano A, et al. Alpha-synuclein expression in the oligodendrocyte lineage: an in vitro and in vivo study using rodent and human models. *Stem Cell Reports*. 2015;5:174-184.
71. Chavarría C, Ivagnes R, Souza JM. Extracellular Alpha-Synuclein: Mechanisms for Glial Cell Internalization and Activation. *Biomolecules.* 2022;12:655.
72. Li JY, Englund E, Holton JL, et al. Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nat Med*. 2008;14:501-503.
73. Kordower JH, Chu Y, Hauser RA, Freeman TB, Olanow CW. Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. *Nat Med*. 2008;14:504-506.
74. Jan A, Gonçalves NP, Vaegter CB, Jensen PH, Ferreira N. The prion-like spreading of alpha-synuclein in Parkinson's disease: update on models and hypotheses. *Int J Mol Sci.* 2021;22:8338.
75. Giusti V, Kaur G, Giusto E, Civiero L. Brain clearance of protein aggregates: a close-up on astrocytes. *Mol Neurodegener*. 2024;19:5.
76. Danzer KM, Kranich LR, Ruf WP, et al. Exosomal cell-to-cell transmission of alpha synuclein oligomers. *Mol Neurodegener*. 2012;7:42.
77. Abounit S, Bousset L, Loria F, et al. Tunneling nanotubes spread fibrillar α-synuclein by intercellular trafficking of lysosomes. *EMBO J*. 2016;35:2120-2138.

78. Loria F, Vargas JY, Bousset L, et al. α-Synuclein transfer between neurons and astrocytes indicates that astrocytes play a role in degradation rather than in spreading. *Acta Neuropathol*. 2017;134:789-808.
79. Lindström V, Gustafsson G, Sanders LH, et al. Extensive uptake of α-synuclein oligomers in astrocytes results in sustained intracellular deposits and mitochondrial damage. *Mol Cell Neurosci*. 2017;82:143-156.
80. Chou TW, Chang NP, Krishnagiri M, et al. Fibrillar α-synuclein induces neurotoxic astrocyte activation via RIP kinase signaling and NF-κB. *Cell Death Dis*. 2021;12:756.
81. Tsunemi T, Ishiguro Y, Yoroisaka A, et al. Astrocytes protect human dopaminergic neurons from α-synuclein accumulation and propagation. *J Neurosci*. 2020;40:8618-8628.
82. Emmanouilidou E, Melachroinou K, Roumeliotis T, et al. Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J Neurosci*. 2010;30:6838-6851.
83. Bliederhaeuser C, Grozdanov V, Speidel A, et al. Age-dependent defects of alpha-synuclein oligomer uptake in microglia and monocytes. *Acta Neuropathol*. 2016;131:379-391.
84. Stefanis L, Emmanouilidou E, Pantazopoulou M, Kirik D, Vekrellis K, Tofaris GK. How is alpha-synuclein cleared from the cell? *J Neurochem*. 2019;150(5):577-590.
85. Ngolab J, Trinh I, Rockenstein E, et al. Brain-derived exosomes from dementia with Lewy bodies propagate α-synuclein pathology. *Acta Neuropathol Commun*. 2017;5:46.
86. Rustom A, Saffrich R, Markovic I, Walther P, Gerdes HH. Nanotubular highways for intercellular organelle transport. *Science*. 2004;303:1007-1010.
87. Rostami J, Mothes T, Kolahdouzan M, et al. Crosstalk between astrocytes and microglia results in increased degradation of α-synuclein and amyloid-β aggregates. *J Neuroinflammation*. 2021;18:124.
88. Scheiblich H, Bousset L, Schwartz S, et al. Microglial NLRP3 inflammasome activation upon TLR2 and TLR5 ligation by distinct α-synuclein assemblies. *J Immunol*. 2021;207:2143-2154.
89. Chakraborty R, Nonaka T, Hasegawa M, Zurzolo C. Tunnelling nanotubes between neuronal and microglial cells allow bi-directional transfer of α-Synuclein and mitochondria. *Cell Death Dis*. 2023;14:329.
90. Yang Y, Qin M, Bao P, Xu W, Xu J. Secretory carrier membrane protein 5 is an autophagy inhibitor that promotes the secretion of α-synuclein via exosome. *PLoS One*. 2017;12:e0180892.
91. Guo M, Wang J, Zhao Y, et al. Microglial exosomes facilitate α-synuclein transmission in Parkinson's disease. *Brain*. 2020;143:1476-1497.
92. Rostami J, Holmqvist S, Lindström V, et al. Human astrocytes transfer aggregated alpha-synuclein via tunneling nanotubes. *J Neurosci*. 2017;37:11835-11853.
93. Ferreira N, Gonçalves NP, Jan A, et al. Trans-synaptic spreading of alpha-synuclein pathology through sensory afferents leads to sensory nerve degeneration and neuropathic pain. *Acta Neuropathol Commun*. 2021;9:31.
94. Laferrière F, He X, Zinghirino F, et al. Overexpression of α-synuclein by oligodendrocytes in transgenic mice does not recapitulate the fibrillar aggregation seen in multiple system atrophy. *Cells.* 2020;9:2371.
95. Pukaß K, Goldbaum O, Richter-Landsberg C. Mitochondrial impairment and oxidative stress compromise autophagosomal degradation of α-synuclein in oligodendroglial cells. *J Neurochem*. 2015;135:194-205.
96. Ihse E, Yamakado H, van Wijk XM, Lawrence R, Esko JD, Masliah E. Cellular internalization of alpha-synuclein aggregates by cell surface heparan sulfate depends on aggregate conformation and cell type. *Sci Rep*. 2017;7:9008.
97. Gustafsson G, Lööv C, Persson E, et al. Secretion and uptake of α-synuclein via extracellular vesicles in cultured cells. *Cell Mol Neurobiol*. 2018;38:1539-1550.
98. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. Alpha-Synuclein is degraded by both autophagy and the proteasome. *J Biol Chem*. 2003;278:25009-25013.
99. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science*. 2004;305:1292-1295.
100. Choi I, Zhang Y, Seegobin SP, et al. Microglia clear neuron-released α-synuclein via selective autophagy and prevent neurodegeneration. *Nat Commun*. 2020;11:1386.
101. di Domenico A, Carola G, Calatayud C, et al. Patient-specific iPSC-derived astrocytes contribute to non-cell-autonomous neurodegeneration in Parkinson's disease. *Stem Cell Reports*. 2019;12:213-229.
102. Schwarz L, Goldbaum O, Bergmann M, Probst-Cousin S, Richter-Landsberg C. Involvement of macroautophagy in multiple system atrophy and protein aggregate formation in oligodendrocytes. *J Mol Neurosci*. 2012;47:256-266.
103. Mavroeidi P, Vetsi M, Dionysopoulou D, Xilouri M. Exosomes in alpha-synucleinopathies: propagators of pathology or potential candidates for nanotherapeutics? *Biomolecules.* 2022;12:957.
104. Iwata A, Maruyama M, Akagi T, et al. Alpha-synuclein degradation by serine protease neurosin: implication for pathogenesis of synucleinopathies. *Hum Mol Genet*. 2003;12:2625-2635.
105. Sung JY, Park SM, Lee CH, et al. Proteolytic cleavage of extracellular secreted {alpha}-synuclein via matrix metalloproteinases. *J Biol Chem*. 2005;280:25216-25224.
106. Guo C, Wang T, Huang H, Wang X, Jiang Y, Li J. Plasminogen degrades α-synuclein, Tau and TDP-43 and decreases dopaminergic neurodegeneration in mouse models of Parkinson's disease. *Sci Rep*. 2024;14:8581.
107. Deyell JS, Sriparna M, Ying M, Mao X. The interplay between α-synuclein and microglia in α-synucleinopathies. *Int J Mol Sci.* 2023;24:2477.
108. Yamanaka H, He X, Matsumoto K, Shiosaka S, Yoshida S. Protease M/neurosin mRNA is expressed in mature oligodendrocytes. *Brain Res Mol Brain Res*. 1999;71:217-224.
109. Suthar SK, Lee SY. Truncation or proteolysis of α-synuclein in Parkinsonism. *Ageing Res Rev*. 2023;90:101978.
110. Zhou T, Lin D, Chen Y, et al. α-synuclein accumulation in SH-SY5Y cell impairs autophagy in microglia by exosomes overloading miR-19a-3p. *Epigenomics*. 2019;11:1661-1677.
111. Tu HY, Yuan BS, Hou XO, et al. α-synuclein suppresses microglial autophagy and promotes neurodegeneration in a mouse model of Parkinson's disease. *Aging Cell*. 2021;20:e13522.
112. Sorrentino ZA, Giasson BI, Chakrabarty P. α-Synuclein and astrocytes: tracing the pathways from homeostasis to neurodegeneration in Lewy body disease. *Acta Neuropathol*. 2019;138:1-21.

113. Angelova PR, Ludtmann MH, Horrocks MH, et al. Ca^{2+} is a key factor in α-synuclein-induced neurotoxicity. *J Cell Sci*. 2016;129:1792-1801.
114. Karikari AA, McFleder RL, Ribechini E, et al. Neurodegeneration by α-synuclein-specific T cells in AAV-A53T-α-synuclein Parkinson's disease mice. *Brain Behav Immun*. 2022;101:194-210.
115. Williams GP, Schonhoff AM, Jurkuvenaite A, Gallups NJ, Standaert DG, Harms AS. CD4 T cells mediate brain inflammation and neurodegeneration in a mouse model of Parkinson's disease. *Brain*. 2021;144:2047-2059.
116. Rostami J, Fotaki G, Sirois J, et al. Astrocytes have the capacity to act as antigen-presenting cells in the Parkinson's disease brain. *J Neuroinflammation*. 2020;17:119.
117. Theodore S, Cao S, McLean PJ, Standaert DG. Targeted overexpression of human alpha-synuclein triggers microglial activation and an adaptive immune response in a mouse model of Parkinson disease. *J Neuropathol Exp Neurol*. 2008;67:1149-1158.
118. Harms AS, Cao S, Rowse AL, et al. MHCII is required for α-synuclein-induced activation of microglia, CD4 T cell proliferation, and dopaminergic neurodegeneration. *J Neurosci*. 2013;33:9592-9600.
119. Sulzer D, Alcalay RN, Garretti F, et al. T cells from patients with Parkinson's disease recognize α-synuclein peptides. *Nature*. 2017;546:656-661.
120. Wissemann WT, Hill-Burns EM, Zabetian CP, et al. Association of Parkinson disease with structural and regulatory variants in the HLA region. *Am J Hum Genet*. 2013;93:984-993.
121. Tansey MG, Wallings RL, Houser MC, Herrick MK, Keating CE, Joers V. Inflammation and immune dysfunction in Parkinson disease. *Nat Rev Immunol*. 2022;22:657-673.
122. Song J, Qin Y, Wang L, et al. Exploring the causal relationship between B lymphocytes and Parkinson's disease: a bidirectional, two-sample Mendelian randomization study. *Sci Rep*. 2024;14:2783.
123. Earls RH, Menees KB, Chung J, et al. NK cells clear α-synuclein and the depletion of NK cells exacerbates synuclein pathology in a mouse model of α-synucleinopathy. *Proc Natl Acad Sci U S A*. 2020;117:1762-1771.
124. Harms AS, Delic V, Thome AD, et al. α-Synuclein fibrils recruit peripheral immune cells in the rat brain prior to neurodegeneration. *Acta Neuropathol Commun*. 2017;5:85.
125. Glenner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun*. 1984;120:885-890.
126. Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. *Science*. 1992;256:184-185.
127. Zhang Y, Chen H, Li R, Sterling K, Song W. Amyloid β-based therapy for Alzheimer's disease: challenges, successes and future. *Signal Transduct Target Ther*. 2023;8:248.
128. De Strooper B, Karran E. The cellular phase of Alzheimer's disease. *Cell*. 2016;164:603-615.
129. Knopman DS, Amieva H, Petersen RC, et al. Alzheimer disease. *Nat Rev Dis Primers*. 2021;7:33.
130. Hampel H, Hardy J, Blennow K, et al. The amyloid-β pathway in Alzheimer's disease. *Mol Psychiatry*. 2021;26:5481-5503.
131. Veeraraghavalu K, Zhang C, Zhang X, Tanzi RE, Sisodia SS. Age-dependent, non-cell-autonomous deposition of amyloid from synthesis of β-amyloid by cells other than excitatory neurons. *J Neurosci*. 2014;34:3668-3673.
132. Frost GR, Li YM. The role of astrocytes in amyloid production and Alzheimer's disease. *Open Biol*. 2017;7:170228.
133. Clarner T, Buschmann JP, Beyer C, Kipp M. Glial amyloid precursor protein expression is restricted to astrocytes in an experimental toxic model of multiple sclerosis. *J Mol Neurosci*. 2011;43:268-274.
134. Jin SM, Cho HJ, Kim YW, Hwang JY, Mook-Jung I. Aβ-induced Ca(2+) influx regulates astrocytic BACE1 expression via calcineurin/NFAT4 signals. *Biochem Biophys Res Commun*. 2012;425:649-655.
135. Zhao J, O'Connor T, Vassar R. The contribution of activated astrocytes to Aβ production: implications for Alzheimer's disease pathogenesis. *J Neuroinflammation*. 2011;8:150.
136. Liang Y, Raven F, Ward JF, et al. Upregulation of Alzheimer's disease amyloid-β protein precursor in astrocytes both in vitro and in vivo. *J Alzheimers Dis*. 2020;76:1071-1082.
137. Lesné S, Docagne F, Gabriel C, et al. Transforming growth factor-beta 1 potentiates amyloid-beta generation in astrocytes and in transgenic mice. *J Biol Chem*. 2003;278:18408-18418.
138. Uddin MS, Lim LW. Glial cells in Alzheimer's disease: From neuropathological changes to therapeutic implications. *Ageing Res Rev*. 2022;78:101622.
139. Shah D, Gsell W, Wahis J, et al. Astrocyte calcium dysfunction causes early network hyperactivity in Alzheimer's disease. *Cell Rep*. 2022;40:111280.
140. Matos M, Augusto E, Machado NJ, dos Santos-Rodrigues A, Cunha RA, Agostinho P. Astrocytic adenosine A2A receptors control the amyloid-β peptide-induced decrease of glutamate uptake. *J Alzheimers Dis*. 2012;31:555-567.
141. Jo S, Yarishkin O, Hwang YJ, et al. GABA from reactive astrocytes impairs memory in mouse models of Alzheimer's disease. *Nat Med*. Aug 2014;20:886-896.
142. Talantova M, Sanz-Blasco S, Zhang X, et al. Aβ induces astrocytic glutamate release, extrasynaptic NMDA receptor activation, and synaptic loss. *Proc Natl Acad Sci U S A*. 2013;110:E2518-2527.
143. Jha MK, Morrison BM. Lactate transporters mediate glia-neuron metabolic crosstalk in homeostasis and disease. *Front Cell Neurosci*. 2020;14:589582.
144. Abramov AY, Canevari L, Duchen MR. Beta-amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase. *J Neurosci*. 2004;24:565-575.
145. Singh N, Das B, Zhou J, Hu X, Yan R. Targeted BACE-1 inhibition in microglia enhances amyloid clearance and improved cognitive performance. *Sci Adv*. 2022;8:eabo3610.
146. Merighi S, Nigro M, Travagli A, Gessi S. Microglia and Alzheimer's Disease. *Int J Mol Sci.* 2022 Oct 27;23(21):12990.
147. Hanslik KL, Ulland TK. The role of microglia and the Nlrp3 inflammasome in Alzheimer's disease. *Front Neurol*. 2020;11:570711.
148. Wang J, Zhen Y, Yang J, Yang S, Zhu G. Recognizing Alzheimer's disease from perspective of oligodendrocytes: Phenomena or pathogenesis? *CNS Neurosci Ther*. 2024;30:e14688.
149. Depp C, Sun T, Sasmita AO, et al. Myelin dysfunction drives amyloid-β deposition in models of Alzheimer's disease. *Nature*. 2023;618:349-357.
150. Michaels TCT, Šarić A, Curk S, et al. Dynamics of oligomer populations formed during the aggregation of Alzheimer's Aβ42 peptide. *Nat Chem*. 2020;12:445-451.

151. Domert J, Rao SB, Agholme L, et al. Spreading of amyloid-β peptides via neuritic cell-to-cell transfer is dependent on insufficient cellular clearance. *Neurobiol Dis*. 2014;65:82-92.
152. Hansen DV, Hanson JE, Sheng M. Microglia in Alzheimer's disease. *J Cell Biol*. 2018;217:459-472.
153. Yamamoto M, Kiyota T, Walsh SM, Liu J, Kipnis J, Ikezu T. Cytokine-mediated inhibition of fibrillar amyloid-beta peptide degradation by human mononuclear phagocytes. *J Immunol*. 2008;181:3877-3886.
154. Lee CY, Landreth GE. The role of microglia in amyloid clearance from the AD brain. *J Neural Transm (Vienna)*. 2010;117:949-960.
155. Reed-Geaghan EG, Savage JC, Hise AG, Landreth GE. CD14 and toll-like receptors 2 and 4 are required for fibrillar A{beta}-stimulated microglial activation. *J Neurosci*. 2009;29:11982-11992.
156. Ulland TK, Colonna M. TREM2 - a key player in microglial biology and Alzheimer disease. *Nat Rev Neurol*. 2018;14:667-675.
157. Yuan P, Condello C, Keene CD, et al. TREM2 haplodeficiency in mice and humans impairs the microglia barrier function leading to decreased amyloid compaction and severe axonal dystrophy. *Neuron*. 2016;90:724-739.
158. Jäntti H, Sitnikova V, Ishchenko Y, et al. Microglial amyloid beta clearance is driven by PIEZO1 channels. *J Neuroinflammation*. 2022;19:147.
159. Koenigsknecht-Talboo J, Landreth GE. Microglial phagocytosis induced by fibrillar beta-amyloid and IgGs are differentially regulated by proinflammatory cytokines. *J Neurosci*. 2005;25:8240-8249.
160. Joshi P, Turola E, Ruiz A, et al. Microglia convert aggregated amyloid-β into neurotoxic forms through the shedding of microvesicles. *Cell Death Differ*. 2014;21:582-593.
161. Trotta T, Panaro MA, Cianciulli A, Mori G, Di Benedetto A, Porro C. Microglia-derived extracellular vesicles in Alzheimer's disease: A double-edged sword. *Biochem Pharmacol*. 2018;148:184-192.
162. Bianco F, Pravettoni E, Colombo A, et al. Astrocyte-derived ATP induces vesicle shedding and IL-1 beta release from microglia. *J Immunol*. 2005;174:7268-7277.
163. Ries M, Sastre M. Mechanisms of Aβ clearance and degradation by glial cells. *Front Aging Neurosci*. 2016;8:160.
164. Jiang Q, Lee CY, Mandrekar S, et al. ApoE promotes the proteolytic degradation of Abeta. *Neuron*. 2008;58:681-693.
165. Krasemann S, Madore C, Cialic R, et al. The TREM2-APOE pathway drives the transcriptional phenotype of dysfunctional microglia in neurodegenerative diseases. *Immunity*. Sep 19 2017;47:566-581.e9.
166. Lau SF, Wu W, Wong HY, et al. The VCAM1-ApoE pathway directs microglial chemotaxis and alleviates Alzheimer's disease pathology. *Nat Aging*. 2023;3:1219-1236.
167. Kanekiyo T, Xu H, Bu G. ApoE and Aβ in Alzheimer's disease: accidental encounters or partners? *Neuron*. 2014;81:740-754.
168. Xia Z, Prescott EE, Urbanek A, et al. Co-aggregation with apolipoprotein E modulates the function of amyloid-β in Alzheimer's disease. *Nat Commun*. 2024;15:4695.
169. Machlovi SI, Neuner SM, Hemmer BM, et al. APOE4 confers transcriptomic and functional alterations to primary mouse microglia. *Neurobiol Dis*. 2022;164:105615.
170. Beretta C, Svensson E, Dakhel A, et al. Amyloid-β deposits in human astrocytes contain truncated and highly resistant proteoforms. *Mol Cell Neurosci*. 2024;128:103916.
171. Sasmita AO, Depp C, Nazarenko T, et al. Oligodendrocytes and neurons contribute to amyloid-β deposition in Alzheimer's disease. *bioRxiv [Preprint].* doi:10.1101/2023.12.11.570514
172. Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med*. 2016;8:595-608.
173. Yoon SS, Jo SA. Mechanisms of amyloid-β peptide clearance: potential therapeutic targets for Alzheimer's disease. *Biomol Ther (Seoul)*. 2012;20:245-255.
174. Yin KJ, Cirrito JR, Yan P, et al. Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-beta peptide catabolism. *J Neurosci*. 2006;26:10939-10948.
175. De Strooper B. Proteases and proteolysis in Alzheimer disease: a multifactorial view on the disease process. *Physiol Rev*. 2010;90:465-494.
176. Pomilio C, Pavia P, Gorojod RM, et al. Glial alterations from early to late stages in a model of Alzheimer's disease: Evidence of autophagy involvement in Aβ internalization. *Hippocampus*. 2016;26:194-210.
177. Cai Z, Qiao PF, Wan CQ, Cai M, Zhou NK, Li Q. Role of blood-brain barrier in Alzheimer's disease. *J Alzheimers Dis*. 2018;63:1223-1234.
178. Quick JD, Silva C, Wong JH, et al. Lysosomal acidification dysfunction in microglia: an emerging pathogenic mechanism of neuroinflammation and neurodegeneration. *J Neuroinflammation*. 2023;20:185.
179. Orre M, Kamphuis W, Dooves S, et al. Reactive glia show increased immunoproteasome activity in Alzheimer's disease. *Brain*. 2013;136:1415-1431.
180. Gericke C, Kirabali T, Flury R, et al. Early β-amyloid accumulation in the brain is associated with peripheral T cell alterations. *Alzheimers Dement*. 2023;19:5642-5662.
181. Dai L, Shen Y. Insights into T-cell dysfunction in Alzheimer's disease. *Aging Cell*. 2021;20:e13511.
182. Su W, Saravia J, Risch I, et al. CXCR6 orchestrates brain CD8(+) T cell residency and limits mouse Alzheimer's disease pathology. *Nat Immunol*. 2023;24:1735-1747.
183. Kim K, Wang X, Ragonnaud E, et al. Therapeutic B-cell depletion reverses progression of Alzheimer's disease. *Nat Commun*. 2021;12:2185.
184. Feng W, Zhang Y, Ding S, et al. B lymphocytes ameliorate Alzheimer's disease-like neuropathology via interleukin-35. *Brain Behav Immun*. 2023;108:16-31.
185. Thériault P, ElAli A, Rivest S. The dynamics of monocytes and microglia in Alzheimer's disease. *Alzheimers Res Ther*. 2015;7:41.
186. Fiala M, Lin J, Ringman J, et al. Ineffective phagocytosis of amyloid-beta by macrophages of Alzheimer's disease patients. *J Alzheimers Dis*. 2005;7:221-232.
187. Zhao Y, Wu X, Li X, et al. TREM2 is a receptor for β-amyloid that mediates microglial function. *Neuron*. 2018;97:1023-1031.e7.
188. Bradshaw EM, Chibnik LB, Keenan BT, et al. CD33 Alzheimer's disease locus: altered monocyte function and amyloid biology. *Nat Neurosci*. 2013;16:848-850.
189. Michaud JP, Bellavance MA, Préfontaine P, Rivest S. Real-time in vivo imaging reveals the ability of monocytes to clear vascular amyloid beta. *Cell Rep*. 2013;5:646-653.
190. Chen X, Holtzman DM. Emerging roles of innate and adaptive immunity in Alzheimer's disease. *Immunity*. 2022;55:2236-2254.
191. Weingarten MD, Lockwood AH, Hwo SY, Kirschner MW. A protein factor essential for microtubule assembly. *Proc Natl Acad Sci U S A*. 1975;72:1858-1862.

192. Samudra N, Lane-Donovan C, VandeVrede L, Boxer AL. Tau pathology in neurodegenerative disease: disease mechanisms and therapeutic avenues. *J Clin Invest.* 2023;133:e168553.
193. Zhang Y, Wu KM, Yang L, Dong Q, Yu JT. Tauopathies: new perspectives and challenges. *Mol Neurodegener*. 2022;17:28.
194. Kovacs GG. Tauopathies. *Handb Clin Neurol*. 2017;145:355-368.
195. Sinsky J, Pichlerova K, Hanes J. Tau protein interaction partners and their roles in Alzheimer's disease and other tauopathies. *Int J Mol Sci*. 2021;22:9207.
196. Reid MJ, Beltran-Lobo P, Johnson L, Perez-Nievas BG, Noble W. Astrocytes in tauopathies. *Front Neurol*. 2020;11:572850.
197. Kovacs GG, Ferrer I, Grinberg LT, et al. Aging-related tau astrogliopathy (ARTAG): harmonized evaluation strategy. *Acta Neuropathol*. 2016;131:87-102.
198. Kovacs GG. Astroglia and tau: new perspectives. *Front Aging Neurosci*. 2020;12:96.
199. Forrest SL, Lee S, Nassir N, et al. Cell-specific MAPT gene expression is preserved in neuronal and glial tau cytopathologies in progressive supranuclear palsy. *Acta Neuropathol*. 2023;146: 395-414.
200. LoPresti P, Szuchet S, Papasozomenos SC, Zinkowski RP, Binder LI. Functional implications for the microtubule-associated protein tau: localization in oligodendrocytes. *Proc Natl Acad Sci U S A*. 1995;92:10369-10373.
201. LoPresti P. Regulation and differential expression of tau mRNA isoforms as oligodendrocytes mature in vivo: implications for myelination. *Glia*. 2002;37:250-257.
202. Gorath M, Stahnke T, Mronga T, Goldbaum O, Richter-Landsberg C. Developmental changes of tau protein and mRNA in cultured rat brain oligodendrocytes. *Glia*. 2001;36:89-101.
203. Setó-Salvia N, Esteras N, de Silva R, et al. Elevated 4R-tau in astrocytes from asymptomatic carriers of the MAPT 10+16 intronic mutation. *J Cell Mol Med*. 2022;26:1327-1331.
204. Jiwaji Z, Tiwari SS, Avilés-Reyes RX, et al. Reactive astrocytes acquire neuroprotective as well as deleterious signatures in response to Tau and Aß pathology. *Nat Commun*. 2022;13:135.
205. Piacentini R, Li Puma DD, Mainardi M, et al. Reduced gliotransmitter release from astrocytes mediates tau-induced synaptic dysfunction in cultured hippocampal neurons. *Glia*. 2017;65:1302-1316.
206. Sidoryk-Wegrzynowicz M, Gerber YN, Ries M, Sastre M, Tolkovsky AM, Spillantini MG. Astrocytes in mouse models of tauopathies acquire early deficits and lose neurosupportive functions. *Acta Neuropathol Commun*. 2017;5:89.
207. Hampton DW, Webber DJ, Bilican B, Goedert M, Spillantini MG, Chandran S. Cell-mediated neuroprotection in a mouse model of human tauopathy. *J Neurosci*. 2010;30:9973-9983.
208. Batenburg KL, Kasri NN, Heine VM, Scheper W. Intraneuronal tau aggregation induces the integrated stress response in astrocytes. *J Mol Cell Biol*. 2023;14:mjac071.
209. Jin M, Shiwaku H, Tanaka H, et al. Tau activates microglia via the PQBP1-cGAS-STING pathway to promote brain inflammation. *Nat Commun*. 2021;12:6565.
210. Kim DW, Tu KJ, Wei A, et al. Amyloid-beta and tau pathologies act synergistically to induce novel disease stage-specific microglia subtypes. *Mol Neurodegener*. 2022;17:83.
211. Perea JR, Llorens-Martín M, Ávila J, Bolós M. The role of microglia in the spread of tau: relevance for tauopathies. *Front Cell Neurosci*. 2018;12:172.
212. Adams JN, Maass A, Harrison TM, Baker SL, Jagust WJ. Cortical tau deposition follows patterns of entorhinal functional connectivity in aging. *Elife*. 2019;8:e49132.
213. Bolós M, Llorens-Martín M, Jurado-Arjona J, Hernández F, Rábano A, Avila J. Direct evidence of internalization of tau by microglia in vitro and in vivo. *J Alzheimers Dis*. 2016;50:77-87.
214. Asai H, Ikezu S, Tsunoda S, et al. Depletion of microglia and inhibition of exosome synthesis halt tau propagation. *Nat Neurosci*. 2015;18:1584-1593.
215. Wang C, Fan L, Khawaja RR, et al. Microglial NF-κB drives tau spreading and toxicity in a mouse model of tauopathy. *Nat Commun*. 2022;13:1969.
216. Perea JR, López E, Díez-Ballesteros JC, Ávila J, Hernández F, Bolós M. Extracellular monomeric tau is internalized by astrocytes. *Front Neurosci*. 2019;13:442.
217. Wang C, Xiong M, Gratuze M, et al. Selective removal of astrocytic APOE4 strongly protects against tau-mediated neurodegeneration and decreases synaptic phagocytosis by microglia. *Neuron*. 2021;109:1657-1674.e7.
218. Saroja SR, Gorbachev K, Julia T, Goate AM, Pereira AC. Astrocyte-secreted glypican-4 drives APOE4-dependent tau hyperphosphorylation. *Proc Natl Acad Sci U S A*. 2022;119:e2108870119.
219. Zhu B, Liu Y, Hwang S, et al. Trem2 deletion enhances tau dispersion and pathology through microglia exosomes. *Mol Neurodegener*. 2022;17:58.
220. Jain N, Ulrich JD. TREM2 and microglia exosomes: a potential highway for pathological tau. *Mol Neurodegener*. Nov 17 2022;17:73.
221. Jain N, Lewis CA, Ulrich JD, Holtzman DM. Chronic TREM2 activation exacerbates Aβ-associated tau seeding and spreading. *J Exp Med*. 2023;220:e20220654.
222. Tang M, Harrison J, Deaton CA, Johnson GVW. Tau clearance mechanisms. *Adv Exp Med Biol*. 2019;1184:57-68.
223. McAlpine CS, Park J, Griciuc A, et al. Astrocytic interleukin-3 programs microglia and limits Alzheimer's disease. *Nature*. 2021;595:701-706.
224. Odfalk KF, Bieniek KF, Hopp SC. Microglia: Friend and foe in tauopathy. *Prog Neurobiol*. Sep 2022;216:102306. doi:10.1016/j.pneurobio.2022.102306
225. Taddei RN, Perbet R, Mate de Gerando A, et al. Tau oligomer-containing synapse elimination by microglia and astrocytes in Alzheimer disease. *JAMA Neurol*. 2023;80:1209-1221.
226. Hablitz LM, Nedergaard M. The glymphatic system: a novel component of fundamental neurobiology. *J Neurosci*. 2021;41:7698-7711.
227. Buccellato FR, D'Anca M, Serpente M, Arighi A, Galimberti D. The role of glymphatic system in Alzheimer's and Parkinson's disease pathogenesis. *Biomedicines*. 2022;10:2261.
228. Ishida K, Yamada K, Nishiyama R, et al. Glymphatic system clears extracellular tau and protects from tau aggregation and neurodegeneration. *J Exp Med*. 2022;219:e20211275.
229. Chen X, Firulyova M, Manis M, et al. Microglia-mediated T cell infiltration drives neurodegeneration in tauopathy. *Nature*. 2023;615:668-677.

Date of submission: July 15, 2024
Date of decision: September 7, 2024
Date of acceptance: September 10, 2024
Date of web publication: September 27, 2024

Behavioral and Brain Functions

RESEARCH

Open Access

Investigation in the cannabigerol derivative VCE-003.2 as a disease-modifying agent in a mouse model of experimental synucleinopathy

Sonia Burgaz[1,2,3], Elisa Navarro[1,2,3], Santiago Rodríguez-Carreiro[1,2,3], Carmen Navarrete[4], Martin Garrido-Rodríguez[5,6,7], Isabel Lastres-Becker[2,8,9,10,11], Julia Chocarro[2,12], José L. Lanciego[2,12], Eduardo Muñoz[5,6,7*] and Javier Fernández-Ruiz[1,2,3*]

Abstract

Background The cannabigerol derivative VCE-003.2, which has activity at the peroxisome proliferator-activated receptor-γ has afforded neuroprotection in experimental models of Parkinson's disease (PD) based on mitochondrial dysfunction (6-hydroxydopamine-lesioned mice) and neuroinflammation (LPS-lesioned mice). Now, we aim to explore VCE-003.2 neuroprotective properties in a PD model that also involves protein dysregulation, other key event in PD pathogenesis.

Methods To this end, an adeno-associated viral vector serotype 9 coding for a mutated form of the α-synuclein gene (AAV9-SynA53T) was unilaterally delivered in the substantia nigra pars compacta (SNpc) of mice. This model leads to motor impairment and progressive loss of tyrosine hydroxylase-labelled neurons in the SNpc.

Results Oral administration of VCE-003.2 at 20 mg/kg for 14 days improved the performance of mice injected with AAV9-SynA53T in various motor tests, correlating with the preservation of tyrosine hydroxylase-labelled neurons in the SNpc. VCE-003.2 also reduced reactive microgliosis and astrogliosis in the SNpc. Furthermore, we conducted a transcriptomic analysis in the striatum of mice injected with AAV9-SynA53T and treated with either VCE-003.2 or vehicle, as well as control animals. This analysis aimed to identify gene families specifically altered by the pathology and/or VCE-003.2 treatment. Our data revealed pathology-induced changes in genes related to mitochondrial function, lysosomal cell pathways, immune responses, and lipid metabolism. In contrast, VCE-003.2 treatment predominantly affected the immune response through interferon signaling.

Conclusion Our study broadens the neuroprotective potential of VCE-003.2, previously described against mitochondrial dysfunction, oxidative stress, glial reactivity and neuroinflammation in PD. We now demonstrate its efficacy against another key pathogenic event in PD as α-synuclein dysregulation. Furthermore, our investigation

*Correspondence:
Eduardo Muñoz
fi1muble@uco.es
Javier Fernández-Ruiz
jjfr@med.ucm.es

Full list of author information is available at the end of the article

© The Author(s) 2024. **Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

sheds light on the molecular mechanisms underlying VCE-003.2 revealing its role in regulating interferon signaling. These findings, together with a favorable ADMET profile, enhance the preclinical interest of VCE-003.2 towards its future clinical development in PD.

Keywords VCE-003.2, Parkinson's disease, alpha-synuclein , Cannabinoids, PPAR-γ

Introduction

Cannabinoids have emerged as promising neuroprotective agents given their ability to work as pleiotropic compounds against the multiple events that affect neural cell homeostasis, integrity and survival in conditions of brain damage and neurodegeneration [31]. This pleiotropism is facilitated by the activity of cannabinoids at multiple pharmacological targets within the endocannabinoid system (e.g., cannabinoid type-1 (CB_1), cannabinoid type-2 (CB_2) and GPR55 receptors, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) enzymes; see [31], [6, 20, 27, 32]. Additionally, cannabinoids interact with other signaling systems, such as transient receptor potential vanilloid type-1 (TRPV1), peroxisome proliferator-activated receptors (PPARs), adenosine and serotonin receptors [31, 70, 73]. This neuroprotective potential has been preclinically investigated in different neurological conditions, such as accidental brain damage (e.g., stroke, brain trauma, spinal cord injury) and in chronic progressive disorders (e.g., Alzheimer's disease and related dementias, amyotrophic lateral sclerosis, Huntington's disease and others; see [4, 23, 30, 55, 65].

One of the disorders that has recruited more research with cannabinoids to date is Parkinson's disease (PD). Several cannabinoids have been found to be able to alleviate specific parkinsonian symptoms such as bradykinesia and immobility [46], tremor [67] and/or L-DOPA-induced dyskinesia [26]. However, a significantly greater interest has been recruited with those cannabinoid treatments active at preserving neuronal integrity then resulting in delayed disease progression [3, 7, 27, 29, 43]. Such neuroprotective properties include cannabinoid receptor-independent antioxidant effects, mainly afforded by the non-psychotropic phytocannabinoid cannabidiol (CBD), whose structure allows this compound to act as scavenger of reactive oxygen species [51], as well as it modulates the antioxidant transcription factor Nuclear Factor (Erythroid-derived 2)-related factor 2 (NRF-2) pathway too [40]. Activating the CB_1 receptor was furtherly proposed as a neuroprotective therapy in experimental PD in a few studies [22, 62], despite it may aggravate akinetic signs in certain circumstances and produce psychoactive effects (reviewed in Fernández-Ruiz [28], . This has limited the studies with this cannabinoid receptor type and redirected the research to non-psychotropic cannabinoid receptors, for example the CB_2 receptor, whose activation with HU-308, Δ^9-tetrahydrocannabivarin (Δ^9-THCV), VCE-004.8 and other agonists entails anti-inflammatory and neuroprotective effects in different experimental models of PD [13, 35, 39, 44, 74]. A similar reduction of inflammatory events, resulting in preservation of nigrostriatal dopaminergic neurons in the basal ganglia, has been found with cannabinoids that are able to target the orphan receptor GPR55, which has been recently associated with the endocannabinoid system [14, 19]. In addition, the activation of PPAR-γ with glitazones [78], but also with some cannabinoids (e.g., VCE-003.2, CBGA-Q, GBGA-Q Salt), have been also associated with neuroprotective effects in different experimental models of PD [12, 15, 36]. Collectively, these studies have situated several cannabinoid compounds in a promising position for serving to generate a cannabinoid-based therapy for specific symptoms and, in particular, for disease progression in patients affected by PD (reviewed recently in Jain et al., [43].

Recent studies have demonstrated that an interesting and clinically promising cannabinoid compound in PD is the non-thiophilic and antioxidant CBG quinone derivative VCE-003.2, which behaves as a PPAR-γ partial agonist with no activity at the CB_1/CB_2 receptors [24]. The compound crosses the blood-brain barrier (BBB) after systemic administration [1, 24] and has been investigated here in relation with other preclinical attributes such as metabolism and toxicology. VCE-003.2 shows a favorable profile and rodent pharmacokinetic data guaranteeing high plasma exposure and rapid excretion after its oral administration, which overall may facilitate further translational studies towards the clinical scenario. In our hands, VCE-003.2 has been found to be active as a neuroprotective agent against inflammation-driven neuronal deterioration in LPS-lesioned mice [12, 36], as well as against mitochondrial impairment and oxidative stress in 6-hydroxydopamine (6-OHDA)-lesioned mice [15]. These cytoprotective effects were confirmed in experiments with cell-based models reproducing in vitro the in vivo models, which also revealed that these benefits were mediated by its binding at a functional alternative site (different from the canonical binding site used by glitazones) in the PPAR-γ receptor [15, 36]. We are now interested in further reinforcing the neuroprotective profile of VCE-003.2 in PD using experimental models based on protein (α-synuclein) dysregulation and accumulation, a key event in PD pathogenesis. α-Synuclein is a key protein in the formation of Lewy bodies in which this protein is a major component [69]. Their mutations have been associated with autosomal dominant or

recessive forms of genetic PD, whereas gene duplications or triplications have been also associated with the genetic parkinsonism (reviewed in Marmion and Kordower [56]. To this end, we used a murine model based on the local overexpression of α-synuclein carrying the A53T mutation, which is one of the most frequent mutation used in experimental models of PD, in the substantia nigra pars compacta (SNpc) through the local application of an adeno-associated viral vector serotype 9 (AAV9) encoding the mutated gene, as previously used in other preclinical studies [18, 45, 48, 66]; see details in a recent review by Björklund and Mattsson [10]. The local overexpression of A53T α-synuclein in the SN leads to a pathological phenotype visible in a short time (2 weeks after viral inoculation) consisting in motor defects, α-synuclein dysregulation, loss of dopaminergic neurons, autophagy induction, and microglial reactivity [18, 66]. Using these mice, we have investigated the neuroprotective potential of a chronic oral administration of VCE-003.2 by analyzing whether this cannabigerol derivative prevents behavioural, biochemical, and histopathological alterations elicited by A53T α-synuclein accumulation. The analysis of nigral dopaminergic neurons was carried out using first classic immunostaining, but the results were furtherly confirmed with a stereological-like procedure based on a deep-learning dedicated algorithm (Aiforia®) that has been successfully validated and published by Penttinen et al. [61]. It presently represents a useful tool with significant advantages (mainly reproducibility, elimination of human error, and fast high-capacity analysis) compared to unbiased stereological analysis. In our hands, it has been used with success in previous studies [21, 63]. Transcriptomic analysis of the striatum identified possible genes and pathways underlying the beneficial effects of VCE-003.2, as well as some of them related to the pathology.

Materials and methods

Viral vector production

A recombinant AAV vector serotype 2/9 expressing the *SNCA* gene with A53T mutation driven by the human synapsin promoter was produced by Vector Builder (www.vectorbuilder.com; catalog No. P211024-1010fpa; lot No. 211221AAVJ03). The virus was formulated in PBS buffer (pH 7.4) supplemented with 200 nM NaCl and 0.001% pluronic F-68. Obtained vector concentration was 9.62×10^{13} GC/ml. Virus purity was determined by SDS-PAGE followed by silver staining, resulting in >80% pure. Plasmid map for pAAV-synapsin-SynA53T and sequence are provided in Supplementary File 1.

Animals and surgical lesions

Male C57BL/6 mice were housed in our animal facilities (CAI-Animalario, Faculty of Medicine, Complutense University, ref. ES280790000086) in a room with a controlled photoperiod (08:00–20:00 light) and temperature (22±1ºC). They had free access to standard food and water and were used at adult age (6–8-month-old; weight=28–33 g). All animal experiments were conducted by researchers having the necessary accreditation and following local, national and European guidelines (directive 2010/63/EU), as well as conformed to ARRIVE guidelines and were approved by our local Ethical Committee for Animal Experimentation (ref: PROEX 201.8/22).

Mice were used for the in vivo overexpression of human α-synuclein with the A53T mutation using recombinant AAV9 encoding for this gene under the control of human synapsin 1 promoter. To this purpose, mice were anaesthetized (ketamine 40 mg/kg+xylazine 4 mg/kg, i.p.) and subjected to unilateral injections of viral particles (AAV9-SynA53T) into the SNpc of the right hemisphere following the procedure described in Lastres-Becker et al. [52]. In brief, 1 µl of a viral suspension containing 3.33×10^{12} GC/ml (selected from a preliminary concentration-response experiment using a range for the viral suspension of 3.33×10^{12} −3.33×10^{13} GC/ml) was unilaterally injected at predefined stereotaxic coordinates: -2.5 mm posterior to bregma, −1.4 mm lateral to midline and −4.5 mm ventral to dura. The volume of 1 µl was pressure-injected in pulses of 0.2 µl/min and, once completed, the needle was left in place for 5 min before withdrawal to minimize reflux of viral suspension through the injection tract. Control animals were sham-operated and injected with 1 µl of a viral suspension of an empty AAV9 (AAV9-null) containing 1.07×10^{12} GC/ml using the same coordinates. The lesions were generated using unilateral inoculation, so that contralateral structures serve as controls for the different analyses (see Fig. 1 for a diagram showing the schedule used).

Pharmacological treatments and sampling

After the application of AAV9-SynA53T or control AAV9-null vectors, animals were distributed into 3 groups in each experiment, and were subjected to a daily treatment with VCE-003.2 (20 mg/kg), or vehicle (sesame oil) by oral administration (see Fig. 1 for a diagram showing the schedule used). The number of animals in each experimental group was: (i) vehicle-treated sham-operated mice: 4–6; (ii) vehicle-treated AAV9-SynA53T mice: 8–10; and (iii) VCE-003.2-treated AAV9-SynA53T mice: 8–10 (ranges are due to elimination of potential outliers in those cases that the statistical analysis recommended to do it). The first administration, in all cases, was done approximately 24 h after the lesion and the treatment was prolonged for two weeks. At the end of the treatment (24 h after the last injection), they were analysed in different behavioural tests just before being killed by

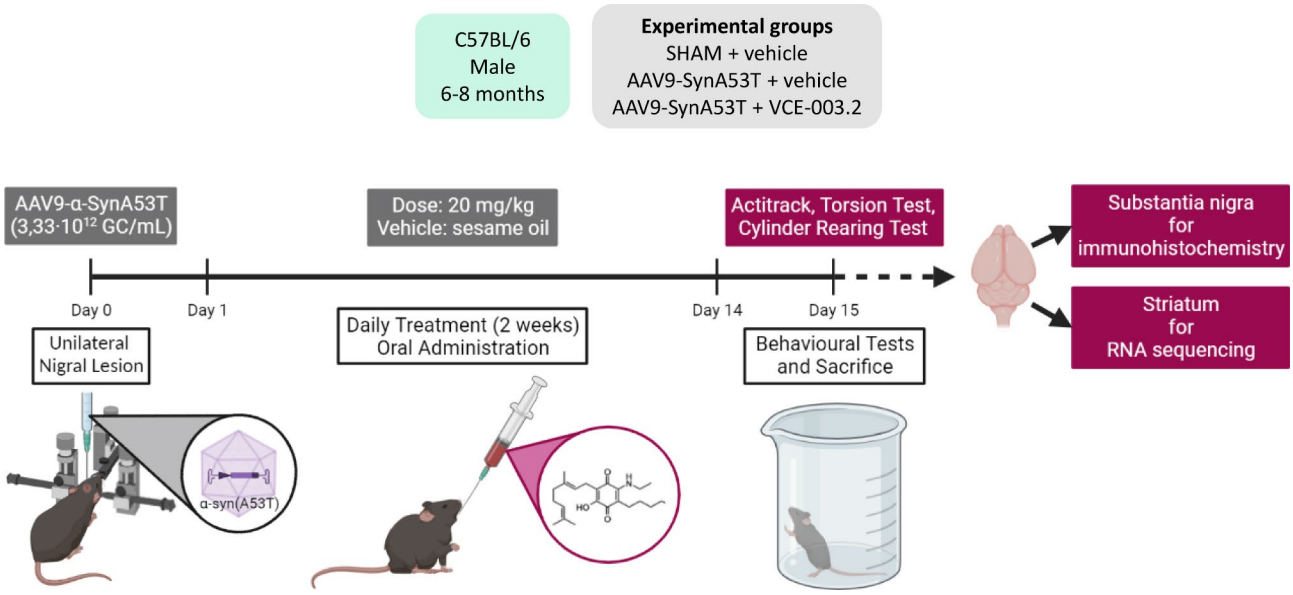


Fig. 1 Representative scheme of the experimental protocol used for the induction of α-synuclein-based PD model used in this study and the treatment with VCE-003.2, with indication of timelines for treatments, behavioral analysis and animal euthanasia

rapid and careful decapitation, and their brains rapidly removed and divided coronally in two parts. The anterior halves were used to dissect the striatum. Tissues were rapidly frozen by immersion in cold 2-methylbutane and stored at -80ºC for RNA sequencing analysis (RNAseq). The posterior halves containing the midbrains were fixed for one day at 4 °C in fresh 4% paraformaldehyde prepared in 0.1 M PBS, pH 7.4. Samples were cryoprotected by immersion in a 30% sucrose solution for a further day, and finally stored at -80 °C for immunohistochemical analysis in the SN.

Pharmacokinetic and ADMET analysis

Pharmacokinetic for oral VCE-003.2 in mice and in vitro off-target binding, transporter interactions, micronucleus assay, and comparative metabolism assays for VCE-003.2 in hepatocytes from different species were performed by Charles River (Edinburgh, UK) and Eurofins Panlabs Discovery Services (New Taipei City, TW), using protocols summarized in Supplementary File 2.

Behavioural tests

Cylinder rearing test (CRT) Given that the lesions were unilateral, this test attempts to quantify the degree of forepaw (ipsilateral, contralateral, or both) preference for wall contacts in rearing movements after placing the mouse in a methacrylate transparent cylinder (diameter: 15.5 cm; height: 12.7 cm; Fleming et al., [33]. Each score was calculated from a 3 min trial with a minimum of 4 wall contacts. See Supplementary File 3 for an example of video recording in the three experimental groups.

Elevated-body swing test (EBST) Mice were placed head-downward hanging by their tail in a vertical axis (about 15 cm from the surface) and recorded for 60 s [8, 11]. A swing was recorded whenever the animal moved its head out of the vertical axis to either side, whereas for the next swing to be counted, the animal must have returned to the vertical position first. See Supplementary File 4 for an example of video recording in the three experimental groups.

Computer-aided Actimeter For the analysis of motor activity, we used a computer-aided actimeter (Actitrack, Panlab, Barcelona, Spain). This apparatus consisted of a 45×45 cm area, with infra-red beams all around, spaced 2.5 cm, coupled to a computerized control unit that analyzes the following parameters: (i) distance run in the actimeter (ambulation); (ii) time spent in inactivity; (iii) frequency of vertical activity (rearing); (iv) mean and maximal velocity developed during the running; and (v) time spent in fast (>5 cm/s) and slow (<5 cm/s) movements. Animals remained for a period of 10 min in the actimeter, but measurements were only recorded during the final 5 min (first 5 min was used only for animal acclimation). These data are presented as Supplementary File 5.

Immunohistochemical procedures

Brains were sliced in coronal sections (containing the SN) in a cryostat (30 µm thick) and collected in antifreeze solution (glycerol/ethylene glycol/PBS, 2:3:5) and stored at -20ºC until being used. Brain sections were mounted on gelatine-coated slides, and once adhered, washed in 0.1 M potassium PBS (KPBS) at pH 7.4. Then,

endogenous peroxidase was blocked by 30 min incubation at room temperature in peroxidase blocking solution (Dako Cytomation, Glostrup, Denmark). After several washes with KPBS, sections were incubated overnight at 4ºC with the following antibodies: (i) monoclonal mouse anti-human α-synuclein (sc-53955; Santa Cruz Biotechnology, Dallas, TX, USA) used at 1:2000; (ii) polyclonal rabbit anti-mouse tyrosine hydroxylase (TH) (AB152; Chemicon-Millipore, Temecula, CA, USA) used at 1:200; (iii) polyclonal rat anti-mouse CD68 antibody (MCA-1957; AbD Serotec, Oxford, UK) used at 1:200; or (iv) polyclonal rabbit anti-mouse GFAP antibody (Z0334; Dako Cytomation, Glostrup, Denmark) used at 1:200. Dilutions were carried out in KPBS containing 2% bovine serum albumin and 0.1% Triton X-100 (Sigma Chem., Madrid, Spain). After incubation, sections were washed in KPBS, followed by 1 hour incubation at room temperature with the corresponding biotinylated secondary antibody (1:1000 for α-synuclein; 1:200 for the rest) (Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. Avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine substrate–chromogen system (Dako Cytomation, Glostrup, Denmark) were used to obtain a visible reaction product. Negative control sections were obtained using the same protocol with omission of the primary antibody. A Leica DMRB microscope and a DFC300FX camera (Leica, Wetzlar, Germany) were used for the observation and photography of the slides, respectively. For quantification of the intensity of α-synuclein, TH, CD68 or GFAP immunostaining in the SN (at both ipsilateral and contralateral sides), we used the NIH Image Processing and Analysis software (ImageJ; NIH, Bethesda, MD, USA) using 4–5 sections, separated approximately by 200 µm, and observed with 5x-20x objectives depending on the immunostaining under quantification. In all sections, the same area of the SNpc was analysed. Analyses were always conducted by experimenters who were blinded to all animal characteristics. Data were expressed as percentage of immunostaining intensity in the ipsilateral (lesioned) side over the contralateral (non-lesioned) side. In addition, in the case of CD68 immunostaining, images at higher magnification were also used for a qualitative analysis of certain morphological characteristics (cell body volume; number, thickness and length of cell processes) that may reflect the state of quiescence (low cell body volume and tiny and long processes) or activation (amoeboid-like morphology: high cell body volume and thick and short processes) of these cells.

In a further analysis, a deep-learning dedicated algorithm was prepared with Aiforia® [61], validated and released (resulting in an error of 4.82%) for quantifying the number of TH-positive neurons in TH-stained, equally-spaced coronal sections covering the whole rostrocaudal extent of the SNpc. The dedicated algorithm was customized to count all positive neurons, independently of the intensity of staining. Sections were scanned at 20x in a slide scanner (Aperio CS2; Leica, Wetzlar, Germany) and uploaded to the Aiforia® cloud. Next, the boundaries of each selected ROI were outlined at low magnification and the dedicated algorithm was then used as a template quantifying the TH-positive neurons. Details on this analysis have been previously published [21, 63].

Transcriptomic analysis

RNA isolation and sequencing RNA was isolated from the striatum samples using the RNeasy Lipid Tissue Mini kit (Qiagen, Barcelona, Spain) following the manufacturer's instructions, including the optional DNase I step. Once RNA was isolated, samples were stored at −80 °C following library preparation. RNA concentration and RNA integrity number (RIN) was assessed using Nanodrop and Bioanalyzer (Centre for Genomic Regulation, Barcelona, Spain). Library preparation and sequencing was outsourced to the Centre for Genomic Regulation (CRG, Barcelona, Spain). Briefly, libraries were prepared using the TruSeq mRNA Library prep following the manufacturer's instructions. mRNA selection was performed using poly(A)-mRNA selection strategies. Libraries were prepared and sequenced by 50 bp sequencing using Illumina NextSeq 2000 run system (Illumina, Inc., San Diego, CA, USA).

RNA-seq data processing and normalization Raw reads were aligned against the GRCm38 genome (v84) from ENSEMBL with HISAT2 (v2.1.0) [47, 59]. This resulted in a ~ 99% alignment rate with 60% of reads aligned to exonic regions. The count matrix was obtained from the alignments using the corresponding ENSEMBL annotation file with featureCounts (v2.0.0) [54]. Then, counts per million (CPM) were calculated using CPM function from the EdgeR packing in R [53]. Lowly expressed genes were filtered out, which were defined as having less than one CPM in at least 25% of the samples leading to a total of 13,980 genes. Principal Component Analysis (PCA) was used to identify sources of variation and outliers. As samples were sequenced in two independent batches, we regressed out sequencing batch to control variation. The final dataset was composed by 17 samples (6 from vehicle-treated sham-operated mice, 5 vehicle-treated AAV9-SynA53T-injected mice, and 6 VCE-003.2-treated AAV9-SynA53T-injected mice).

Differential expression analysis Differential expression analysis was performed using the R package limma [64]. EdgeR was used for data normalization using TMM values and followed by voom transformation. *P*-values were corrected using the Benjamini-Hochberg false discovery rate (FDR).

Pathway enrichment analysis. We used Gene Set Enrichment Analysis (GSEA) focusing on Hallmark gene sets [72]. To perform pathway analysis, ranked DEGs in terms of t-value were used as input. We used the fGSEA R-package [49]. Only pathways with adjusted *P*-value < 0.05 were considered.

Transcription factor enrichment analysis For exploring if genes were enriched for specific transcription factors, we used DoRothEA [37, 41]. Regulons were filtered at confidence levels "A" and "B". Regulon enrichment was runned using fgsea with preranked data in terms of t value. Only regulons with adjusted *P*-value < 0.05 were considered.

Statistics

Data were assessed using one-way ANOVA, followed by the Tukey test using GraphPad Prism, version 8.00 for Windows (GraphPad Software, San Diego, CA, USA). A p value lower than 0.05 was used as the limit for statistical significance. The sample sizes in the different experimental groups were always ≥ 5.

Results

Pharmacokinetic, metabolic, and toxicological properties of VCE-003.2

We have previously shown that VCE-003.2 crossed the BBB after oral administration in rats [1]. Now, we extended our PK studies in mice, found that plasma VCE-003.2 levels peaked at 4 h (Tmax) and then slowly declined to basal levels at 24 h. Oral VCE-003.2 resulted in 69% bioavailability (F) in mice (Supplementary Table 1), which was significantly higher than the one observed in rats (F = 13,77%) [1]. Moreover, VCE-003.2 did not significantly inhibit the activity of relevant cytochrome P450 isoforms, did not inhibit hERG channel activity and it was not genotoxic as assessed by AMES assays [1]. To complement these studies, we interrogated for potential off-targets and genotoxicity of VCE-003.2. Using radioligand binding assays, we explored the interaction with a panel of 172 receptors and channels, and we found that VCE-003.2 did not show a significant binging activity to these targets (Supplementary File 2). The potential genotoxicity of VCE-003.2 was investigated using micronucleus assays either in the presence or absence of a metabolic activation system (S9-mix), in cultured human lymphocytes (in vitro) and in rat bone marrow cells (in vivo). We observed that VCE-003.2 is not clastogenic or aneugenic either in human lymphocytes or in the bone marrow micronucleus test of male rats up to a dose of 2000 mg/kg (Supplementary File 2).

We also investigated the metabolic stability and metabolite profiles of VCE-003.2 using suspensions of cryopreserved hepatocytes from CD-1 mouse, Wistar rat, Beagle dog, Göttingen minipig, cynomolgus monkey and human. The rates of metabolism and the metabolite profiles were compared across the species tested. Twenty-six metabolites of VCE-003.2 were found in the hepatocyte incubations of the six species investigated. Based on the MS peak intensities, no human specific metabolites were observed and five major metabolites of VCE-003.2 were detected in at least four of the six species tested. Metabolic reactions observed included oxidations, the loss of C_3H_6 in combination with two oxidations, and a desaturation in combination with one or two oxidations. The postulated major metabolic pathway VCE-003.2 was also predicted (Supplementary File 2).

Finally, we explored the in vitro interaction of VCE-003.2 with human BCRP, BSEP and MDR1 efflux (ABC) transporters, and with human solute carrier (SLC) transporters MATE1, MATE2-K, OAT1, OAT3, OATP1B1, OATP1B3, OCT1 and OCT2. VCE-003.2 did not inhibit SLC transported and up to 22.5 µM showed inhibitory activity of the ABC transporters (BCR = 89% inhibition, BSEP = less than 50% inhibition; MDR1 = 81% inhibition) (Supplementary File 2). Altogether, VCE-003.2 represents an excellent ligand for studying the relevance of PPAR-γ activation in experimental models of PD, as has been demonstrated in previous studies [12, 15, 36] and situates this compound in a clear advantage for future translational development.

Behavioural readouts of treatment with VCE-003.2 in mice injected with AAV9-SynA53T

To explore the neuroprotective potential of an oral administration of VCE-003.2 against the overexpression of neuronal (A53T)α-synuclein, we first analysed the motor performance of these animals using CRT and EBTS. These motor tests discriminate the unilateral hemiparesis and were assessed 24 h after the last VCE-003.2 administration, avoiding any acute effect of the drug. CRT showed a preference for the ipsilateral right paw (controlled by the non-lesioned contralateral SN) in mice-injected with AAV9-SynA53T, which was prevented by the treatment with VCE-003.2 ($F(2,17) = 11.50$, $p < 0.001$; Fig. 2 and Supplementary File 3). Also, we noticed a general mobility increase, reflected in the total number of rearings, in the VCE-003.2-treated animals, which tended to be elevated with respect to both vehicle-treated sham-operated and AAV9-SynA53T-injected mice ($F(2,18) = 2.99$, $p = 0.075$; Fig. 2).

Next, we performed EBST, which is useful to detect the side more frequent in the torsion movements depending on the lesioned side. We found that vehicle-treated AAV9-SynA53T-injected mice tended to show a preference for contralateral turns, which was not attenuated by the treatment with VCE-003.2, even it tended to be enhanced ($F(2,19) = 1.49$, ns; Fig. 2 and Supplementary File 4). However, as in CRT, we observed a higher number

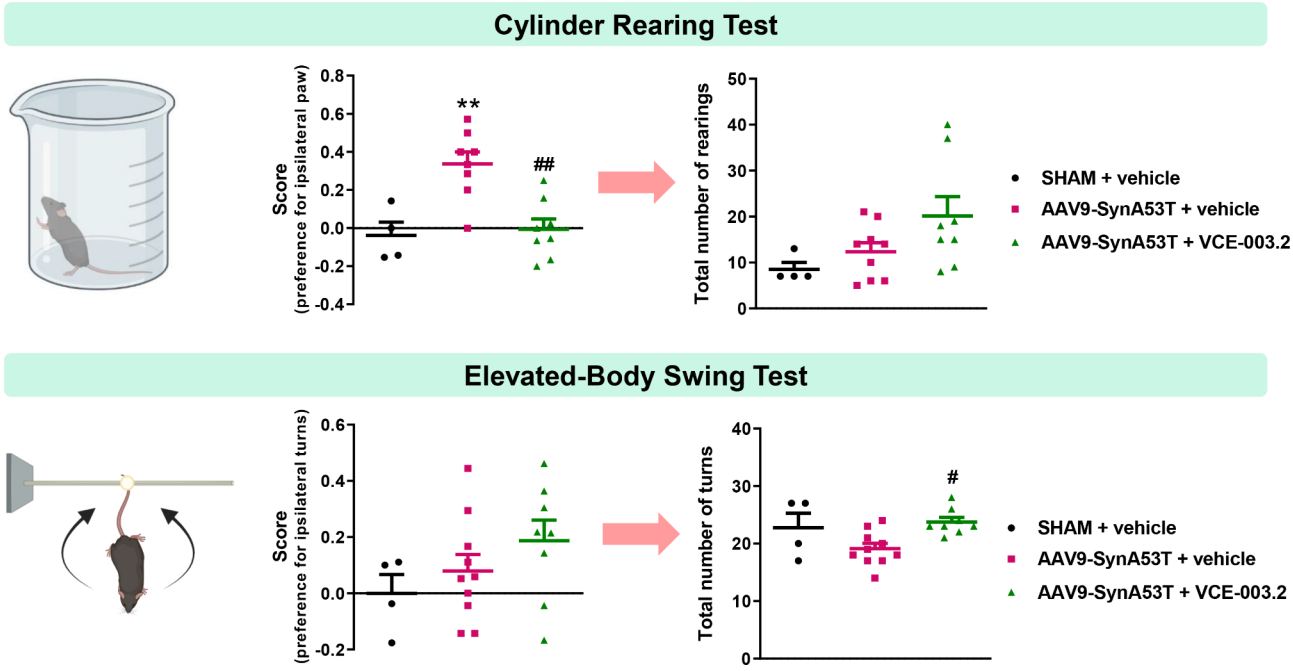


Fig. 2 Behavioural analysis in the Cylinder Rearing Test (CRT) and in the Elevated-Body Swing Test (EBST) of vehicle-treated control (sham-operated) adult male mice and unilaterally AAV9-SynA53T-injected adult male mice treated with vehicle or VCE-00.2 (20 mg/kg) given orally. Treatments were daily and prolonged for 2 weeks. Data (preference score (left) and total number of movements (right)) corresponded to 24 h after the last dose and were expressed as means ± SEM of more than 4 animals *per* group. They were analyzed by one-way ANOVA followed by the Tukey test (**$p < 0.01$ *versus* vehicle-treated sham-operated mice; #$p < 0.05$, ##$p < 0.01$ *versus* vehicle-treated AAV9-SynA53T-injected mice)

of total turns (irrespective of the side) shown during the test performance ($F(2,19) = 5.13$, $p < 0.05$; Fig. 2), demonstrating a greater activity in the treated animals.

Such elevated motor activity elicited by VCE-003.2 in AAV9-SynA53T-injected mice in comparison with the other two groups was also visible in the data obtained in a computer-aided actimeter (actitrack), with statistically significant increases in locomotor parameters such as general activity ($F(2,19) = 4.39$, $p < 0.05$), ambulation ($F(2,19) = 4.55$, $p < 0.05$), mean and maximal velocities ($F(2,19) = 4.14$, $p < 0.05$ and $F(2,19) = 5.53$, $p < 0.05$, respectively), and frequency of slow movements ($F(2,19) = 5.82$, $p < 0.01$). This increase remained as a mere numerical trend in parameters as vertical activity ($F(2,19) = 1.56$, $p = 0.24$) and frequency of fast movements ($F(2,19) = 2.65$, $p = 0.096$), whereas the opposite (lower values) was detected, as expected, for resting time ($F(2,19) = 5.13$, $p < 0.05$), then confirming that VCE-003.2-treated AAV9-SynA53T-injected mice frequently exhibited a greater motor activity (see these data in the Supplementary File 5).

Histopathological readouts of treatment with VCE-003.2 in mice injected with AAV9-SynA53T

Immunohistochemical analysis demonstrated an AAV9-driven overexpression of human α-synuclein all over the midbrain in the lesioned mice, in particular in the SNpc ($F(2,18) = 19.42$, $p < 0.0001$; see Supplementary File 6) [18, 66]. Furthermore, the enhanced neuronal expression of mutated α-synuclein was associated with a significant reduction (around 35%) of TH immunoreactivity in the SNpc ($F(2,21) = 20.67$, $p < 0.0001$; Fig. 3). Treatment with VCE-003.2 of mice injected with AAV9-SynA53T did not alter the immunoreactivity levels of α-synuclein (Supplementary File 6). However, it prevented the reduction in TH immunoreactivity to the same value detected in sham-operated mice (Fig. 3). This observation supports the idea that VCE-003.2 treatment preserves the integrity of TH-positive neurons in the SNpc against the enhanced neuronal expression of mutated α-synuclein, despite our analysis only served to measure immunoreactivity levels. In a further analysis, using a stereological-like analysis based on a deep-learning dedicated algorithm (Aiforia®), we could confirm that the reduction in TH measured in the SNpc of mice injected with AAV9-SynA53T, as well as the recovery detected after the treatment with VCE-003.2, reflected both a loss and a preservation in the number of TH-positive neurons, respectively ($F(2,21) = 6.72$, $p < 0.01$; see Supplementary File 7). This preservation may contribute to the behavioral improvements indicated in the previous subsection.

The neuronal overexpression of mutated α-synuclein in the SN also induced robust glial reactivity in both microglia (CD68: $F(2,17) = 27.58$, $p < 0.0001$; Fig. 4), and

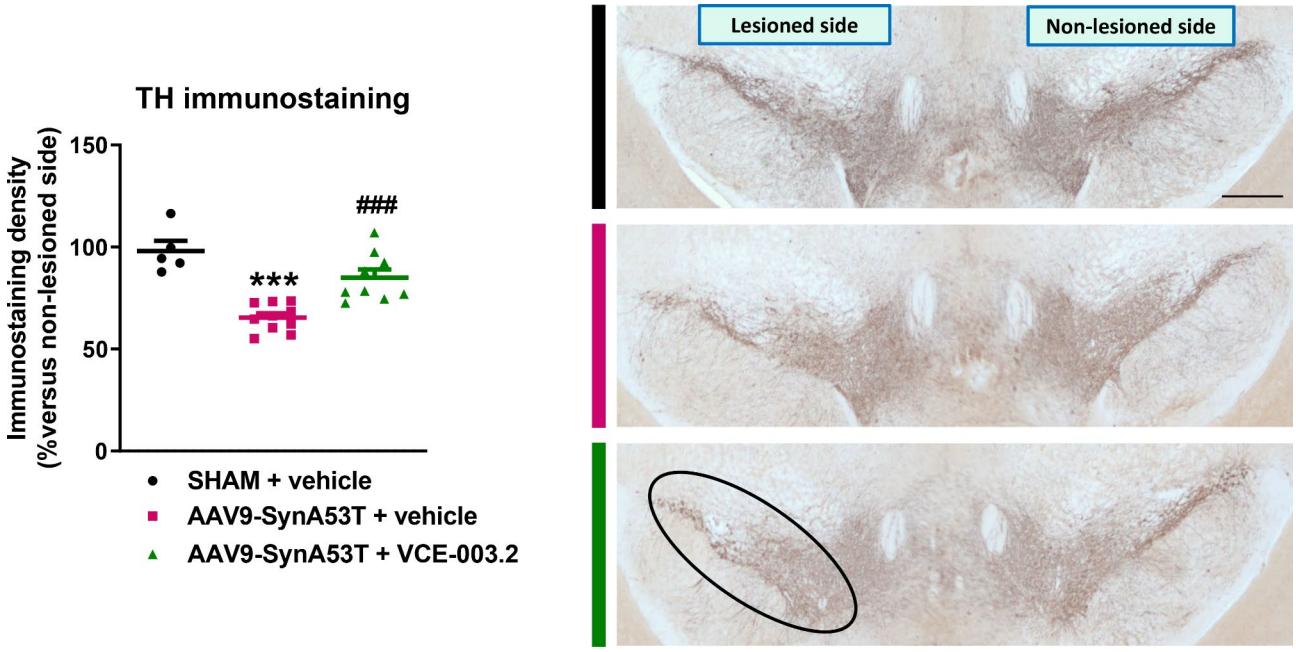


Fig. 3 Intensity of the immunostaining for TH measured in a selected area of the SNpc of vehicle-treated control (sham-operated) adult male mice and unilaterally AAV9-SynA53T-injected adult male mice treated with vehicle or VCE-00.2 (20 mg/kg) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to 24 h after the last dose and were expressed as means ± SEM of more than 5 animals *per* group. They were analyzed by one-way ANOVA followed by the Tukey test (***$p < 0.005$ *versus* vehicle-treated sham-operated mice; ###$p < 0.005$ *versus* vehicle-treated AAV9-SynA53T-injected mice). Representative immunostaining images for each experimental group, with indication of the approximate area quantified, are shown in right panels (scale bar = 200 µm)

astrocytes (GFAP: $F(2,21) = 26.17$, $p < 0.0001$; Fig. 4 and representative images in Supplementary File 8). In addition, a simple morphological analysis of CD68-labelled cells revealed a higher presence of cells with apparently amoeboid aspect (greater cell body volume and thick and short cells branches) in the SN of vehicle-treated AAV9-SynA53T mice compared to vehicle-treated sham-operated mice (Supplementary File 9), which is compatible with an activated state of these cells. VCE-003.2 treatment attenuated both responses, with a statistically significant effect in CD68 immunostaining (Fig. 4) and a numerical trend in GFAP immunostaining (Fig. 4 and representative images in Supplementary File 8). VCE-003.2 treatment of AAV9-SynA53T mice also reduced the number of CD68-labelled cells with apparently activated phenotype to a more quiescent state (smaller cell bodies and more, tiny and longer processes) (Supplementary File 9).

Transcriptomic readouts of treatment with VCE-003.2 in mice injected with AAV9-SynA53T

Having established the neuroprotective efficacy of VCE-003.2 in this α-synuclein model of PD, and recognizing that functional recovery likely involves the survival of nigrostriatal terminals arising the striatum, we aimed sought to delve into downstream cellular pathways through transcriptomic analysis of this specific brain region. To this end, we carried out RNA-Seq analysis in the right (lesioned) striatum of mice from the three different experimental groups (Supplementary Table 2). We first examined the impact of α-synuclein lesion on gene expression, comparing the AAV9-SynA53T-injected mice *versus* sham-operated animals, all treated with vehicle. We did observe a strong transcriptomic dysregulation with 1055 differentially expressed genes (DEGs) at FDR < 0.05 (50.8% up-regulated and 49.2% down-regulated) (Fig. 5, left panel; Supplementary Table 3). The oral administration of VCE-003.2 in the AAV9-SynA53T-injected mice produced a much more modest effect, with only 14 genes (*Irf2*, *Ifitm3*, *Ifit1*, *Ifit3*, *Oasl2*, *H2-Q6*, *Mir6236*, *Lgals9*, *Samd9*, *B2m*, *Oas2*, *H2-K1*, *H2-D1* and *ligp1*) becoming significantly altered from their expression in vehicle-treated AAV9-SynA53T-injected mice, most of them up-regulated, with 9 additional genes being close to be statistically significant (Fig. 5, right panel; Supplementary Table 4). Interestingly, these 23 genes appear to be part of a network of proteins related to interferon biology, as confirmed through Local Network Cluster Analysis using STRING (data not shown). The treatment with VCE-003.2 in AAV9-SynA53T-injected mice did not alter the expression of genes encoding for cannabinoid receptors or for those enzymes involved in the endocannabinoid synthesis and degradation in comparison with those animals treated with vehicle, despite

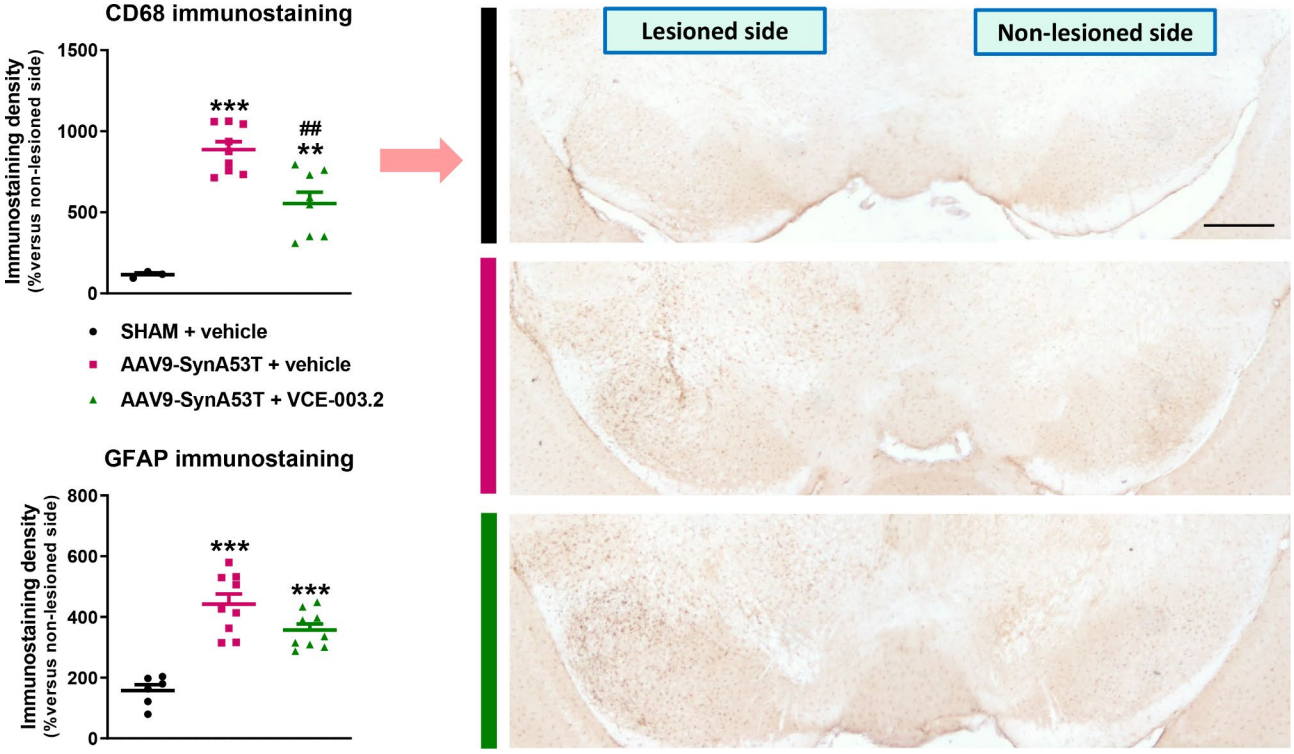


Fig. 4 Intensity of the immunostaining for CD68 and GFAP measured in a selected area of the SN of vehicle-treated control (sham-operated) adult male mice and unilaterally AAV9-SynA53T-injected adult male mice treated with vehicle or VCE-00.2 (20 mg/kg) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to 24 h after the last dose and were expressed as means ± SEM of more than 5 animals *per* group. They were analyzed by one-way ANOVA followed by the Tukey test (**$p < 0.01$, ***$p < 0.005$ *versus* vehicle-treated sham-operated mice; ##$p < 0.01$ *versus* vehicle-treated AAV9-SynA53T-injected mice). Representative CD68 immunostaining images for each experimental group are shown in right panels (scale bar = 200 µm)

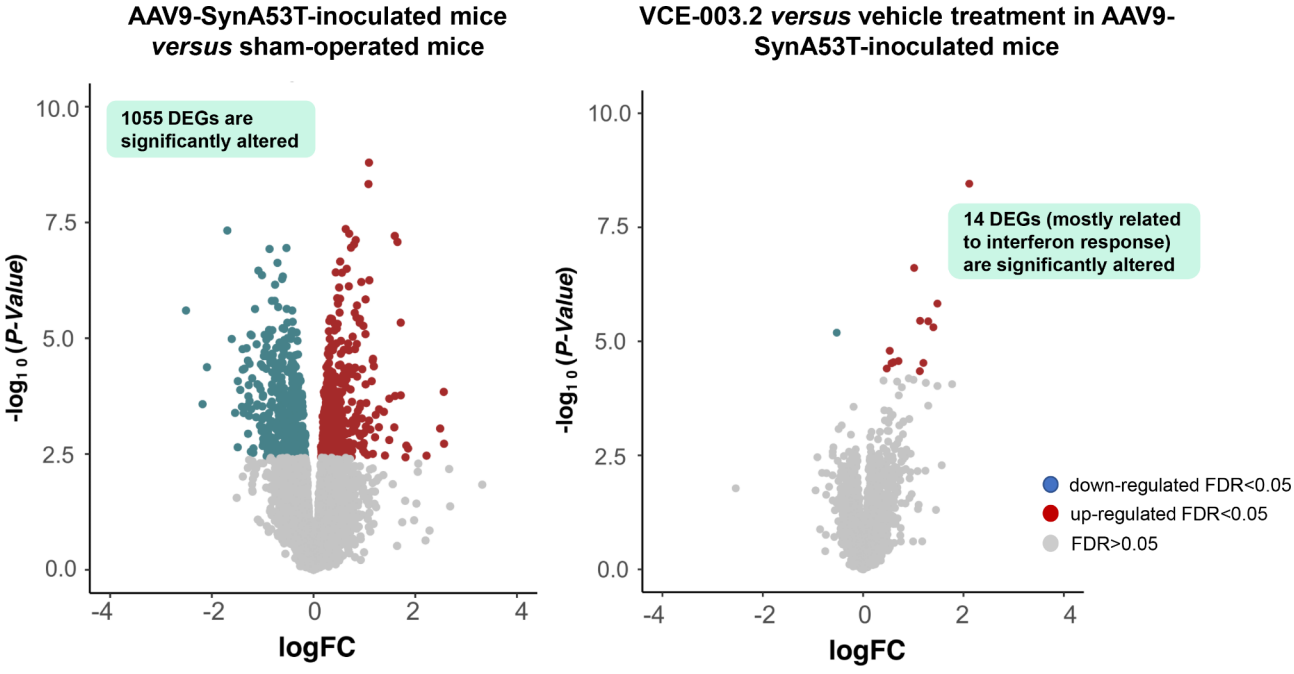


Fig. 5 Volcano plots showing differentially expressed genes (DEGs). X-axis shows the logFC and Y-axis shows -log(P-value). Every dot represents a specific gene (black are genes at FDR > 0.05 and red are genes at FDR < 0.05). Left panel: Volcano plot of the DEGs of AAV9-SynA53T-injected mice *versus* sham-operated mice, both treated with vehicle. Right panel: Volcano plot of the DEGs of VCE-003.2-treated AAV9-SynA53T-injected mice *versus* those treated with vehicle. DEGs were observed at FDR < 0.05

some of them did result altered by the local injection of AAV9-SynA53T into the SN. This was the case of the *Cnr1* (down-regulated: logFC=-1.068; FDR=0.007) and the *Gpr55* (up-regulated: logFC=+1.14; FDR=0.006) encoding the CB_1 and GPR55 receptors, respectively, as well as the *Faah* gene (down-regulated: logFC=-0.47; FDR=0.029) encoding the FAAH enzyme (Supplementary Tables 3 and 4). As regards to some PD-related proteins (e.g., α-synuclein, parkin, leucine-rich repeat kinase-2 (LRRK2)), our data indicated an important and expected up-regulation of *Snca* gene (logFC=0.48; FDR=0.0058) by the local injection of AAV9-SynA53T into the SN, but not in *Prkn* or *Lrrk2*, and no effect of the VCE-003.2 in any of these three genes (Supplementary Tables 3 and 4).

To understand the biological implications of the transcriptomic dysregulation, we performed GSEA using Hallmark Pathways from Gene Ontology. Pathway analysis revealed that DEGs affected by mutated α-synuclein overexpression were enriched for pathological hallmarks (see Supplementary Table 5) such as an impaired mitochondrial function affecting the oxygen species pathway and the oxidative phosphorylation (Fig. 6 and Supplementary Table 5). We also observed down-regulation in pathways associated with autophagy and lysosomal function (e.g., mTORC1 signaling), as well as lipid metabolism (e.g., fatty acid metabolism, adipogenesis) and the interferon response (Fig. 6 and Supplementary Table 5). In contrast, treatment with VCE-003.2 led to a significant increase in the expression of genes related to the interferon signaling, which was the opposite response when compared with the one detected after the delivery of AAV9-SynA53T (Fig. 6 and Supplementary Table5).

To determine whether the observed transcriptomic dysregulation was orchestrated by specific transcription factors, we conducted regulon enrichment analysis using DoRothEA [37, 41]. In a first set of analysis, we detected a significant enrichment in several regulons, which were down-regulated in vehicle-treated AAV9-SynA53T-injected mice with respect to vehicle-treated sham-operated animals (Fig. 7; see Supplementary Table 6). Among them was the finger family transcription factor Specificity

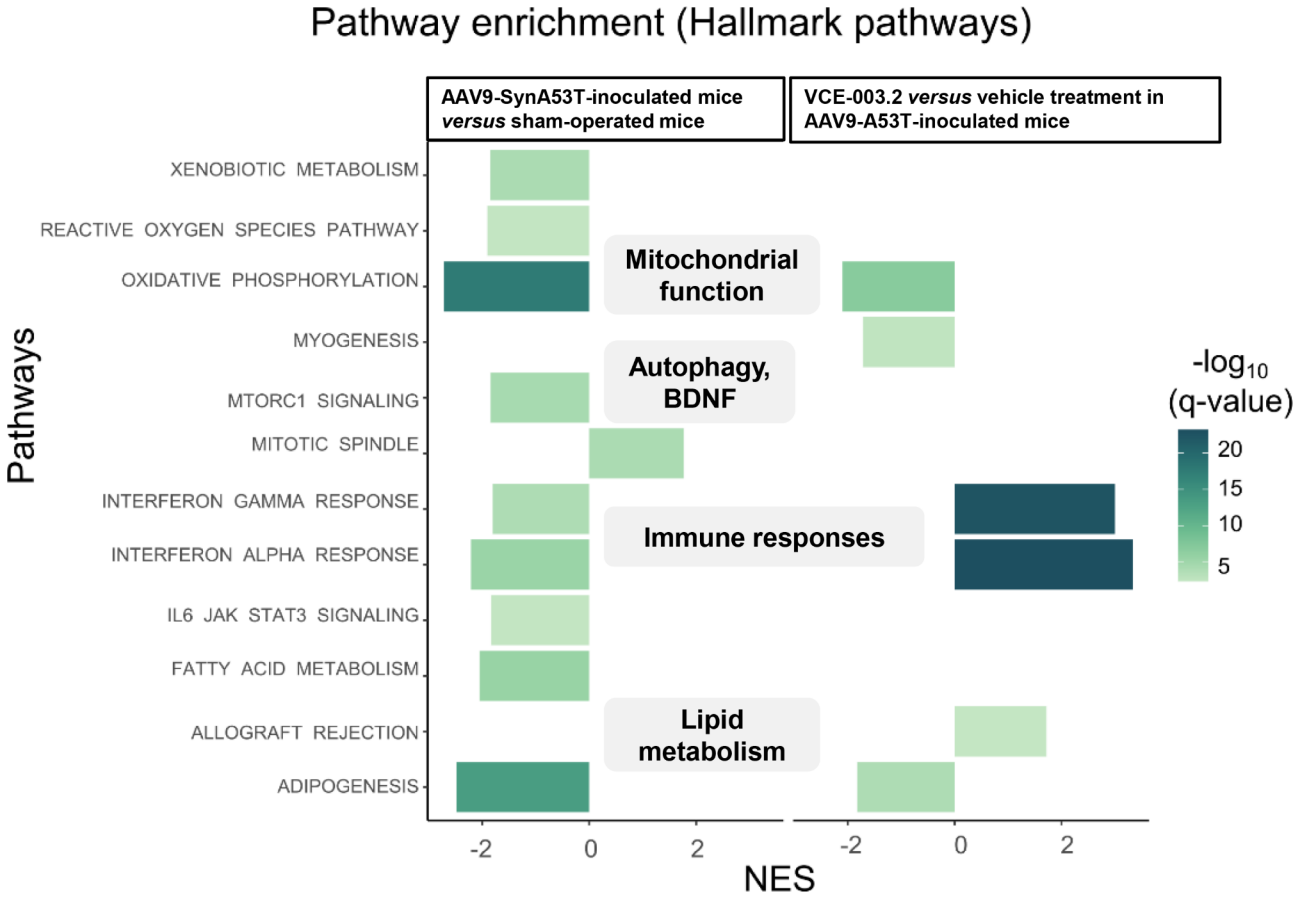


Fig. 6 Pathway enrichment analysis using Hallmark Pathways from GO using pre-ranked GSEA. Pathways represented are significant at q-value < 0.05. X-axis shows the directionality and Y-axis the biological pathway, with the green color tone reflecting the levels of significance. The significant enrichment for the comparison of AAV9-SynA53T-injected mice *versus* sham-operated mice, both treated with vehicle, is presented on the left, whereas the one for the comparison of VCE-003.2-treated AAV9-SynA53T-injected mice *versus* those treated with vehicle is presented on the right

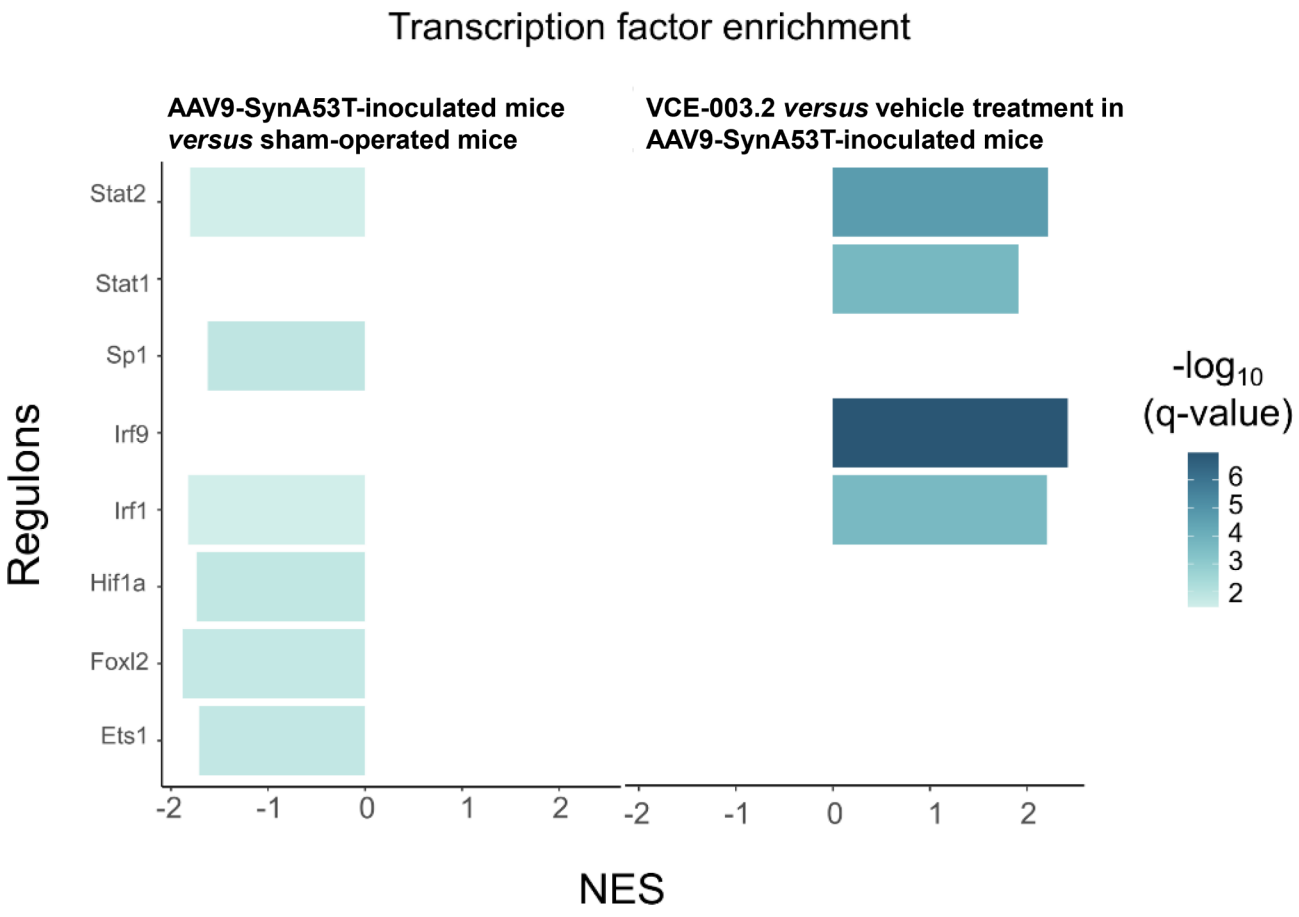


Fig. 7 Transcription factor enrichment analysis using DoRothEA showing the altered regulons that were significant at q-value < 0.05. X-axis shows the directionality and Y-axis the different regulons, with the green color tone reflecting the levels of significance. The significant enrichment for the comparison of AAV9-SynA53T-injected mice *versus* sham-operated mice, both treated with vehicle, is presented on the left, whereas the one for the comparison of VCE-003.2-treated AAV9-SynA53T-injected mice *versus* those treated with vehicle is presented on the right

protein-1 (*Sp1*), the tumorigenic factor Forkhead box I2 (*FoxI2*), and others such as Erythroblast transformation specific-1 (*Ets1*) and Hypoxia-inducible factor-1a (*Hif1a*) (Fig. 7 and Supplementary Table 6). Other down-regulated regulon of interest is Interferon regulatory factor-1 (*Irf1*), along with other transcription factors such as Signal transducer and activator of transcription-1 (*Stat1*) and −2 (*Stat2*), the latter also down-regulated (Fig. 7 and Supplementary Table 6). When we compared VCE-003.2-treated AAV9-SynA53T-injected mice with the same animals treated with vehicle, we observed up-regulation for *Stat1*, *Stat2*, *Irf*1 and *Irf9* (Fig. 7 and Supplementary Table 6). This suggests that VCE-003.2 may reverse the transcriptomic deficiencies in these factors induced by the neuronal overexpression of mutated α-synuclein (Fig. 7). Whether this mechanism of action is a key event in the ability of VCE-003.2 to preserve the structure and functionality of the nigrostriatal projection in the basal ganglia, or whether a mere consequence of such neuroprotective effect exerted by VCE-003.2 will require further research. Nevertheless, this finding is a novel and significant result of our transcriptomic analysis. All raw RNA-Seq data have been uploaded to ArrayExpress repository to make them available for further analysis (accession number: E-MTAB-13768).

Discussion

The non-thiophilic CBG quinone derivative VCE-003.2 administered i.p. has been intensely investigated in different experimental models of PD with positive results as a potential disease modifying agent [15, 36]. Its efficacy has also been confirmed through alternative routes of administration (e.g., oral) that may facilitate its further clinical development [12, 15]. However, the in vitro and in vivo models employed in these studies (e.g., LPS-lesioned mice, 6-OHDA-exposed SH-SY5Y cells, 6-OHDA-lesioned mice) fail to reproduce the dysregulation and aggregation of α-synuclein, a seminal event in PD pathogenesis [16]. In this study, our aim was to assess the potential efficacy of the orally administered VCE-003.2 in a model based on α-synuclein dysregulation and accumulation, which would contribute to positioning

this cannabinoid derivative for a promising translation to the clinical scenario. Moreover, we have investigated some additional pharmacological characteristics of VCE-003.2 in relation with its ADMET, pharmacokinetics, and secondary pharmacology properties that demonstrate that it counts with high druglikeness and a solid preclinical characterization, which reinforce its promising translational potential for this and similar pathological conditions.

To achieve this major objective of our study, we used an experimental model of PD based on the intraparenchymal delivery of AAV9-SynA53T in the SN. This model, previously employed in similar studies evaluating various neuroprotective compounds [18, 66], has demonstrated robustness in recapitulating the expected PD-pathogenic events such as motor defects, elevated levels of α-synuclein and loss of TH-positive neurons in the SNpc, and the activation of astrocytes and microglial cells. Using this model, we show how the oral administration of VCE-003.2 resulted in preservation of nigral TH-containing neurons, attenuation of glial (microglia and astrocytes) reactivity, and improved performance in the motor tests used. Notably, there was a significant recovery in the CRT and, overall, an increased mobility with respect to mice injected with AAV9-SynA53T. While we anticipated that VCE-003.2 treatment may also reduce α-synuclein overexpression, given its potential effects by enhancing autophagic and/or lysosomal functions [12], no changes were observed in α-synuclein levels in the SNpc of mice injected with AAV9-SynA53T after administration of VCE-003.2. Further research is needed to investigate if VCE-003.2 may prevent aggregation leading to the neuroprotection observed.

To shed light in the molecular pathways underlying the biological effects, especially the functional recovery, we performed RNA-Seq analysis of the striatum where we shall observe the consequences of preserving nigrostriatal innervation. We found a substantial transcriptomic change driven by this mutant α-synuclein overexpression, which fit with some expected changes in gene families encoding for proteins associated, for example, with the oxygen species pathway and the oxidative phosphorylation, features importantly associated to PD [34]. We also observed alterations in pathways associated with autophagy and lysosomal function (e.g., mTORC1 signaling), as well as lipid metabolism (e.g., fatty acid metabolism, adipogenesis), inflammation and the interferon response, all of them previously implicated in the development of PD [2, 5, 42, 50, 58, 68]. Our analysis also included some important PD-related genes such as *Prkn* and *Lrrk2*, which were not altered, and also *Snca* gene which exhibited an important and expected up-regulation derived from the local injection of AAV9-SynA53T into the SN. These findings reinforce the suitability of the experimental model for preclinical studies in PD in relation with the overexpression of SynA53T, which it is expected that may also reach the caudate-putamen (e.g., through the axons of transfected nigral neurons) despite the AAV9-SynA53T was injected in the SNpc. In fact, the possibility that the AAV9-SynA53T could also reach the caudate-putamen and transfect non-dopaminergic neurons (e.g., striatal projection neurons, striatal interneurons) is an option that cannot be discarded with the present experimental approach, and that may, in part, may explain the singularity in some of changes observed in transcriptomic data (e.g., interferon response). To confirm this possibility will require additional research. Furthermore, we also identified novel groups of genes naïve for PD that would require further analysis to unravel their implication and relevance in the context of PD (Supplementary Tables 2–5). Our transcriptomic analysis also revealed changes in a few genes related to the endocannabinoid system (i.e., *Cnr1*, *Gpr55*, *Faah*) which were exclusively associated with the overexpression of mutated α-synuclein into the SN, remaining unaltered by the treatment with VCE-003.2. However, what we did observe was a down-regulation of genes involved in interferon signaling pathways driven by α-synuclein overexpression, the main responsible for anti-viral responses [57]. This effect was reverted by the treatment with VCE-003.2, presumably through the activation of the PPAR-γ receptor, according to our previous findings [36]. The PPAR-γ receptor is known to be one of the most crucial mediators in the inflammatory response in the brain [9] and also outside the brain (e.g., immune system; Straus and Glass [71], , so it is likely that VCE-003.2 may be active in modulating interferon response. In this case, the pharmacological correction caused by this treatment seems to be orchestrated by Irf1 and Irf9 transcription factors, and their coregulators Stat1 and Stat2, responsible for immune and anti-viral responses [57], as their downstream genes were significantly activated after VCE-003.2 treatment. As indicated above, whether this mechanism of action is a key event in the ability of VCE-003.2 to preserve the structure and functionality of the nigrostriatal projection in the basal ganglia, or whether a mere consequence of such neuroprotective effect exerted by VCE-003.2 will require further research. Nevertheless, this finding is a novel and significant result of our transcriptomic analysis. It is also important to remark that the present findings align with existing evidence that highlights an interaction between the signaling pathways of the PPAR-γ receptor and type-1 interferons together with their protective effect even observed in an oncological context [25]. When we correlate these upregulated pathways with the diminished expression observed in glial markers, particularly CD68, it may indicate a microglial phenotypic shift characterized by less

reactive microglia and different inflammatory processes [17]. In other words, we hypothesize that the activation of PPAR-γ is triggering a protective inflammatory response, which may contribute to the safeguarding of dopaminergic neurons, which we will delve into in future experiments. In support of this idea, our transcriptomic analysis revealed that one of the up-regulated genes by the local overexpression of SynA53T was *Ppargc1b* (logFC=+0.44; FDR=0.04; Supplementary Tables 3 and 4), whereas *Ppargc1a* resulted down-regulated (logFC=-0.42; FDR=0.006; Supplementary Tables 3 and 4). These genes encode for proteins that function as coactivators of PPAR-γ, a response triggered in our study by the AAV9-SynA53T-mediated lesion. These results could be interpreted as an endogenous protective response involving PPAR-γ signaling upon AAV9-SynA53T inoculation. Treatment with VCE-003.2 may strengthen this signaling pathway through its ability to bind the regulatory site in the PPAR-γ receptor, although this will require further research.

Lastly, we also detected a significant enrichment in several regulons of interest in relation with the context (neurodegeneration) of this study, which were down-regulated in vehicle-treated AAV9-SynA53T-injected mice with respect to vehicle-treated sham-operated animals. This was the case of Specificity protein-1 (*Sp1*), which is a zinc finger family transcription factor that can regulate a number of genes that influence cell survival and proliferation [77]. Similar changes were detected for: (i) the tumorigenic factor Forkhead box I2 (*FoxI2*), which has been associated with the control of cellular identity [75]; (ii) Erythroblast transformation specific-1 (*Ets1*), which is expressed at high levels mainly in immune tissues [38], but has been also linked to cellular senescence [76]; and (iii) hypoxia-inducible factor-1a (*Hif1a*), which appears to play an important role in conditions of oxygen deprivation also in chronic neurodegeneration [60].

Conclusion

Our results extend the neuroprotective potential of VCE-003.2, already described against mitochondrial dysfunction, oxidative stress, glial reactivity and neuroinflammation in PD, to other key pathogenic event in this disease, as α-synuclein dysregulation and accumulation, and identified some relevant pathways of interest (e.g., interferon signaling) for further research in relation with the molecular mechanisms underlying these beneficial effects. It is true that this potential was particularly evident in relation with behavioral responses and histopathological markers, but the results at the trancriptomic level were not so conclusive, affecting mainly the aforementioned alterations in interferon signaling. This fact opens the door to other regulatory processes such as post-transcriptional modifications or protein activity modulation that will be investigated in future projects. In any case, these results, together with a favorable absorption, distribution, metabolism, excretion, and toxicology (ADMET) profile, reinforce the preclinical interest of VCE-003.2 towards its future clinical development in PD.

Abbreviations

AAV9-SynA53T	Adeno-associated viral vector serotype 9 encoding (A53T) α-synuclein
ADMET	Absorption, distribution, metabolism, excretion, and toxicology
BBB	Blood-brain barrier
CB_1 receptor	Cannabinoid type-1 receptor
CB_2 receptor	Cannabinoid type-2 receptor
CBG	Cannabigerol
CPM	Counts per million
CRT	Cylinder rearing test
Δ^9-THCV	Δ^9-tetrahydrocannabivarin
DEGs	Differentially expressed genes
EBST	Elevated-body swing test
FAAH	Fatty acid amide hydrolase
FDR	False discovery rate
GFAP	Glial fibrillary acidic protein
LRRK2	Leucine-rich repeat kinase-2
MAGL	Monoacylglycerol lipase
NRF2	Nuclear Factor (Erythroid-derived 2)-related factor 2
6-OHDA	6-hydroxydopamine
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PD	Parkinson's disease
PK	Pharmacokinetics
PPAR-γ	Peroxisome proliferator-activated receptor-γ
RNAseq	RNA sequencing analysis
SNpc	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
$TRPV_1$	Transient receptor potential vanilloid type-1

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12993-024-00256-9.

Supplementary Material 1: Plasmid map for pAAV-synapsin-SynA53T and sequence.

Supplementary Material 2: Chemical structure, in vitro pharmacology data, physicochemical and early ADME properties with relevance for good in vivo performance, and pharmacokinetic profile of VCE-003.2.

Supplementary Material 3: Representative video recordings of the response in the Cylinder Rearing Test (CRT) of vehicle-treated control (sham-operated) adult male mice and unilaterally AAV9-SynA53T-injected adult male mice treated with vehicle or VCE-00.2 (20 mg/kg) given orally. Treatments were daily and prolonged for 2 weeks. Video recordings were obtained at 24 h after the last dose.

Supplementary Material 4: Representative video recordings of the response in the Elevated-Body Swing Test (EBST) of vehicle-treated control (sham-operated) adult male mice and unilaterally AAV9-SynA53T-injected adult male mice treated with vehicle or VCE-00.2 (20 mg/kg) given orally. Treatments were daily and prolonged for 2 weeks. Video recordings were obtained at 24 h after the last dose.

Supplementary Material 5: Computer-aided actimeter (actitrack) analysis of vehicle-treated control (sham-operated) adult male mice and unilaterally AAV9-SynA53T-injected adult male mice treated with vehicle or VCE-00.2 (20 mg/kg) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to 24 h after the last dose and were expressed as means ± SEM of more than 5 animals *per* group. They were analyzed by one-way ANOVA followed by the Tukey test (*$p < 0.05$ *versus* vehicle-treated sham-operated mice; #$p < 0.05$ *versus* vehicle-treated AAV9-SynA53T-

injected mice).

Supplementary Material 6: Intensity of the immunostaining for α-synuclein measured in a selected area of the SNpc of vehicle-treated control (sham-operated) adult male mice and unilaterally AAV9-SynA53T-injected adult male mice treated with vehicle or VCE-00.2 (20 mg/kg) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to 24 h after the last dose and were expressed as means ± SEM of more than 5 animals *per* group. They were analyzed by one-way ANOVA followed by the Tukey test (***$p < 0.005$ *versus* vehicle-treated sham-operated mice). Representative immunostaining images for each experimental group, with indication of the approximate area quantified, are shown in right panels (scale bar = 200 µm, and 4x magnification (50 µm) in inlets).

Supplementary Material 7: Number of TH-positive neurons measured, using a deep-learning dedicated algorithm (Aiforia®), in a selected area of the SNpc of vehicle-treated control (sham-operated) adult male mice and unilaterally AAV9-SynA53T-injected adult male mice treated with vehicle or VCE-00.2 (20 mg/kg) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to 24 h after the last dose and were expressed as means ± SEM of more than 5 animals *per* group. They were analyzed by one-way ANOVA followed by the Tukey test (**$p < 0.005$ *versus* vehicle-treated sham-operated mice; #$p < 0.05$ *versus* vehicle-treated AAV9-SynA53T-injected mice).

Supplementary Material 8: Representative GFAP immunostaining images for each experimental group, corresponding to the data presented in the Fig. 4 (scale bar = 200 µm).

Supplementary Material 9: Representative CD68 immunostaining images for each experimental group corresponding to higher magnification images selected from those used to generate the data presented in the Fig. 4, with the purpose to identify morphological characteristics (cell body volume, number, thickness and length of cell branches) that may reflect the quiescent or activated states of CD68-labelled cells (scale bar = 100 µm).

Supplementary Material 10: Pharmacokinetic parameters of VCE-003.2 in plasma following a single intravenous (IV) (10 mg/kg) and oral (50 mg/kg) dose in CD-1 mice.

Supplementary Material 11: Excel tables showing the data of the RNA-Seq analysis in the striatum of vehicle-treated control (sham-operated) adult male mice and unilaterally AAV9-SynA53T-injected adult male mice treated with vehicle or VCE-00.2 (20 mg/kg) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to 24 h after the last dose and were expressed as means ± SEM of more than 5 animals *per* group.

Acknowledgements
Authors are indebted to Yolanda García-Movellán for administrative assistance and to CAI-Animalario, Complutense University for animal housing and care.

Author contributions
J.F-R. wrote the different manuscript drafts, which were revised and approved by all authors). J.F-R. and E.M. designed, coordinated and supervised the study, and were involved in funding acquisition. S.B., I.L-B., J.L.L. and S.R-C. were involved in the development of the mouse model. S.B., S.R-C. and J.Ch. carried out the behavioural and histopathological analyses, whereas E.N., M.G-R. and C.N. conducted the transcriptomic analysis. C.N. and E.M. coordinated the VCE-003.2 synthesis and its pharmacological characterization. J.F-R. and EN carried out all statistical analysis of the data.

Funding
This work has been supported by grants from CIBERNED (CB06/05/0089 to JF-R), MICINN (PID2021-128906OB-I00 to JF-R, PID2022-139936OA-I00 to EN and PID2022-137065OB-I00 to ILB) with FEDER funds and Michael J. Fox Foundation (MJFF-022552 to JF-R). These agencies had no further role in study design, the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication. Sonia Burgaz was a predoctoral fellow supported by the Complutense University, whereas Santiago Rodríguez-Carreiro was supported by the Ministry of University (FPU Programme).

Data availability
Raw RNA-Seq data have been uploaded to ArrayExpress repository to make them available for further analysis (accession number: E-MTAB-13768). Further information and reasonable request for resources and reagents should be directed to and will be fulfilled by the corresponding authors, Eduardo Muñoz (fi1muble@uco.es) and Javier Fernández-Ruiz (jjfr@med.ucm.es).

Ethical approval
All animal experiments were conducted according to local and European rules (directive 2010/63/EU), as well as conformed to ARRIVE guidelines. They were approved by the ethical committees of our university and the regulatory institution (ref. PROEX 201.8/22).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Universitario de Investigación en Neuroquímica, Universidad Complutense, Madrid, Spain
[2]Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain
[3]Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain
[4]Emerald Health Pharmaceuticals, San Diego, USA
[5]Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Córdoba, Spain
[6]Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Córdoba, Spain
[7]Hospital Universitario Reina Sofía, Córdoba, Spain
[8]Instituto de Investigaciones Biomédicas Sols-Morreale (IIBSM), UAM-CSIC, Madrid, Spain
[9]Departamento de Bioquímica, Facultad de Medicina, Universidad Autónoma de Madrid (UAM), Madrid, Spain
[10]Instituto de Investigación del Hospital Universitario de La Paz (IdiPAZ), Madrid, Spain
[11]Institute Teófilo Hernando for Drug Discovery, Universidad Autónoma de Madrid, Madrid, Spain
[12]CNS Gene Therapy Department, Center for Applied Medical Research (CIMA), Universidad de Navarra, Pamplona, Spain

Received: 20 May 2024 / Accepted: 18 October 2024
Published online: 01 November 2024

References

1 Aguareles J, Paraíso-Luna J, Palomares B, Bajo-Grañeras R, Navarrete C, Ruiz-Calvo A, García-Rincón D, García-Taboada E, Guzmán M, Muñoz E, Galve-Roperh I. Oral administration of the cannabigerol derivative VCE-003.2 promotes subventricular zone neurogenesis and protects against mutant huntingtin-induced neurodegeneration. Translational Neurodegeneration. 2019;8:9.
2 Anand S, Azam Ansari M, Kumaraswamy Sukrutha S, Alomary MN, Anwar Khan A, Elderdery AY. Resolvins lipid mediators: potential therapeutic targets in Alzheimer and Parkinson disease. Neuroscience. 2022;507:139–48.
3 Antonazzo M, Botta M, Bengoetxea H, Ruiz-Ortega JA, Morera-Herreras T. Therapeutic potential of cannabinoids as neuroprotective agents for damaged cells conducing to movement disorders. Int Rev Neurobiol. 2019;146:229–57.
4 Aymerich MS, Aso E, Abellanas MA, Tolon RM, Ramos JA, Ferrer I, Romero J, Fernández-Ruiz J. Cannabinoid pharmacology/therapeutics in chronic degenerative disorders affecting the central nervous system. Biochem Pharmacol. 2018;157:67–84.
5 Basurco L, Abellanas MA, Ayerra L, Conde E, Vinueza-Gavilanes R, Luquin E, Vales A, Vilas A, Martin-Uriz PS, Tamayo I, Alonso MM, Hernaez M, Gonzalez-Aseguinolaza G, Clavero P, Mengual E, Arrasate M, Hervás-Stubbs S, Aymerich MS. Microglia and astrocyte activation is region-dependent in the α-synuclein mouse model of Parkinson's disease. Glia. 2022;71:571–87.

6 Bajaj S, Jain S, Vyas P, Bawa S, Vohora D. The role of endocannabinoid pathway in the neuropathology of Alzheimer's disease: can the inhibitors of MAGL and FAAH prove to be potential therapeutic targets against the cognitive impairment associated with Alzheimer's disease? Brain Res Bull. 2021;174:305–22.
7 Baul HS, Manikandan C, Sen D. Cannabinoid receptor as a potential therapeutic target for Parkinson's disease. Brain Res Bull. 2019;146:244–52.
8 Bentea E, Van der Perren A, Van Liefferinge J, El Arfani A, Albertini G, Demuyser T, Merckx E, Michotte Y, Smolders I, Baekelandt V, Massie A. Nigral proteasome inhibition in mice leads to motor and non-motor deficits and increased expression of Ser129 phosphorylated αsynuclein. Front Behav Neurosci. 2015;9:68.
9 Bernardo A, Minghetti L. PPAR-γ agonists as regulators of microglial activation and brain inflammation. Curr Pharm Design. 2006;12:93–109.
10 Björklund A, Mattsson B. (2024) The AAV-α-synuclein model of Parkinson's disease: An update. J Parkinsons Dis, in press.
11 Borlongan CV, Sanberg PR. Elevated body swing test: a new behavioral parameter for rats with 6-hydroxydopamine-induced hemiparkinsonism. J Neurosci. 1995;15:5372–8.
12 Burgaz S, García C, Gómez-Cañas M, Muñoz E, Fernández-Ruiz J. Development of an oral treatment with the PPAR-γ-acting cannabinoid VCE-003.2 against the inflammation-driven neuronal deterioration in experimental Parkinson's disease. Molecules. 2019;24:2702.
13 Burgaz S, García C, Gómez-Cañas M, Rolland A, Muñoz E, Fernández-Ruiz J. Neuroprotection with the cannabidiol quinone derivative VCE-004.8 (EHP-101) against 6-hydroxydopamine in cell and murine models of Parkinson's disease. Molecules. 2021a;26:3245.
14 Burgaz S, García C, Gonzalo-Consuegra C, Gómez-Almería M, Ruiz-Pino F, Unciti JD, Gómez-Cañas M, Alcalde J, Morales P, Jagerovic N, Rodríguez-Cueto C, de Lago E, Muñoz E, Fernández-Ruiz J. Preclinical investigation in neuroprotective effects of the GPR55 ligand VCE-006.1 in experimental models of Parkinson's disease and amyotrophic lateral sclerosis. Molecules. 2021b;26:7643.
15 Burgaz S, García C, Gómez-Cañas M, Navarrete C, García-Martín A, Rolland A, Del Río C, Casarejos MJ, Muñoz E, Gonzalo-Consuegra C, Muñoz E, Fernández-Ruiz J. Neuroprotection with the cannabigerol quinone derivative VCE-003.2 and its analogs CBGA-Q and CBGA-Q-Salt in Parkinson's disease using 6-hydroxydopamine-lesioned mice. Mol Cell Neurosci. 2021c;110:103583.
16 Calabresi P, Mechelli A, Natale G, Volpicelli-Daley L, Di Lazzaro G, Ghiglieri V. α-Synuclein in Parkinson's disease and other synucleinopathies: from overt neurodegeneration back to early synaptic dysfunction. Cell Death Dis. 2023;14:176.
17 Carta AR, Pisanu A. Modulating microglia activity with PPAR-γ agonists: a promising therapy for Parkinson's disease? Neurotox Res. 2013;23:112–23.
18 Castro-Sánchez S, García-Yagüe AJ, López-Royo T, Casarejos MJ, Lanciego JL, Lastres-Becker I. Cx3cr1-deficiency exacerbates α-synuclein-A53T induced neuroinflammation and neurodegeneration in a mouse model of Parkinson's disease. Glia. 2018;66:1752–62.
19 Celorrio M, Rojo-Bustamante E, Fernández-Suárez D, Sáez E, Estella-Hermoso de Mendoza A, Müller CE, Ramírez MJ, Oyarzábal J, Franco R, Aymerich MS. GPR55: a therapeutic target for Parkinson's disease? Neuropharmacology. 2017;125:319–32.
20 Chen C. Inhibiting degradation of 2-arachidonoylglycerol as a therapeutic strategy for neurodegenerative diseases. Pharmacol Ther. 2023;244:108394.
21 Chocarro J, Rico AJ, Ariznabarreta G, Roda E, Honrubia A, Collantes M, Peñuelas I, Vázquez A, Rodríguez-Pérez AI, Labandeira-García JL, Vila M, Lanciego JL. Neuromelanin accumulation drives endogenous synucleinopathy in non-human primates. Brain. 2023;146:5000–14.
22 Chung YC, Bok E, Huh SH, Park JY, Yoon SH, Kim SR, Kim YS, Maeng S, Park SH, Jin BK. Cannabinoid receptor type 1 protects nigrostriatal dopaminergic neurons against MPTP neurotoxicity by inhibiting microglial activation. J Immunol. 2011;187:6508–17.
23 Coles M, Steiner-Lim GZ, Karl T. Therapeutic properties of multi-cannabinoid treatment strategies for Alzheimer's disease. Front NeuroSci. 2022;16:962922.
24 Díaz-Alonso J, Paraíso-Luna J, Navarrete C, Del Río C, Cantarero I, Palomares B, Aguareles J, Fernández-Ruiz J, Bellido ML, Pollastro F, Appendino G, Calzado MA, Galve-Roperh I, Muñoz E. VCE-003.2, a novel cannabigerol derivative, enhances neuronal progenitor cell survival and alleviates symptomatology in murine models of Huntington's disease. Sci Rep. 2016;6:29789.
25 Dicitore A, Caraglia M, Gaudenzi G, Manfredi G, Amato B, Mari D, Persani L, Arra C, Vitale G. Type I interferon-mediated pathway interacts with peroxisome proliferator activated receptor-γ (PPAR-γ): at the cross-road of pancreatic cancer cell proliferation. Biochim Biophys Acta. 2014;1845:42–52.
26 Espadas I, Keifman E, Palomo-Garo C, Burgaz S, García C, Fernández-Ruiz J, Moratalla R. Beneficial effects of the phytocannabinoid Δ9-THCV in L-DOPA-induced dyskinesia in Parkinson's disease. Neurobiol Dis. 2020;141:104892.
27 Fernández-Moncada I, Eraso-Pichot A, Dalla Tor T, Fortunato-Marsol B, Marsicano G. An enquiry to the role of CB1 receptors in neurodegeneration. Neurobiol Dis. 2023;184:106235.
28 Fernández-Ruiz J. The endocannabinoid system as a target for the treatment of motor dysfunction. Br J Pharmacol. 2009;156:1029–40.
29 Fernández-Ruiz J, Moreno-Martet M, Rodríguez-Cueto C, Palomo-Garo C, Gómez-Cañas M, Valdeolivas S, Guaza C, Romero J, Guzmán M, Mechoulam R, Ramos JA. Prospects for cannabinoid therapies in basal ganglia disorders. Br J Pharmacol. 2011;163:1365–78.
30 Fernández-Ruiz J, Moro MA, Martínez-Orgado J. Cannabinoids in neurodegenerative disorders and stroke/brain trauma: from preclinical models to clinical applications. Neurotherapeutics. 2015;12:793–806.
31 Fernández-Ruiz J. The biomedical challenge of neurodegenerative disorders: an opportunity for cannabinoid-based therapies to improve on the poor current therapeutic outcomes. Br J Pharmacol. 2019;176:1370–83.
32 Ferrisi R, Ceni C, Bertini S, Macchia M, Manera C, Gado F. Medicinal chemistry approach, pharmacology and neuroprotective benefits of CB_2R modulators in neurodegenerative diseases. Pharmacol Res. 2021;170:105607.
33 Fleming SM, Ekhator OR, Ghisays V. Assessment of sensorimotor function in mouse models of Parkinson's disease. J Visualized Experiments. 2013;76:50303.
34 Ganjam GK, Bolte K, Matschke LA, Neitemeier S, Dolga AM, Höllerhage M, Höglinger GU, Adamczyk A, Decher N, Oertel WH, Culmsee C. Mitochondrial damage by α-synuclein causes cell death in human dopaminergic neurons. Cell Death Dis. 2019;10:865.
35 García C, Palomo-Garo C, García-Arencibia M, Ramos JA, Pertwee RG, Fernández-Ruiz J. Symptom-relieving and neuroprotective effects of the phytocannabinoid Δ^9-THCV in animal models of Parkinson's disease. Br J Pharmacol. 2011;163:1495–506.
36 García C, Gómez-Cañas M, Burgaz S, Palomares B, Gómez-Gálvez Y, Palomo-Garo C, Campo S, Ferrer-Hernández J, Pavicic C, Navarrete C, Bellido ML, García-Arencibia M, Pazos MR, Muñoz E, Fernández-Ruiz J. Benefits of VCE-003.2, a cannabigerol quinone derivative, against inflammation-driven neuronal deterioration in experimental Parkinson's disease: possible involvement of different binding sites at the PPARγ receptor. J Neuroinflamm. 2018;15:19.
37 Garcia-Alonso L, Holland CH, Ibrahim MM, Turei D, Saez-Rodríguez J. Benchmark and integration of resources for the estimation of human transcription factor activities. Genome Res. 2019;29:1363–75.
38 Garrett-Sinha LA. An update on the roles of transcription factor Ets1 in autoimmune diseases. WIREs Mech Dis. 2023;15:e1627.
39 Gómez-Gálvez Y, Palomo-Garo C, Fernández-Ruiz J, García C. Potential of the cannabinoid CB2 receptor as a pharmacological target against inflammation in Parkinson's disease. Progress Neuropsychopharmacol Biol Psychiatry. 2016;64:200–8.
40 Gugliandolo A, Pollastro F, Grassi G, Bramanti P, Mazzon E. (2018) In Vitro model of neuroinflammation: efficacy of cannabigerol, a non-psychoactive cannabinoid. International Journal of Molecular Sciences 19, 1992.
41 Holland CH, Szalai B, Saez-Rodríguez J. Transfer of regulatory knowledge from human to mouse for functional genomics analysis. Biochim Biophys Acta. 2020;1863:194431.
42 Iba M, McDevitt RA, Kim C, Roy R, Sarantopoulou D, Tommer E, Siegars B, Sallin M, Kwon S, Sen JM, Sen R, Masliah E. Aging exacerbates the brain inflammatory micro-environment contributing to α-synuclein pathology and functional deficits in a mouse model of DLB/PD. Mol Neurodegeneration. 2022;17:60.
43 Jain V, Behl T, Sehgal A, Singh S, Sharma N, Makeen HA, Albratty M, Meraya AM, Najmi A. Therapeutic molecular insights into the active engagement of cannabinoids in the therapy of Parkinson's disease: a novel and futuristic approach. Neurotox Res. 2023;41:85–102.
44 Javed H, Azimullah S, Haque ME, Ojha SK. Cannabinoid type 2 (CB2) receptors activation protects against oxidative stress and neuroinflammation associated dopaminergic neurodegeneration in rotenone model of Parkinson's disease. Front NeuroSci. 2016;10:321.
45 Kelly R, Bemelmans AP, Joséphine C, Brouillet E, McKernan DP, Dowd E. Time-course of alterations in the endocannabinoid system after viral-mediated overexpression of α-synuclein in the rat brain. Molecules. 2022;27:507.
46 Kelsey JE, Harris O, Cassin J. The CB1 antagonist rimonabant is adjunctively therapeutic as well as monotherapeutic in an animal model of Parkinson's disease. Behav Brain Res. 2009;203:304–7.

47 Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods. 2015;12:357–60.
48 Ko WKD, Bezard E. Experimental animal models of Parkinson's disease: a transition from assessing symptomatology to α-synuclein targeted disease modification. Exp Neurol. 2017;298:172–9.
49 Korotkevich G, Sukhov V, Budin N, Shpak B, Artyomov MN, Sergushichev A. Fast gene set enrichment analysis. BioRχiv (doi. 2021. https://doi.org/10.1101/060012).
50 Kuo SH, Tasset I, Cheng MM, Diaz A, Pan MK, Lieberman OJ, Hutten SJ, Alcalay RN, Kim S, Ximénez-Embún P, Fan L, Kim D, Ko HS, Yacoubian T, Kanter E, Liu L, Tang G, Muñoz J, Sardi SP, Li A, Gan L, Cuervo AM, Sulzer D. Mutant glucocerebrosidase impairs α-synuclein degradation by blockade of chaperone-mediated autophagy. Sci Adv. 2022;8:eabm6393.
51 Lastres-Becker I, Molina-Holgado F, Ramos JA, Mechoulam R, Fernández-Ruiz J. Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: relevance to Parkinson's disease. Neurobiol Dis. 2005;19:96–107.
52 Lastres-Becker I, Ulusoy A, Innamorato NG, Sahin G, Rábano A, Kirik D, Cuadrado A. α-Synuclein expression and Nrf2 deficiency cooperate to aggravate protein aggregation, neuronal death and inflammation in early-stage Parkinson's disease. Hum Mol Genet. 2012;21:3173–92.
53 Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12:323.
54 Liao Y, Smyth GK, Shi W. FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics. 2014;30:923–30.
55 Lins BR, Anyaegbu CC, Hellewell SC, Papini M, McGonigle T, De Prato L, Shales M, Fitzgerald M. Cannabinoids in traumatic brain injury and related neuropathologies: preclinical and clinical research on endogenous, plant-derived, and synthetic compounds. J Neuroinflamm. 2023;20:77.
56 Marmion DJ, Kordower JH. α-Synuclein nonhuman primate models of Parkinson's disease. J Neural Transm. 2018;125:385–400.
57 Muhuri M, Maeda Y, Ma H, Ram S, Fitzgerald KA, Tai PW, Gao G. (2021) Overcoming innate immune barriers that impede AAV gene therapy vectors. J Clin Invest 131, e143780.
58 Nascimento AC, Erustes AG, Reckziegel P, Bincoletto C, Ureshino RP, Pereira G, Smaili SS. α-Synuclein overexpression induces lysosomal dysfunction and autophagy impairment in human neuroblastoma SH-SY5Y. Neurochem Res. 2020;45:2749–61.
59 Navarro E, Udine E, de Paiva Lopes K, Parks M, Riboldi G, Schilder BM, Humphrey J, Snijders GJL, Vialle RA, Zhuang M, Sikder T, Argyrou C, Allan A, Chao MJ, Farrell K, Henderson B, Simon S, Raymond D, Elango S, Ortega RA, Shanker V, Swan M, Zhu CW, Ramdhani R, Walker RH, Tse W, Sano M, Pereira AC, Ahfeldt T, Goate AM, Bressman S, Crary JF, de Witte L, Frucht S, Saunders-Pullman R, Raj T. Dysregulation of mitochondrial and proteolysosomal genes in Parkinson's disease myeloid cells. Nat Aging. 2021;1:850–63.
60 Ogunshola OO, Antoniou X. Contribution of hypoxia to Alzheimer's disease: is HIF-1alpha a mediator of neurodegeneration? Cell Mol Life Sci. 2009;66:3555–63.
61 Penttinen AM, Parkkinen I, Blom S, Kopra J, Andressoo JO, Pitkänen K, Voutilainen MH, Saarma M, Airavaara M. Implementation of deep neural networks to count dopamine neurons in substantia nigra. Eur J Neurosci. 2018;48:2354–61.
62 Pérez-Rial S, García-Gutiérrez MS, Molina JA, Pérez-Nievas BG, Ledent C, Leiva C, Leza JC, Manzanares J. Increased vulnerability to 6-hydroxydopamine lesion and reduced development of dyskinesias in mice lacking CB1 cannabinoid receptors. Neurobiol Aging. 2011;32:631–45.
63 Rico AJ, Corcho A, Chocarro J, Ariznabarreta G, Roda E, Honrubia A, Arnaiz P, Lanciego JL. Development and characterization of a non-human primate model of disseminated synucleinopathy. Front Neuroanat. 2024;18:1355940.
64 Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. (2015) Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43, e47.
65 Rodríguez-Cueto C, García-Toscano L, Santos-García I, Gómez-Almería M, Gonzalo-Consuegra C, Espejo-Porras F, Fernández-Ruiz J, de Lago E. Targeting the CB_2 receptor and other endocannabinoid elements to delay disease progression in amyotrophic lateral sclerosis. Br J Pharmacol. 2021;178:1373–87.
66 Rodríguez-Pérez AI, Sucunza D, Pedrosa MA, Garrido-Gil P, Kulisevsky J, Lanciego JL, Labandeira-García JL. Angiotensin type 1 receptor antagonists protect against α-synuclein-induced neuroinflammation and dopaminergic neuron death. Neurotherapeutics. 2018;15:1063–81.
67 Sañudo-Peña MC, Patrick SL, Khen S, Patrick RL, Tsou K, Walker JM. Cannabinoid effects in basal ganglia in a rat model of Parkinson's disease. Neurosci Lett. 1998;248:171–4.
68 Siddiqui T, Bhatt LK. (2023) Emerging autophagic endo-lysosomal targets in the management of Parkinson's disease. Rev Neurol (Paris), in press.
69 Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature. 1997;388:839–40.
70 Stone NL, Murphy AJ, England TJ, O'Sullivan SE. A systematic review of minor phytocannabinoids with promising neuroprotective potential. Br J Pharmacol. 2020;177:4330–52.
71 Straus DS, Glass CK. Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. Trends Immunol. 2007;28:551–8.
72 Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA. 2005;102:15545–50.
73 Tambe SM, Mali S, Amin PD, Oliveira M. Neuroprotective potential of cannabidiol: molecular mechanisms and clinical implications. J Integr Med. 2023;21:236–44.
74 Wang G, Ma W, Du J. β-Caryophyllene (BCP) ameliorates MPP + induced cytotoxicity. Biomed Pharmacother. 2018;103:1086–91.
75 Wijchers PJ, Hoekman MF, Burbach JP, Smidt MP. Cloning and analysis of the murine Foxi2 transcription factor. Biochim Biophys Acta. 2005;1731:133–8.
76 Xiao FH, Yu Q, Deng ZL, Yang K, Ye Y, Ge MX, Yan D, Wang HT, Chen XQ, Yang LQ, Yang BY, Lin R, Zhang W, Yang XL, Dong L, He Y, Zhou J, Cai WW, Li J, Kong QP. (2022) ETS1 acts as a regulator of human healthy aging via decreasing ribosomal activity. Science Advances 8, eabf2017.
77 Yu Q, Liu W, Chen Z, Zhang M. Specificity protein 1: a protein with a two-sided role in ischemic stroke. Front Cell Neurosci. 2021;15:757670.
78 Zamanian MY, Terefe EM, Taheri N, Kujawska M, Tork YJ, Abdelbasset WK, Shoukat S, Opulencia MJC, Heidari M, Alesaeidi S. Neuroprotective and anti-inflammatory effects of pioglitazone on Parkinson's disease: a comprehensive narrative review of clinical and experimental findings. CNS Neurol Disorders Drug Targets. 2023;22:1453–61.

Publisher's note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.