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TESIS DOCTORAL

Cannabioids as disease modifiers in multiple sclerosis and amyotrophic lateral sclerosis
Los cannabinoides como agentes modificadores de la enfermedad en la esclerosis múltiple y la esclerosis lateral amiotrófica

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PRESENTADA POR

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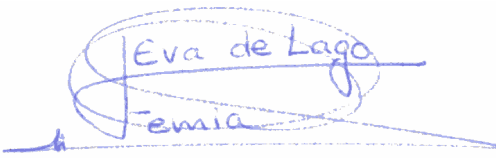
CERTIFICAN:

Que la presente Tesis Doctoral titulada "*Los cannabinoides como agentes modificadores de la enfermedad en la esclerosis múltiple y la esclerosis lateral amiotrófica*" presentada por Miguel Moreno Martet, Licenciado en Biología, para optar al grado de Doctor por la Universidad Complutense de Madrid, ha sido realizada bajo nuestra dirección y reúne todos los requisitos necesarios para ser juzgada.

Y para que conste y a los efectos oportunos, firman el presente certificado en Madrid a 23 de Mayo de 2014.



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RESUMEN

Introducción

Las enfermedades neurodegenerativas son un desafío clave en este siglo XXI para la investigación clínica. En la actualidad existen millones de pacientes afectados en el mundo, y en los próximos años las cifras no harán sino incrementar debido al envejecimiento de la población y la mayor esperanza de vida de los países desarrollados. El conocimiento sobre estas enfermedades es muy amplio pero a pesar de ello la cura para alguna de ellas enfermedades está aún por descubrirse. En esta tesis doctoral nos hemos centrado en la esclerosis multiple (EM) y la esclerosis lateral amiotrófica (ELA), dos enfermedades que afectan a las neuronas localizadas en la médula espinal produciendo alteraciones en los músculos inervados por estas células. En estas dos enfermedades hemos estudiado la implicación del sistema cannabinoide endógeno (SCE) como modulador del transcurso de la enfermedad y su utilidad como diana terapéutica para el desarrollo de terapias que modifiquen el desarrollo de estas enfermedades.

La EM es la primera causa de afectación neurológica en jóvenes adultos. En España afecta a 50 de cada 100000 habitantes y los costos para el Sistema Nacional de Salud rondan los 30000-40000 € al año según datos estadísticos de la UE (Sobocki et al., 2007). La EM es una enfermedad crónica inflamatoria, desmielinizante y neurodegenerativa del sistema nervioso central (SNC) cuyo origen aún se desconoce (Noseworthy et al., 2000). El principal signo patológico de la EM son placas de desmielinización que aparecen en áreas de materia blanca y gris a lo largo del SNC (Kutzelnigg and Lassmann, 2006). Según la localización de estas placas de desmielinización, el paciente sufrirá síntomas que abarcan desde pérdida de la visión, debilidad muscular, disfunción de la vejiga, rigidez y espasmos musculares, afectación en el habla, temblor, vértigo y depresión. Algunas drogas han dado resultados esperanzadores en neuroprotección en diferentes modelos (ver revisión de Luessi et al., 2012) y se han llevado a ensayos clínicos. Lamentablemente en humanos no han sido efectivas (Kapoor et al., 2010, Kamm et al., 2012) por lo que el tiempo apremia en la búsqueda de nuevos medicamentos que retrasen o impidan el deterioro clínico en la EM

La ELA es una enfermedad neurodegenerativa donde mueren tanto las moto-neuronas superiores como inferiores localizadas en la corteza motora, tallo cerebral y médula espinal. La muerte progresiva de estas moto-neuronas causa debilidad muscular, fasciculaciones dolorosas, atrofia y finalmente parálisis muscular que conllevará la muerte del paciente al afectar a los músculos implicados en la respiración. Según donde se manifiesten los síntomas, se clasifica la ELA como espinal cuando afecta a las extremidades y bulbar cuando afecta a los músculos orofaríngeos. La esperanza de vida en los pacientes de ELA es de solo 3-5 años tras el diagnóstico. Numerosos procesos están implicados en la patogénesis de la ELA: excitotoxicidad, estrés oxidativo, agregación de proteínas, disfunción mitocondrial y desregulación del metabolismo de ARN (Bento-Abreu et al., 2010; Boillée et al., 2006; Gendron et al., 2014). En la actualidad no existe cura para tratar la ELA. El único medicamento aprobado es el Rilutek©, que atenúa la excitotoxicidad, aunque su eficacia sólo incrementa en unos pocos meses la esperanza de vida de los pacientes (Bensimon et al., 1994; Gurney et al., 1996). El resto de tratamientos para los pacientes de ELA consiste en terapias paliativas para incrementar su calidad de vida, drogas para reducir la fatiga, aliviar los calambres musculares y controlar la espasticidad en las etapas iniciales de la enfermedad hasta terapia respiratoria asistida una vez los músculos respiratorios están afec-

tados (Bede et al., 2011).

La EM y la ELA comparten subpoblaciones neuronales afectadas así como mecanismos patogénicos que contribuyen al daño neuronal: excitotoxicidad, estrés oxidativo y neuroinflamación. Estos mecanismos patogénicos alteran la homeostasis celular y potencian el daño causado por el entorno neuronal. Existen mecanismos fisiológicos que pueden aliviar estos procesos de toxicidad y mantener la homeostasis celular. El SCE es uno de estos sistemas. Descubierto en los años 90 del siglo XX, el SCE está implicado en una amplia variedad de procesos fisiológicos ayudando a las células a volver a la correcta homeostasis tras un daño agudo o crónico. Por tanto los diferentes elementos del SCE (por ejemplo receptores cannabinoides, endocannabinoides y enzimas de síntesis y degradación de endocannabinoides) ofrecen dianas terapéuticas noveles muy interesantes para el desarrollo de fármacos. Además los cannabinoides son muy interesantes desde este punto de vista ya que actúan a través de múltiples dianas implicadas en la supervivencia celular. En la EM y la ELA los cannabinoides pueden tener efectos anti-excitotoxicos, actuar como antioxidantes y modular la activación glial así como la inflamación. Estudios preclínicos manipulando el SCE han dado resultados esperanzadores en modelos pre-clínicos de enfermedades neurodegenerativas como enfermedad de Alzheimer (Bisogno and Di Marzo, 2008), enfermedad de Parkinson (García-Arencibia et al., 2009), enfermedad de Huntington(Pazos et al., 2008), EM (Baker and Pryce, 2008; Pertwee, 2007) y ELA (Bilsland and Greensmith, 2008; Carter et al., 2010).

Objetivos

Esta tesis doctoral se propone estudiar la aplicación de los cannabinoides como tratamiento para la EM y la ELA. A pesar de los numerosos avances en la investigación del SCE relacionados con la EM y la ELA aún hay aspectos susceptibles de ser investigados. En la EM están por elucidar el papel que juegan los receptores CB1 y CB2 en efectos que alteran el transcurso de la enfermedad, así como el uso de terapias basadas en fitocannabinoides que activen estas dos dianas. En el caso de la ELA, es necesario identificar los posibles cambios que afectan al SCE durante el transcurso de la enfermedad, especialmente los referidos a las enzimas de síntesis y degradación de endocannabinoides, así como el uso de combinaciones de fitocannabinoides como terapias modificadoras de la enfermedad. Por tanto los objetivos propuestos para esta tesis son los siguientes:

1- Estudio de los mecanismos subyacentes a la mejora de la progresión del cannabinoide sintético WIN 55,212-2 en el modelo de encefalomielitis autoinmune experimental (EAE) en ratón, prestando especial atención al papel de los receptores CB_1 y CB_2.

2- Estudio del uso de los fitocannabinoides Δ^9-THC y CBD, así como su combinación similar al medicamento Sativex como agentes modificadores de la EM en los modelos de enfermedad desmielinizante inducida por el virus de la encefalopatía de Theiler (TMEV-IDD) y la EAE.

3- Caracterización del SCE en un modelo celular y en el modelo de ELA de ratones

transgénicos para la proteína SOD1^{G93A} y estudio del uso de una combinación de Δ^9-THC y CBD como agente modificador de la ELA.

4- Estudio del bloqueo de la expresión de los receptores cannabinoides en un modelo de ELA en pez cebra (*Danio rerio*).

Para llevar a cabo estos objetivos hemos empleado diversas técnicas de biología molecular así como modelos in vitro e in vivo. En esta Tesis doctoral desarrollamos los siguientes objetivos en cada uno de los capítulos resumidos a continuación:

Capítulo 1: Estudio de los mecanismos subyacentes a la mejora de la progresión del cannabinoide sintético WIN 55,212-2 en el modelo de encefalomielitis autoinmune experimental (EAE), prestando especial atención al papel de los receptores CB_1 y CB_2.

En este capítulo evaluamos el efecto de un tratamiento crónico de dosis de 5 mg/kg de WIN 55,212-2 en el modelo de la EAE en ratón. Nos centramos en los posibles efectos mediados por receptores CB_1 y CB_2 así como la relevancia de estos receptores para controlar respuestas anti-excitotoxicas y anti-inflamatorias.

Los resultados de este capítulo se encuentran recogidos en la siguiente publicación:

de Lago, E., Moreno-Martet, M., Cabranes, A., Ramos, J.A., Fernández-Ruiz, J. *Cannabinoids ameliorate disease progression in a model of multiple sclerosis in mice, acting preferentially through CB1 receptor-mediated anti-inflammatory effects.* Neuropharmacology 2012, 62(7):2299-2308

Capítulo 2: Estudio del uso de los fitocannabinoides Δ^9-THC y CBD, así como su combinación similar al medicamento Sativex como agentes modificadores de la EM en los modelos de enfermedad desmielinizante inducida por el virus de la encefalopatía de Theiler (TMEV-IDD) y la EAE.

En este capítulo evaluamos el efecto de los fitocannabinoides Δ^9-THC y CBD así como de la combinación de ambos en los modelos de TMEV-IDD y de EAE. En la TMEV-IDD, la combinación de ambos y el tratamiento con CBD mostró mayor eficacia que el Δ^9-THC para mejorar los síntomas motores de la enfermedad. Los ratones tratados con una combinación similar al Sativex (medicamento aprobado para el tratamiento de la espasticidad y el dolor neuropático en EM) mostraron una reducción del número de infiltrados celulares en el SNC, menor reactividad microglial, reducción en el daño axonal y restauración de la morfología de la mielina. Commprobamos que el Δ^9-THC actuaba a través de receptores CB_1 y CB_2 y el CBD a través de receptores nucleares PPARγ. En el modelo de EAE, el Δ^9-THC y la combinación similar al Sativex mejoraron el deterioro neurológico mientras que el CBD no tuvo efecto. Experimentos realizados con antagonistas mostraron que los efectos del Δ^9-THC en la EAE son mediados a por el receptor CB1 y no a través de otras dianas anti-inflamatorias como el receptor PPARγ.

Los resultados de este capítulo se encuentran recogidos en las siguientes publicaciones:

Feliú, A., Moreno-Martet, M., Mecha, M., Carrillo-Salinas, F. J., Fernández-Ruiz J., de Lago, E., Guaza C.
SATIVEX-LIKE COMBINATION OF PHYTOCANNABINOIDS AS A DISEASE-MODI- FYING THERAPY IN A VIRAL AUTOIMMUNE MODEL OF MULTIPLE SCLEROSIS. (pendiente de publicación).

Moreno-Martet, M., Feliú, A., Guaza, C., Fernández-Ruiz J., de Lago, E.
PHYTOCANNABINOID SATIVEX-LIKE COMBINATION AS A DISEASE-MODIFYING THERAPY IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS. (pendiente de publicación).

Capítulo 3: Caracterización del SCE en un modelo celular y en el modelo de ELA de ratones transgénicos para la proteína SOD1^{G93A} y estudio del uso de una combinación de Δ^9-THC y CBD como agente modificador de la ELA.

En este capítulo hemos identificado por primera vez los componentes del SCE (receptores CB_1 y CB_2 y enzimas de síntesis y degradación de endocannabinoides NAPE-PLD, DAGL, FAAH y MAGL) en la línea celular NSC-34 y en el modelo de ELA de ratones transgénicos para la SOD1^{G93A}. En las células vimos un incremento del receptor CB_1 y la enzima FAAH en células diferenciadas, indicando importancia de estos elementos en el mantenimiento de la homeostasis celular. En los ratones transgénicos vimos un incremento del receptor cannabinoide CB_2 tanto en machos y hembras en estadios finales de la enfermedad, y un incremento en la expresión de la enzima de síntesis NAPE-PLD en machos transgénicos.
Además hemos hecho un estudio del uso terapéutico de una combinación similar al Sativex tanto en machos como en hembras del modelo de ELA de ratones transgénicos para la SOD1^{G93A}. El tratamiento tras la aparición de los primeros síntomas mejoró el deterioro neurológico en las primeras semanas. Aunque el efecto se perdió con el tiempo y no vimos una mejora en la supervivencia de los animales tratados respecto a los tratados con vehículo, al analizar secciones de médula espinal teñidas por tinción de Nissl, apreciamos mayor número de moto-neuronas en el asta dorsal, indicando un posible efecto neuroprotector de la combinación de cannabinoides.

Los resultados de este capítulo se encuentran recogidos en las siguientes publicaciones:

Moreno-Martet, M., Mestre, L., Loría, F., Guaza, C., Fernández-Ruiz, J., de Lago, E.
Identification of receptors and enzymes for endocannabinoids in NSC-34 cells: re- levance for in vitro studies with cannabinoids in motor neuron diseases. Neuroscience Letters 2012, 508(2):67-72.

Moreno-Martet, M., Espejo-Porras, F., Fernández-Ruiz, J., de Lago, E.
Changes in Endocannabinoid Receptors and Enzymes in the Spinal Cord of SOD1 G93A-Transgenic Mice and Evaluation of a Sativex ®-like Combination of Phyto- cannabinoids: Interest for Future Therapies in Amyotrophic Lateral Sclerosis. CNS Neuroscience & Therapeutics 2014, (artículo aceptado, en prensa).

Capítulo 4: Estudio del bloqueo de la expresión de los receptores cannabinoides en un modelo de ELA en pez cebra (*Danio rerio*).

En este capítulo hemos investigado los efectos del bloqueo de la expresión de los genes que codifican para los receptores cannabinoides *CNR1* y *CNR2* y como afectan a la axonopatía causada por la sobreexpresión de proteínas relacionadas con la ELA en el pez cebra (Danio rerio). Datos preliminares muestran como el bloqueo de la expresión de *CNR1* podría tener un efecto positivo en la mutación $SOD1^{A4V}$.

Conclusiones

Las conclusiones derivadas de los anteriores objetivos son las siguientes:

1-WIN 55,212-2 ejerce efectos anti-glutamatergicos y anti-inflammatorios a través de los receptores CB_1 en las etapas inciales de la EAE en ratones.

2- Nuestros datos apoyan el uso potencial del Sativex® como terapia capaz de frenar el avance de la EM en los modelos de TMEV-IDD y EAE.

3- El SCE sufre alteraciones durante la progresión de la ELA en un modelo de ELA en ratón. El uso de una combinación de THC y CBD en estos ratones tiene efectos beneficiosos en las primeras semanas de enfermedad.

4- El bloqueo de la expresión del gen *CNR1* en el modelo de ELA en pez cebra puede revertir la axonopatía causada por la mutación $SOD1^{A4V}$.

Aportaciones fundamentales de esta tesis doctoral

Como hemos comprobado en diversos modelos de EM, los cannabinoides pueden alterar el curso de la enfermedad a través de los receptores CB_1 y CB_2 así como de los receptores nucleares PPAR, disminuyendo el daño neurológico, impidiendo la entrada de agregados celulares al SNC, restaurando la morfología de la mielina y disminuyendo la activación microglial y la inducción e COX2, TNFa e iNOS. En la ELA el receptor CB_2 está incrementado en estadios finales de la enfermedad en ratones transgénicos y Sativex disminuye el deterioro neurológico de la ELA en un modelo de ratón. Además hemos comprobado el efecto del bloqueo de los receptores cannabinoides en un modelo de ELA en pez cebra. Todos estos datos aportados en esta Tesis doctoral fundamentan el paso del uso de terapias basadas en cannabinoides de los modelos pre-clinicos al uso de ensayos clínicos para intentar aliviar los síntomas que actualmente no tienen cura en EM y ELA.

LIST OF ACRONYMS USED IN THIS THESIS

2-AG 2-Arachidonoylglycerol
ABHD α/β hydrolase domain
ACEA Arachidonoyl 2-chloroethylamide
ACPA Arachidonylcyclopropylamide
AEA N-arachidonoylethanolamine
ALS Amyotrophic lateral sclerosis
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC Antigen-presenting cell
ATP Adenosine triphosphate
BDS Botanical drug substance
BBB Blood-brain barrier
cAMP cyclic adenosine monophosphate
CAMS Cannabinoids in Multiple Sclerosis
CB_1R Cannabinoid receptor type 1
CB_2R Cannabinoid receptor type 2
CBC Cannabichromene
CBD Cannabidiol
CBG Cannabigerol
CBR Cannabinoid receptors
CBN Cannabinol
CNS Central nervous system
CSF Cerebrospinal fluid
COX Cyclooxygenase
CUPID Cannabinoid Use in Progressive Inflammatory brain Disease
DAGL Diacylglycerol lipase
DALN Desacetyllevonantradol
DNA Desoxyribonucleic acid
DSE Depolarization-induced suppression of excitation
DSI Depolarization-induced suppression of inhibition
Δ^9-THC delta-9-tetrahydrocannabinol
Δ^9-THCV delta-9-tetrahydrocannabivarin
EAAT Excitatory amino-acid transporter
EAE Experimental autoimmune encephalomyelitis
EBV Epstein-Barr virus
ECS Endocannabinoid system
FAAH Fatty acid amide hydrolase
FUS Fused in sarcoma

GPCR / GPR G protein-coupled receptor
HHV Human herpes virus
HPA hypothalamic-pituitary-adrenal
ICAM intercellular adhesion molecule
IFN Interferon
IL Interleukin
JNK c-Jun N-terminal kinases
LTD Long-term depression
LTP Long-term potentiation
MAGL Monoacylglycerol lipase
MAPK Mitogen-activated protein kinases
MBP Myelin basic protein
MOG Myelin oligodendrocyte glycoprotein
MS Multiple sclerosis
NAGly N-arachidonyl glycine
NAPE-PLD N-acyl phosphatidylethanolamine specific phospholipase D
NMDA N-methyl-D-aspartate
NO Nitric oxyde
OEA N-oleylethanolamine
OPC Olygodendrocyte precursor cells
PEA Palmythoylethanolamine
PI3K Phosphoinositide 3-kinase
PLC Phospholipase C
PPAR Peroxisome proliferators-activated receptors
RNA Ribonucleic acid
RNS Reactive nitrogen species
ROS Reactive oxygen species
SOD Superoxide dismutase
TDP Tar-DBP Protein
TGF Tumour growth factor
TMEV-IDD Theiler's murine encephalomyelitis virus-induced demyelinating disease
TNF Tumour necrotic factor
TRPA Transient receptor potential ankyrin
TRPV Transient receptor potential vanilloid
VCAM vascular cell adhesion molecule

ABSTRACT

Neurodegenerative diseases are the XXI century challenge for health systems in developed countries. In this thesis, we focus in multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS), two different diseases that affect neural cells within the spinal cord producing alterations in the muscles innerved by spinal nerves. MS is an autoimmune and demyelinating disease that affects spinal nerves and damage spinal axons. ALS is associated with the direct death of motor neurons causing paralysis of the muscles. In both disorders we have studied the involvement of the endocannabinoid system (ECS) as a modulator of the clinical course of the disease and in particular as a target for developing potential disease-modifying therapies. The ECS is an intercellular communication system involved in the maintenance of cell homeostasis that plays a role in cell survival through cytoprotective mechanisms.

Our results show that the elements of the ECS are valuable therapeutic targets that can modify the course of MS and ALS. First we have investigated the role that cannabinoids play in MS. We have elucidated the role of the cannabinoid receptor CB_1 in the effect caused by the synthetic cannabinoid WIN 55,212-2 in a mice model of MS. We have tested the use of a Sativex-like combination of the phytocannabinoids Δ^9-THC and CBD in two mice models of MS. We show that these phytocannabinoids reduce the neurological decline and the disease progression of these models of MS through cannabinoid receptors and PPARγ nuclear receptors.

Second we have characterized the changes in the ECS in an in vitro cell line and an in vivo transgenic $SOD1^{G93A}$ mice model of ALS. There are changes in the ECS related with the disease progression that point to valuable therapeutic targets. We have activated these targets with a Sativex-like combination of Δ^9-THC and CBD in the $SOD1^{G93A}$ transgenic mice. The Sativex-like combination improved the neurological decline in the first weeks after symptoms onset in treated animals. Although there was no significant effect over survival, Nissl stainings of spinal cord sections showed a higher number of motor neurons in the treated animals, pointing to an effect neuroprotector effect of the Sativex-like combination. Finally we have studied the effect of blocking the expression of the cannabinoid receptors in a zebrafish (*Danio rerio*) model of ALS. Our results show a possible effect that *CNR1* blockade reverts the toxic effect of $SOD1^{A4V}$ mutations.

Our data supports the increasing amount of evidence pointing to a possible neuroprotective effect derived of the manipulation of the ECS in MS and ALS and encourage research to take a step further from pre-clinical research to clinical trial with patients.

INTRODUCTION

Introducción

INTRODUCTION

Central nervous system (CNS) pathologies involving the death of specific groups of neurons, the so-called progressive neurodegenerative disorders, have become a key challenge for biomedical research. There are millions of people affected throughout the world and the number of patients affected with a neurodegenerative disease will increase in the next years due to aging of population in developed countries. Although knowledge improves each day regarding the pathogenic events that causes neurons to die, the cure for any neurodegenerative disease is yet to be found. In this thesis, we focus in multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS), two different diseases that affect neural cells within the spinal cord producing alterations in the muscles innerved by spinal nerves. MS is an autoimmune and demyelinating disease that affects spinal nerves and damage spinal axons. ALS is associated with the direct death of motor neurons causing paralysis of the muscles. In both disorders we have studied the involvement of the endocannabinoid system (ECS) as a modulator of the clinical course of the disease and in particular as a target for developing potential disease-modifying therapies. Following this brief summary, the key etiologic, neuropathological and pharmacological characteristics of both disorders will be reviewed, followed by a description of the biochemical, physiological and pharmacological aspects of the cannabinoids and the ECS, in particular those aspects that support their neuroprotective properties.

MULTIPLE SCLEROSIS

MS is the first cause of young adult neurological impairment. In Spain around 40-60 people in 100000 inhabitants are affected and according to European statistics the economical cost for the Spanish National Health System is 30000-40000€/year per patient (Sobocki et al., 2007). MS is a chronic inflammatory, demyelinating and neurodegenerative disease that affects different areas of the central nervous system (CNS) and has unknown aetiology (Noseworthy et al., 2000). The main pathological hallmark of MS is plaques of demyelination that appear mainly in white matter areas but also in grey matter areas (Kutzelnigg and Lassmann, 2006). Depending on the location of the demyelination, the damage will cause a broad range of clinical symptoms e.g. loss of vision, weakness, bladder dysfunction, stiffness and painful spasms, impaired speech, tremor, vertigo and depression. There are different types of MS according to the evolution of the disease: relapsing remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS). 80% of MS cases start with a single acute episode of inflammation that causes neurological symptoms that partially or totally remit when the inflammation is controlled. In the remaining 20% of the patients MS is progressive

Box 1

Spinal cord

The spinal cord hosts the main pathway for information linking the brain and the peripheral nervous system. It goes down the skull through the foramen magnum to the lumbar region and is protected by the vertebral column. In the spinal cord the grey matter is located centrally, surrounded by bunches of mielynated axons that form the white matter. These groups of axons are called fiber tracts or pathways, and there are two kinds: the sensory ascendant pathway (afferent) and the motor descendant pathway (efferent). These two pathways are organized in different areas:

Somatosensory organization:

Sensory pathways are located into the dorsal column-medial lemniscus (DCML) tract and the anterolateral system. The sensory information travels from sensory receptors located outside the central nervous system (CNS) to the primary sensory cortex in the brain trough three different neurons named primary, secondary and tertiary sensory neurons. In both pathways the primary motor neuron soma is located in the dorsal root ganglion and innervates their main axon into the spinal cord. In the DCML the primary axons synapse with the secondary neuron in the nucleus gracillis or in the nucleus cuneatus, were secondary axons are bundled into the internal arcuate fibres, that decussate and synapse with tertiary sensory neurons in the thalamic ventral postero lateral (VPL) nucleus until the primary sensory cortex. In the anterolateral system the secondary neuron is located in the substantia gelatinosa and then sensory fibres decussate and travel up to the VPL in the spinothalamic tract.

Motor organization:

The motor pathway is formed by upper motor neurons that send signals to the spinal cord where lower motor neurons that innerve muscles are located. Voluntary movement originates in the motor cortex (Brodmann areas 1,2,3,4 and 6). The axons of the large pyramidal cells located in the layer V of the motor cortex form the corticospinal tract that travels down through the internal capsule and the medullary pyramids, where about 90% of the axons decussate to the contralateral side and travel down the spinal cord in the lateral corticospinal tract. The remaining axons that do not decussate form the ventral corticospinal tract. The upper motor neurons synapse with lower motor neurons directly or though interneurons. In the brainstem, these lower motor neurons are located in the motor cranial nuclei: oculomotor, trochlear, motor nucleus of the trigeminal nerve, abducens, facial nerve, accessory, and hypoglossal. In the spinal cord the lower motor neurons are located in the ventral horn. In both cases, the lower motor neurons innervate muscles and mediate muscle contraction.

Physical injury, demyelination, inflammation and neurodegeneration that occur in spinal cord will cause different symptoms ranging from pain, muscle weakness, dystonia, paraplegia, tetraplegia or death if respiratory muscles are affected. For the purpose of this Thesis we have focused in two different diseases that affect spinal cord: multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS).

since onset. During RRMS clinical episodes alternate with total or partial remission but with time the recovery from each episode will be incomplete and persistent symptoms will accumulate. 65% of these patients will enter the SPMS phase (Compston and Coles, 2008). Both genetic and environmental factors have a strong influence in MS. The Class II human leukocyte antigen *HLA-DRB1* gene (Barcellos et al., 2002) and the interleukin 7 receptor α chain *IL7R* gene (Gregory et al., 2007) where the first genetic factors that implied MS susceptibility, but the exact mechanism is still undefined (Hauser and Oksenberg, 2006). Environmental factors such as differential distribution of MS in a North-South gradient distribution, migration during childhood (Kurtzke, 1993) and infection with different virus (e.g. human herpesvirus 6 (HHV-6) (Moore and Wolfson, 2002) and Epstein-Barr Virus (EBV) (Owens and Bennett, 2012)) during youth are as well related

with MS pathogenesis.

It is thought that MS onset is an autoreactive process from peripheral lymphocytes that cross the blood-brain-barrier (BBB). The initial important event in MS pathogenesis in susceptible individuals is the activation of $CD4^+$ T cells in the periphery (Sospedra and Martin, 2005). The autoreactive $CD4^+$ T cells cross the BBB with the help of adhesion molecules (e.g. vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM)) and enter the CNS. Inside the CNS $CD4^+$T cells secrete chemokynes such as interferon gamma (IFNγ) and tumor necrotic factor alpha (TNFα) following reactivation by antigen presenting cells (APC). This inflammatory process will activate resident microglia and recruit $CD8^+$ cells, B-cells, granulocytes and mast cells in the active lesion area. This inflammatory reaction aims proteins present in the myelin sheath, e.g. myelin basic protein (MBP) or myelin olygodendrocyte glycoprotein (MOG). After the destruction of the myelin sheath olygodendrocytes

Box 2

MS and ALS *in vivo* models used in this Thesis.

MS mice models

There are two main models that recapitulate different key events of MS in rodents: experimental autoimmune encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD). In EAE the mice are induced with an autoimmune disease. It can be passive EAE, when Th1-cells against myelin peptides from an immunized mouse are transferred to naïve mice. In active EAE mice are immunized against a myelin protein, myelin basic protein (MBP) or myelin olygodendrocytic protein (MOG) being the most common. In this thesis we have focused on a particular form or EAE called chronic relapsing EAE (CREAE). In this model we use C57BL6/J mice, where the MOG_{35-55} fragment is co-injected with a complete Freund's adjuvant (CFA) and Bordetella pertussis toxin to accelerate the immunizing effect and keep the effect of the immunized antigen. The immunization promotes IFNγ production that activates MHC-II-$CD4^+$ Th1 cells that focally infiltrate the spinal cord producing inflammation and demyelination. Around 12 days after immunization, mice develop tail weakening and a progressive paralysis that worsens until reaching its peak, followed by incomplete recovery.

The TMEV-IDD is generated injecting Theiler's murine encephalomyelitis virus, a picorna virus, intracerebrally into susceptible mice. The virus induces a biphasic disease, with an acute phase driven by $CD4^+$ T-cells infiltration into the spinal cord followed by a chronic demyelinating phase 30 to 35 days post-inoculation. Pro-inflammatory cytokines recruit macrophages and monocytes inside the CNS demyelinating axons.

ALS models

*B6SJL-Tg(SOD1*G93A)1Gur/J*

The main *in vivo* model used in ALS research is the $SOD1^{G93A}$ transgenic mouse (Gurney et al., 1994; Sugiura et al., 1995). The overexpression of SOD 1 with the G93A mutation reproduces features of ALS in mice. First symptoms appear around day 60, where tremor and loss of strength in the hindlimb is followed by abnormal gait and progressive paralysis that leads to death when mice are 130 days old. SOD1 gain-of-toxic function drives motor neuron death. This model also reproduces gliosis and microglial activation around 90 days.

Zebrafish

The zebrafish (*Danio rerio*) is a nice model organism to study neurodegenerative diseases (Paquet et al., 2009). Zebrafish are easy to maintain, breed, the development of their embryo is transparent, they have a complete CNS and their genetic expression can be easily manipulated (Laird and Robberecht, 2011). Zebrafish embryos injected with ALS-related genes mRNA such as *SOD1* (Lemmens et al., 2007) or *TDP-43* (Kabashi et al., 2009; Laird et al., 2010) develop an axonopathy in the caudal region characterized by shortened axons and aberrant branching in the motor neurons that innerve caudal muscles.

and Schwann cells dye as well. During the remitting phases of the disease, there is a partial re-myelination and the neurological symptoms totally or partially resolve. In the progressive phase the progression of the disability occurs gradually over time and is due to irreversible axonal loss and neurodegenerative processes.

The therapies to treat MS focus in two aspects: alleviation of symptoms, based on improving everyday life masking the symptoms (e.g. spasticity, bladder dysfunction, pain) (Krupp and Rizvi, 2002) and disease modifying agents that reduce the number of relapses and inflammation. These disease modifying agents are focused on relieve inflammation and the activation of the immune system (Luessi et al., 2012). β-interferon is used to inhibit the activation and migration of the T-cells and modulates the release of anti-inflammatory and pro-inflammatory cytokines (IFNB Multiple Sclerosis Study Group, 2001). Mitoxantrone supresses B and T cells activation and inhibits T-cell migration (Bastianello et al., 1994). Finally, natalizumab inhibits the migration of leukocytes across the BBB (Miller et al., 2003). These treatments for MS are all immunomodulatory or anti-inflammatory, and have limited effects on the neurodegeneration that drives clinical deterioration. There are no licensed neuroprotective agents that slow, stop or reverse neurodegeneration. Some compounds have succeed as neuroprotective agents in MS models (reviewed in (Luessi et al., 2012)) and have entered clinical trials. Unfortunately the result of these trials does not show a positive effect in MS in the parameters measured, (e.g lamotrigine (Kapoor et al., 2010), statins (Kamm et al., 2012)) except for amiloride (Arun et al., 2013). Thus there is an urge to find novel drugs that delay MS progression and treat neurodegeneration to avoid clinical deterioration.

AMYOTROPHIC LATERAL SCLEROSIS

ALS is a neurodegenerative disease that affects upper and lower motor neurons in motor cortex, brainstem and spinal cord. The progressive selective death of motor neurons causes muscle weakening, fasciculation, atrophy and paralysis that lead to death when it affects muscles involved in breathing. Depending on the onset of the disease, ALS can be classified as spinal ALS, when the limb muscles are the first muscles affected or bulbar ALS, when the onset reflects the loss of brainstem motor neurons that innerve oropharyngeal muscles. Usually, life expectancy in ALS is 3-5 years after the first symptoms appear. 10% of the ALS cases are classified as familiar ALS (fALS), when ALS patients cluster in families, due to different mutations in a wide variety of genes. The remaining 90% of the ALS cases are of unknown origin and are classified as sporadic ALS (sALS). The most prevalent cause of ALS is an intronic GGGGCC hexanucleotide expansion in the *C9ORF72* gene (DeJesus-Hernandez et al., 2011; Renton et al., 2011) that accounts for 40% of fALS and 7% of sALS, followed by mutations in the superoxide dismutase 1 (*SOD1*) gene (Rosen et al., 1993) that are involved in 12% of fALS. Alterations in the RNA binding proteins FUS (Kwiatkowski et al., 2009; Vance et al., 2009) and *TDP-43* (Neumann et al., 2006) encoded by the *FUS* and *TARDBP* genes are responsible of 4% of fALS cases each.

Several processes contribute to the pathogenesis of ALS: excitotoxicity, oxidative stress, defects in glia-neuron crosstalk, protein aggregation, mitochondrial dysfunction, impairment in endosomal trafficking and dysregulation of RNA transcription and processing (Bento-Abreu et al., 2010; Boillée et al., 2006; Gendron et al., 2014). Ubiquitin positive-cytoplasmic protein aggregates are one of the pathological hallmarks in ALS. De-

pending on the proteins involved, aggregates can be classified as positive-SOD1, positive-TDP-43 and positive FUS inclusions. The role that these aggregates play in ALS is yet to be found, as for other neurodegenerative diseases (Polymenidou and Cleveland, 2011). Independently of the protein altered, the degenerative events start with the retraction of the axon from the neuromuscular joint and the consequent denervation of the muscle. The muscle suffers from cramps, fasciculations, i.e. painful contraction of single fibres, atrophy and finally paralysis. The retraction of the axon is accompanied by impairment in the neuron-glia crosstalk. Microglia gets activated, releasing toxic factors e.g. TNFα, interleukin-1β (IL-1β) and nitric oxyde (NO), and astrocytes become reactive too (Bilsland et al., 2008; Henkel et al., 2009; Nagai et al., 2007). Olygodendrocytes become dysfunctional aswell (Philips et al., 2013).

There is no effective treatment to delay or stop ALS progression. The only licensed drug approved so far is Rilutek®, that contains riluzole, which is supposed to act inhibiting excitotoxicity (Bensimon et al., 1994; Gurney et al., 1996). However, the clinical effect of the drug is a mild increase in 2-3 months in lifespan of ALS patients. The other clinical approximation to treat ALS patients are designed to relieve symptoms and improve the quality of life with support of drugs to help reduce fatigue, ease muscle cramps, control spasticity in the first stages of the disease or the use of mechanical respirators to assist breathing once breathing muscles are affected (Bede et al., 2011)

COMMON PATHOGENIC MECHANISMS IN MS AND ALS

MS and ALS share not only neuronal subpopulations affected but also have common pathogenic mechanisms that contribute to the neuronal damage: excitotoxicity, oxidative damage and neuroinflammation.

Excitotoxicity:

Excitotoxicity is the damage caused by overactivation of glutamate ionotropic receptors. These receptors are the N-methyl-D-aspartate (NMDA) receptor and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. Glutamate is the main excitatory amino acid transmitter in the CNS. In the synaptic cleft glutamate is washed out by astrocytes through excitatory aminoacid transporters (EAAT), mainly EAAT2. Excitotoxicity leads to alteration in intracellular Ca^{2+} homeostasis, adenosine triphosphate (ATP) production and mitochondrial impairment, proteolytic enzyme activation and reactive oxygen species (ROS) production that generates a “vicious circle” that contributes to neuronal death. There are high levels of glutamate in MS cerebrospinal fluid (CSF) (Sarchielli et al., 2003) and brain (Cianfoni et al., 2007). MS patients have increased ionotropic glutamate receptors levels (Newcombe et al., 2008) and impaired glutamate re-uptake by EAAT transporters as well (Vallejo-Illarramendi et al., 2006). Thus, in an experimental model of MS, blockade of glutamate receptors suppressed neurological symptoms and the release of pro-inflammatory cytokines (Sulkowski et al., 2013). Motor neurons have low calcium buffering proteins and AMPA permeable to Ca^{2+}, which makes them sensible to AMPA mediated toxicity (van den Bosch et al., 2006). In ALS patients there are elevated glutamate levels in spinal cord (Pioro et al., 1999) and altered glutamate transport (Rothstein et al., 1992; 1995; Sasaki et al., 2000). Besides, the only licensed drug to treat ALS is riluzole, which has anti-excitotoxic properties (Bensimon et al., 1994).

Oxidative stress:

Reactive oxygen species (ROS) are a by-product of metabolism in healthy cells. They derive from mitochondrial respiration, due to

leakage of electrons that result in an incomplete reduction of molecular O_2. This gives as a result the superoxide radical anion $[O_2]^{*-}$ and hydrogen peroxide (H_2O_2). These two molecules are not highly reactive, but if not properly detoxified $[O_2]^{*-}$ can react with nitric oxide (NO) to produce peroxinitrite $[ONOO]^{*-}$ and H_2O_2 can slowly be decomposed to hydroxyl radical $[OH]^{*-}$ in a process catalysed by reduced metal ions called Fenton reaction. $[ONOO]^{*-}$ and $[OH]^{*-}$ are highly energetic molecules harmful to nucleic acid, lipids and proteins. In homeostasis, antioxidant mechanisms (e.g. glutathione, SOD, antioxidant scavengers) keep ROS under control, but misbalance due to increased ROS production or reduced antioxidant mechanisms lead to oxidative stress (Kalyanaraman, 2013). The accumulation of oxidative stress in non-replicating neurons can determine their sensibility facing a toxic insult e.g. a mutated protein and can compromise their survival. In MS patients plaques, infiltrated macrophages and activated microglia produce a vast amount of ROS that damage DNA and lipids (Haider et al., 2011). This oxidative stress damages also mitochondrial DNA and impairs the mitochondrial respiratory chain (Lu et al., 2000; Mahad et al., 2008) and this effect has been related with axonal loss (Mahad et al., 2009). Antioxidant molecules such as sulforaphane or mitoQ succeeded improving the symptoms in a MS mouse model (Bin Li et al., 2013; Mao et al., 2013).

There is evidence of elevated oxidative stress damage in ALS patients as seen in CSF (Tohgi et al., 1999), and post-mortem tissue of familial and sporadic ALS patients in spinal cord and motor cortex (Ferrante et al., 1997; Shaw et al., 1995). This increased oxidative stress damage is also found in animal models of the disease (Liu et al., 2007). The normal activity of Cu-Zn-SOD1 is the detoxification of $[O2]^{*-}$ into H_2O_2, that will be detoxified to O_2 and H_2O by catalases. It is tempting to suppose that mutations in the SOD1 enzyme will exacerbate oxidative stress damage that may be harmful to motor neurons, therefore the relation between SOD1 mutations and ALS. However, despite of the presence of mutations in Cu-Zn-SOD1, the exact pathogenic mechanism is unknown. The loss of dismutase activity in a mouse model did not result in ALS (Reaume et al., 1996). A gain of toxic function derived from mutations that expose the Cu^{2+} in SOD1 and therefore favours the production of $[OH]^{*-}$ through the Fenton reaction has also been proposed. However, not all the SOD1 mutations leading to ALS have the Cu^{2+} exposed, and the depletion of Cu^{2+} does not affect onset, progression nor survival in mice models of ALS (Subramaniam et al., 2002). Thus, oxidative stress is thought to exacerbate other pathogenic mechanisms present in ALS such as protein aggregation, excitotoxicity, neuroinflammation, impaired axonal transport and RNA metabolism (see (Barber and Shaw, 2010) for a review). Despite the success of some antioxidant therapies delaying disease progression in animal models of ALS, such as vitamin E (Gurney et al., 1996) or the coenzyme Q_{10} derivate MitoQ (Miquel et al., 2014) to name a few, their use in clinical trials with human patients has shown no overall effect (Barber and Shaw, 2010).

Neuroinflammation:

Infiltration of lymphocytes to the CNS and activation of CNS resident macrophages are key events both in MS and ALS. This cells drive white matter damage in MS patients (Lassmann, 2014) and the activation of microglial cells has been correlated with outcome in MS progressive patients (Giannetti et al., 2014). In ALS patients there is also presence of activated microglia and infiltrated lymphocytes (Engelhardt et al., 1993; Henkel et al., 2004) in the regions of motor neuron damage (Ince et al., 1996; Kawamata et al., 1992). This activated microglia, depending on its activation phenotype, can have

an anti-inflammatory gene expression profile (M2 phenotype) or pro-inflammatory gene expression profile (M1 phenotype). This pro-inflammatory microglia secretes the cytokynes TNFα, IL-β, produce oxidative ROS and reactive nitrogen species (RNS) and supress the supply of trophic factors to neurons (Cunningham, 2013; Henkel et al., 2009). This inflammatory reaction sustained chronically over time contributes to neurodegeneration (Perry and Holmes, 2014). Both in ALS and MS there is astrogliosis, and reactive astrocytes secrete prostaglandin E2, leukotriene B4, and nitric oxide that contribute to the neuronal damage (Sofroniew, 2009). In models of mutant SOD-1, astrocytes drive motor neuron degeneration (Nagai et al., 2007). This effect has recently been reproduced in human cells, where astrocytes generated from sALS patients drive motor neuron degeneration through a caspase-independent apoptosis called necroptosis (Re et al., 2014).

These common pathogenic mechanisms alter the neuronal homeostasis and exacerbate the damage caused by the neuronal entourage. Physiologically there are systems that can cope with these toxic stimuli to maintain cellular homeostasis. One of these mechanisms discovered in the 90s of the XX century is the endocannabinoid system (ECS). ECS is implicated in a wide variety of physiological processes helping cells to come back to homeostasis after an acute or chronic injury. Thus the different elements of the ECS (i.e. cannabinoid receptors, endocannabinoids and synthesis and degradation enzymes) offer novel therapeutic targets very attractive from the point of view of drug development. This is particularly interesting as most cannabinoids are multi-target compounds that act through combination of different mechanisms involved in neuronal survival. These novel drugs can overthrow the situation where neurodegenerative diseases that have no cure nowadays may have successful therapeutic interventions that delay or stop degeneration in the future.

CANNABINOIDS AND THE ENDOCANNABINOID SYSTEM

The ECS is an intercellular communication system involved in the maintenance of cell homeostasis that plays a role in cell survival through cytoprotective mechanisms. The discovery of the ECS is a consequence of the molecular and pharmacological characterization of the mechanisms of action of a large family of molecules called cannabinoids. The name includes compounds derived from *Cannabis sativa* but also all the molecules that interact with the endocannabinoid system. Cannabinoids were initially identified in the weed plant *Cannabis sativa*. In 1963, Mechoulam & Shvo isolated and described for the first time the structure for the cannabidiol (CBD) (Mechoulam and Shvo, 1963). One year later, they isolated and described the structure of the main psychoactive compound in *Cannabis sativa*, the Δ^9-tetrahydrocannabinol (Δ^9-THC) (Gaoni and Mechoulam, 1964). These findings lead to the search of the endogenous mediators responsible for the psychoactive effects of Δ^9-THC. In 1988 the group of Allyn Howlett discovered the first endogenous receptor for cannabinoids, which was called cannabinoid receptor type 1 (CB_1R) (Devane et al., 1988). In 1993 a different receptor was discovered and was named cannabinoid receptor type 2 (CB_2R) (Munro et al., 1993). The presence of these receptors activated by Δ^9-THC suggested the existence of endogenous ligands. These endogenous cannabinoids, called endocannabinoids, were discovered as a novel family of fatty acids derived molecules. The arachidonoylethanolamide (AEA) was the first endocannabinoid discovered in 1992 by

the group of Raphael Mechoulam and named anandamide (Devane et al., 1992). The second endocannabinoid to be found was the 2-arachidonoylglycerol (2-AG) in 1995 (Mechoulam et al., 1995). The endocannabinoids, their endogenous receptors plus the synthesis and degradation enzymes for the endocannabinoids form what we call the endocannabinoid system (ECS).

Cannabinoid receptors:

The cannabinoid receptors (CBR) belong to the G protein-coupled receptors (GPCRs). GPCRs are seven trans-membrane domain receptors that integrate different extracellular stimuli into intracellular signalling pathways through the interaction between the receptor and G-proteins.

CB_1 receptor:

The CB_1R was the first cannabinoid receptor to be discovered, initially using the radiolabeled cannabinoid ligand [^{3}H]CP-55,940 in rat (Devane et al., 1988) and then cloned in rat and humans (Gérard et al., 1991; Matsuda et al., 1990). The CB_1R is a 473 amino acid protein encoded by the *CNR1* gene that in humans is located in the locus 6q14-q15 (Hoehe et al., 1991). The *CNR1* is widely conserved through evolution and is found in mammals, reptiles, birds, fish (Lam et al., 2006; Valenti et al., 2005) as well as some invertebrate clade (McPartland et al., 2006).
The CB_1R is widely expressed in the CNS, in neurons as well as in astrocytes, olygodendrocytes and neural precursors (Aguado et al., 2005; Molina-Holgado et al., 2002). The basal ganglia (substantia nigra pars reticulata, globus pallidus and striatum), brain cortex, cerebellum and hippocampus are the brain regions where CB_1R is more expressed whereas the expression of CB_1R is lower in the periaqueductal gray, brainstem, amygdala, some thalamic nuclei and the dorsal horn of the spinal cord.

Outside the CNS the CB_1R is also expressed, as it has been found in adrenal and pituitary gland, heart, liver, spleen, gastrointestinal tract, and urinary and reproductive structures (Galiègue et al., 1995; Gérard et al., 1991; Mackie, 2005).

CB_2 receptor:

The CB_2R detection was first reported in spleen in 1993 (Munro et al., 1993). The CB_2R is a 360 amino acid protein encoded by the *CNR2* gene that in humans is located in the locus 1p36 (Karsak et al., 2005). The homology between CB_1R and CB_2R is around 44 %, but between the transmembrane domains this homology rises up to 70 % (Montero et al., 2005). The CB_2R is expressed in different kinds of peripheral immune cells: macrophages, $CD4^+$ T cells, $CD8^+$ T cells, B cells, natural killer cells, monocytes and polymorphonuclear neutrophils (Derocq et al., 1995; Galiègue et al., 1995; Mechoulam et al., 1995). During inflammation and degeneration, CB_2R is also expressed in microglia, the resident macrophages of the CNS, depending on their activation state (Carlisle et al., 2002). CB_2R is not only expressed in peripheral immune cells. It has been described in pulmonary endothelial cells (Zoratti et al., 2003), gastrointestinal tract (Hillsley et al., 2007; Storr et al., 2002), and modulating bone formation and turnover (Ofek et al., 2006). CB_2R is also involved in meiosis in spermatogonia (Grimaldi et al., 2009), it appears in cirrhotic liver but not in normal liver (Julien et al., 2005), in cardiomiocytes (Shmist et al., 2006) and in mature and precursor adipocytes (Roche et al., 2006). The presence of the CB_2R in the CNS has been a controversial issue, as the first reports did not found CB_2R expression in the CNS (Derocq et al., 1995; Galiègue et al., 1995), but some authors have described its presence in neurons in DRG sensory neurons cultures (Anand et al., 2008; Ross et al., 2001), basal ganglia (Lanciego et al., 2011), brainstem (Van Sickle et al., 2005), cerebe-

Box 3

Non-cannabinoid receptors activated by cannabinoids

The physiological and pharmacological effects of cannabinoid compounds cannot be fully explained by the activation of CB_1R and CB_2R. For instance, the cannabinoid "tetrad" (hypothermia, analgesia, hypolocomotion and catalepsy) produced by Δ^9-THC and other cannabinoid agonists is blocked by rimonabant and absent in $CB1^{-/-}$ mice. However, in the case of AEA, these effects are still present in $CB1^{-/-}$ mice and are not blocked by rimonabant as in the case of hypothermia. This suggests the existence of additional receptors or intracellular targets that mediate the action of certain cannabinoids. Several *in vitro* and *in vivo* experiments have recently shown that these cannabinoids can activate other GPCRs, nuclear receptors and ion channels:

GPCRs

Some orphan GPCRs (i.e. GPR18, GPR55 and GPR119) are activated by fatty acids derived molecules and certain cannabinoids. GPR18 is an orphan receptor that is activated by N-arachidonyl glycine, which is derived from AEA and inhibits FAAH enzyme (Kohno et al., 2006). Besides N-arachidonyl glycine, Δ^9-THC and abn-CBD also activate GPR18 (McHugh et al., 2012). GPR55 is the endogenous receptor proposed for the bioactive lipid lysophosphatidilinositol. It is also activated by different cannabinoids such as Δ^9-THC, HU-210, noladin eter, R(+)-methanandamide, JWH-015, AM-251 and AM-281, although the evidence is only *in vitro* and controversial and the significance of GPR55 *in vivo* as a cannabinoid receptor is still to be demonstrated (Ross, 2009). N-oleylethanolamine (OEA), another fatty acid derived molecule synthesized by NAPE enzyme, activates GPR119 (Overton et al., 2006).

PPARs

Peroxisome proliferators-activated receptors (PPARs) are nuclear receptors that once activated form heterodimers with the retinoid X receptor (RXR) and become transcription factors for genes related with cell differentiation, development and metabolism (Heneka and Landreth, 2007). PPARs can be activated by certain cannabinoids. For example, PPARα is activated by anandamide, WIN55-212,2, noladin eter, OEA and N-palmythoylethanolamine (PEA) (Citraro et al., 2013; Sun et al., 2007). PPARγ is activated by Δ^9-THC and WIN55-212,2 (Mestre et al., 2009; O'Sullivan et al., 2005), leading to the activation of genes involved in anti-inflammatory response (O'Sullivan and Kendall, 2010).

TRPs

Transient receptor potential channels (TRPs) are ion channels located in the cell membrane that regulate extreme sensations of pain, temperature and pressure. The TRPs activated by cannabinoids belong to two sub-groups: the vanilloid group (TRPVs) responsible of the pain/hot sensations and activated by capsaicine and piperine present in chilli peppers, and the ankyrin (TRPAs) responsible of pressure responses. AEA, methanandamide and ACEA can mimic the capsaicin activation of TRPV1 channels (Di Marzo et al., 1998a; Price et al., 2004; Zygmunt et al., 1999). CBD, Δ^9-THC and cannabinol can activate TRPV2 channels (Qin et al., 2008) whereas TRPA1 channels are activated by Δ^9-THC and cannabinol (Jordt et al., 2004).

llum (Gong et al., 2006; Onaivi et al., 2008; Rodríguez-Cueto et al., 2014), hippocampal formation (Brusco et al., 2008; Gong et al., 2006; Onaivi et al., 2008) and also in neural precursors during neurogenesis (Molina-Holgado et al., 2007; Palazuelos et al., 2006).

Besides CB_1R and CB_2R there are other receptors activated by cannabinoids (reviewed in box 3). These receptors include GPCRs such as GPR18, GPR55, GPR119 but also other receptors as the ion channel family TRPV or the nuclear receptor PPAR.

Endocannabinoids:

The endogenous ligands for the CB_1R and CB_2R are known as endocannabinoids. These lipid messengers are molecules derived from long chain fatty acids that are not stored in vesicles as other neurotransmitters. Instead, different stimuli trigger the synthesis of endocannabinoids where and when they are needed. So far, there are 4 physiologi-

cally relevant endocannabinoids described: AEA (Devane et al., 1992), 2-AG (Mechoulam et al., 1995), 2-arachidonyl glyceryl ether (noladin ether) (Hanus et al., 2001) and the endogenous antagonist virhodamine (O-arachydonoylethanolamine) (Porter et al., 2002).

The mechanisms of synthesis and degradation of AEA and 2-AG have been extensively studied and are better known than those for the other endocannabinoids, so they will be addressed in the following sections.

Synthesis and degradation of AEA:

The first endocannabinoid was discovered in 1992 (Devane et al., 1992). This N-arachidonoylethanolamide was named anandamide, a word derived from the Sanskrit *ānanda*, which means bliss, and the amide bond characteristic of the chemical structure. It is a CB_1R partial agonist and a weak CB_2R agonist. AEA is mainly synthetized when an increase of intracellular Ca^{2+} activates a N-acyltransferase enzyme that transfers arachidonic acid to phosphatidylethanolamine to form N-arachidonoyl phosphatidylethanolamine (NAPE). This NAPE is then hydrolysed by the specific phospholipase NAPE-PLD, also Ca^{2+} inducible, producing AEA (Di Marzo et al., 1994).

AEA concentrations in CNS are lower than other endocannabinoids (Bisogno et al., 1999), and it has a short half-life due to its hydrolysation by the fatty acid amide hydrolase enzyme (FAAH) (Maurelli et al., 1995; Willoughby et al., 1997). The FAAH is the main degrading enzyme that hydrolyzes AEA to arachidonic acid and ethanolamine. However FAAH is not a specific enzyme for AEA, as it also uses other N-acyltethanolamines as a substrate. FAAH is even active on other major endocannabinoids as 2-AG. The distribution of this enzyme is associated with the presence of AEA and the CB_1R and it is localised post-synaptically (Egertová et al., 2003).

Synthesis and degradation of 2-AG:

The next physiologically relevant endocannabinoid to be found was the 2-arachidonoylglycerol in 1995 (Mechoulam et al., 1995). It was first isolated in canine gut and later found in brain where it is present in higher concentrations than AEA (Sugiura et al., 1995). 2-AG synthesis is independent from AEA. In this case, phospholipase C generates 1,2-diacylglycerol (1,2-DAG) from phosphatidylinositol. Then the diacylgycerol lipase (DAGL) enzyme hydrolyzes the 1,2-DAG to 2-AG. This reaction is also stimulated by an increase in intracellular Ca^{2+}. 2-AG is degraded by the monoacylglycerol lipase (MAGL) and FAAH to arachidonic acid and glycerol (Di Marzo et al., 1998b; Goparaju et al., 1999). The distribution of MAGL correlates with CB_1R presence, but it is located in the pre-synaptic neuron (Dinh et al., 2002). Besides MAGL and FAAH, 2-AG is degraded by two serine hidrolases, ABHD6 and ABHD12, with different subcellular locations than MAGL, thus providing different 2-AG pools in the cell (Blankman et al., 2007).

AEA and 2-AG as precursors of other bioactive lipids:

In addition to synthetic and degradative metabolisms of AEA and 2-AG, both endocannabinoids may be metabolized by other key enzymes involved in lipid metabolism. This is the case of COX-2, which in addition to arachidonic acid, may also use AEA and 2-AG as substrates to generate prostamides and prostaglandin glycerol-esters, respectively. These oxygenated derivatives are biologically active and their generation from endocannabinods may account for some CB_1R-CB_2R independent mechanism. The synthesis of these oxygenated derivatives may explain some paradoxical effects obtained when the biosynthesis of endocannabinoids is inhibited (Valdeolivas et al., 2013).

Pharmacology of the endocannabinoid system:

The research in the endocannabinoid system field has led to the discovery of different tools to manipulate the endocannabinoid system. In general, the different molecules that are used nowadays that modulate the endocannabinoid system can be classified as cannabinoid receptors agonists, cannabinoid receptors antagonists and endocannabinoid tone enhancers, including allosteric regulators of receptors and enzyme inhibitors.

Cannabinoid receptors agonists:

The cannabinoid receptors agonists are molecules that activate CB_1R, CB_2R or both, with different potency and affinity. They can be classified according to their structure in four groups: Classic cannabinoids, non-classic cannabinoids, aminoalkylindoles and eicosanoids.

Classic cannabinoids

The classic cannabinoids are compounds derived from a dibenzopyrane molecule with different lateral chains and degrees of unsaturation. The phytocannabinoids present in the hemp plant *Cannabis sativa* belong to this group, although not all of them are cannabinoid receptor agonists or even cannabinoid receptor ligands. The most abundant cannabinoid and the psychoactive molecule responsible for the marijuana "high" is the Δ^9-THC (Gaoni and Mechoulam, 1964). Δ^9-THC is a non-selective cannabinoid agonist, binding both CB_1R and CB_2R. The second most abundant cannabinoid is the cannabidiol (CBD) (Mechoulam and Shvo, 1963). CBD is a non-psychoactive cannabinoid that albeit having a low affinity for CB_1R and CB_2R deserves to be mentioned here. It is proposed that CBD inhibits CB_1R/CB_2R *in vitro* (Thomas et al., 2007) and can also inhibit the AEA cellular re-uptake (Bisogno et al., 2001). However, it is thought that the broad biological effects of CBD are derived from its antioxidant structure and its interaction with enzymes outside the ECS such as 5-HT_{1A} serotonin receptors (Espejo-Porras et al., 2013), PPARγ nuclear receptors (Esposito et al., 2011) or adenosine A2 receptors (Mecha et al., 2013). Besides Δ^9-THC and CBD, there are other phytocannabinoids in lower proportion in *Cannabis sativa* that have can interact with the cannabinoid receptors or other receptors. This is the case of the CB_1R/CB_2R agonist cannabinol (CBN), the CB_2R agonist and CB_1R antagonist and Δ^9-tetrahidrocannabivarin (Δ^9-THCV) or the non-psychoactive compounds cannabigerol (CBG) and cannabichromene (CBC) (Pertwee, 2008).

Some of the synthetic compounds used in research have a dibenzopyrene analogue structure as 11-hidroxi-Δ^8-THC-dimethylheptyl (HU-210) (Mechoulam et al., 1988), 11-hidroxi-hexahydrocannabinol-dimethylheptyl (HU-243) or nabilone (Lemberger and Rowe, 1975).

Non-classic cannabinoids

This group is formed by synthetic bicyclic and tricyclic Δ^9-THC analogues that lack the pyrane ring. The most important and used compound in this group is CP-55,940 (Koe et al., 1985). This bicyclic molecule is 5 times more potent than Δ^9-THC and binds both CB_1R and CB_2R. The radioactive labelled CP-55,940 has been widely used for cannabinoid receptor binding assays. Other compounds in this group are CP-55,244 (Koe et al., 1985), CP-50,556 (levonantradol) (Milne et al., 1979) and desacetyllevonantradol (DALN) (Yaksh, 1981).

Aminoalkylindoles

This group of compounds derived from pravadoline structures are not related with the dibenzopyrene structure. The most used aminoalkylindole is WIN55,212-2 (D'Ambra et al., 1992; Haycock et al., 1990), with a very high affinity for both CB_1R and CB_2R.

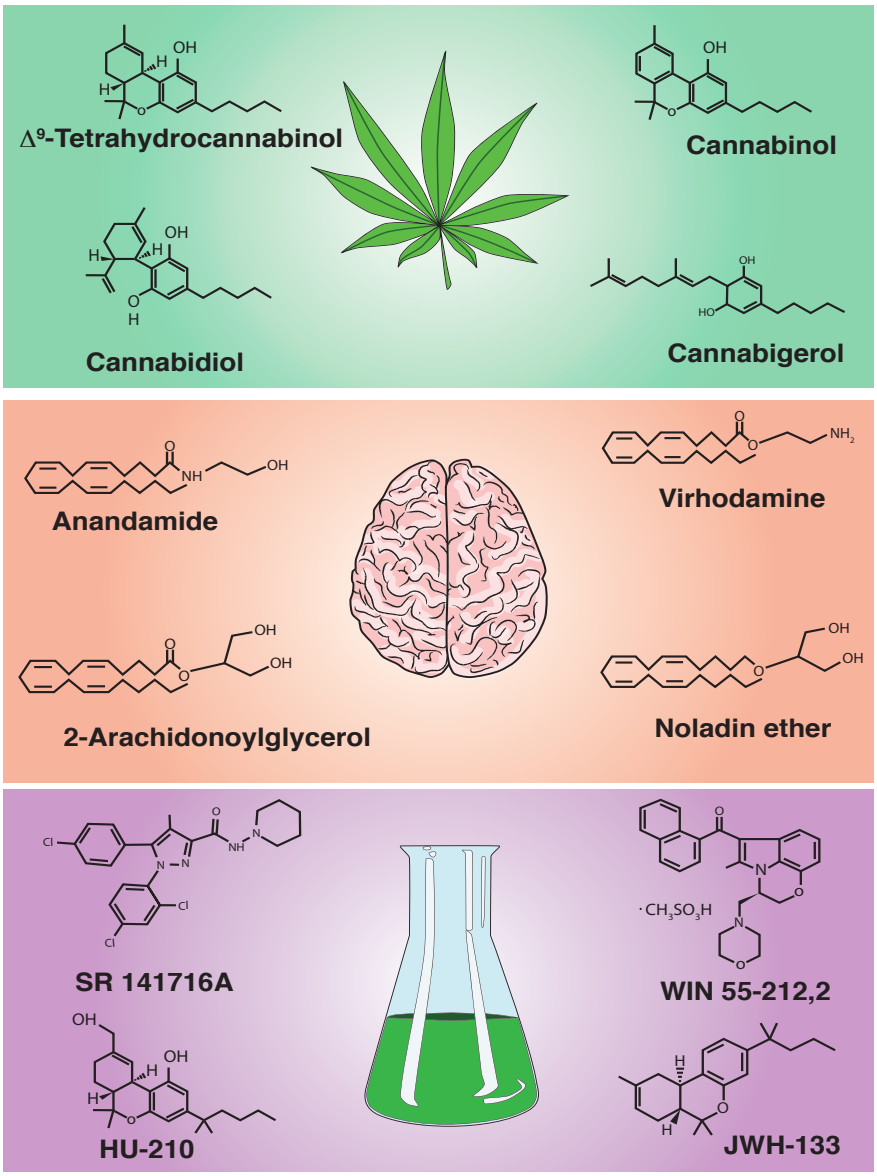

Figure 1: Different cannabinoid-related structures. The classic cannabinoid dibenzopyrane derived structure present in phytocannabinoids, the eicosanoid structure of endocannabinoids and some synthetic cannabinoids with different structures

Some CB_2R selective molecules are derived from WIN55,212-2 such as JWH-015 (Griffin et al., 1997) and L-768,242 (Huffman, 2000).

Eicosanoids

These compounds derive from arachidonic acid and share a similar structure with endocannabinoids. The AEA related structures enable the synthesis of CB_1R selective agonists, as methanandamide (AM-356), more potent than AEA due to its higher resistance to FAAH degradation (Abadji et al., 1994). Other CB_1R selective agonists in this group are arachidonoyl-2-chloroethylamide (ACEA), arachidonoylcyclopropylamide (ACPA) (Hillard et al., 1999), O-1812 (Di Marzo et al., 2001a), O-585 and O-689 (Showalter et al., 1996).

Cannabinoid receptor antagonists:
The use of molecules that selectively block the action of the cannabinoid receptors has been a major tool in the study of the endocannabinoid system. These molecules had allowed the discrimination between CB_1R or CB_2R-mediated pathways. Together with the use of transgenic knockout mice models, cannabinoid antagonists have allowed a deeper knowledge into the cannabinoid signalling.

SR141716A (rimonabant) was the first discovered and is a selective CB_1R antagonist (Rinaldi-Carmona et al., 1994). Other selective CB1R antagonists are derived from rimonabant, such as the AM-251 or the AM-281 (Gatley et al., 1996; 1998).

The most used CB_2R antagonists are SR141528 (Rinaldi-Carmona et al., 1998) and AM630 (Hosohata et al., 1997a; 1997b).

Endocannabinoid tone enhancers:
Inhibitors of endocannabinoids degrading enzymes increase the endocannabinoid tone. This strategy offers an interesting therapeutic approach due to the specific activation of the CBR at the site were endocannabinoids are needed and therefore synthetized. The use of these specific inhibitors block the degradation of AEA or 2-AG allowing its accumulation so endocannabinoids can activate CBRs prolonged over time. The effect of AEA can be enhanced using reversible FAAH inhibitors such as URB-597 (Mor et al., 2004) , OL-135, OL-92 (Boger et al., 2005), BMS-1(Lichtman, 2004), the more selective SA-47 (Zhang et al., 2007), or the more potent PF-750 (Ahn et al., 2007). JZL184 is a selective irreversible MAGL lipase inhibitor used to increase endogenous 2-AG (Long et al., 2008). Inhibitors for serine hydrolases have been also developed such as Piperidyl-1,2,3-triazole ureas to block DAGL (Hsu et al., 2013b) thus blocking 2-AG synthesis or ABHD6 (Hsu et al., 2013a) thus blocking 2-AG degradation.

A similar enhancing action over the endocannabinoid tone can be elicited using inhibitors of the endocannabinoid transporter, despite being the existence of this protein still controversial. A proof of support for the existence of this protein is the development of different inhibitors such as AM-404 (Beltramo et al., 1997), VDM11 (de Petrocellis et al., 2000), UCM-707 (López-Rodríguez et al., 2001), OMDM1 and OMDM2 (Ortar et al., 2003) and many other derivatives that increase the concentration of endocannabinoids. They may have the same application as FAAH and MAGL inhibitors.

Another mechanism to enhance cannabinoid signalling is the use of allosteric enhancers for the CBR, although by mechanisms not yet fully understood (Baillie et al., 2013). For example, the anti-inflammatory lipid lipoxin A4 was proposed to enhance the affinity of AEA at CB_1R both *in vivo* and *in vitro* (Pamplona et al., 2012).

Cannabinoid signalling:

The cannabinoid receptors (CBR) are integral membrane proteins with seven transmembrane domain proteins that are coupled to heterotrimeric G-proteins on the intracellular face of the membrane. The seven membrane-spanning helices are linked with three intracellular and extracellular loops. The binding site in the CBR is embedded in the transmembrane helices of the receptor. To activate the CBR, cannabinoid ligands latterally diffuse in the lipid membrane and interact with the receptor in a hydrophobic groove formed by transmembrane helixes 3 and 6 (Makriyannis et al., 2005). This interaction creates a conformational change in the transmembrane core and a rearrangement of the TM6 and the associated intracellular loop that activates the G protein and triggers signal transduction. Canonically, the CBR associate with $G_{i/0}$ proteins, and after its activation, the Gα-subunit inhibits the adenylate cyclase enzyme, inhibiting the cy-

clic adenosine monophosphate (cAMP) production. The Gβγ subunit can then activate different signalling pathways downstream as the phosphatidylinositol-3-kinase (PI3K)-Akt and the mitogen activated protein kinases (MAPK) p38 MAPK (Herrera et al., 2005; Liu et al., 2000) and Jun-N-terminal Kinase (JNK) (Rueda et al., 2000). In some circumstances however, CBR activation can increase cAMP production, by coupling to G_s and they can even couple G_q and G_{13} (Bonhaus et al., 1998; Lauckner et al., 2005). CB_1R but not CB_2R can inhibit ionic channels. When CB_1R is activated and interacts with the heterotrimeric G protein, the βγ subunit inhibits the N, P and Q Ca^{2+} channels and the D K^+ channels. On the other hand, $G_{i/0}$ proteins activate A K^+ channels and inwardly rectifying K^+ channels (Mackie et al., 1995). Finally, CB_2R activation increases intracellular Ca^{2+} levels through the activation of the phospholipase C (PLC) pathway (Zoratti et al., 2003) .

Canonical cannabinoid receptor signalling is altered by the formation of dimers or oligomers with other membrane receptors. In any case, this dimerization will alter the signalling compared to the activation of a single receptor. CB_1R can form homodimers with itself and heteromers with D2 receptors, opioid receptors, CB_2R and GPR55 (Callen et al., 2012; Kargl et al., 2012; Rios et al., 2006; Wager-Miller et al., 2002). The formation of the heteromers imply the enhancement or the reversion of the signalling mediated by a single receptor, but the physiological relevance of these heteromers need further investigation.

ECS in the nervous system:

The endocannabinoids are important modulators of synaptic transmission in the CNS. These effects are mainly mediated by the CB_1R, which is the most abundant GPCR in the brain. Endocannabinoid signalling mediates synaptic plasticity. In a few words, the efficacy of a synapse can be altered by increasing or decreasing the amount of neurotransmitter presynaptically released across the synapse or by increasing or decreasing the amount of receptors present post-synaptically, thereby altering that synapse sensitivity. The changes at one synapse will affect the entire network of synapses to which that neuron is connected. The decrease in GABA or glutamate release are called depolarization-induced suppression of inhibition (DSI) and depolarization-induced

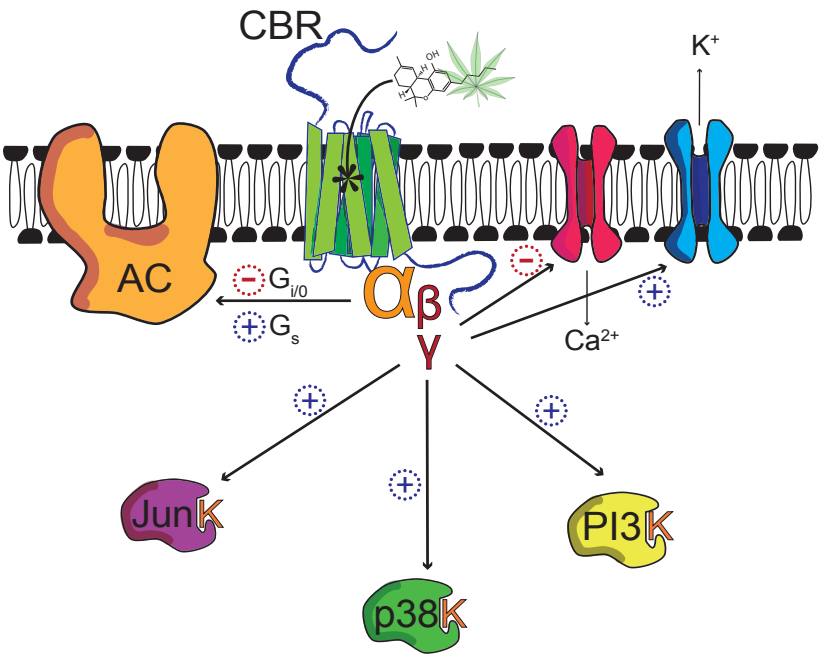

Figure 2:
Signalling driven by cannabinoid receptors.
When the agonist (e.g. Δ^9-THC) binds the CBR, the Gα subunit, depending on the G protein, will inhibit or stimulate adenylate cyclase. The Gβγ subunit will activate different MAPK such as JunK, p38K and PI3K. When CB_1R is activated the Gβγ will also close Ca^{2+} channels and open K^+ channels

suppression of excitation (DSE) respectively and are mediated by CB_1R and endocannabinoid retrograde signalling (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001). The increase or decrease in the amounts of post-synaptic receptors is called long-term potentiation (LTP) and long-term depression (LTD) respectively. The endocannabinoid signalling is mainly involved in a wide variety of LTD (see (Castillo et al., 2012) for a complete review). These modulatory effects in the central nervous system are exerted through different mechanisms: retrograde signalling, non-retrograde signalling and neuron-astrocyte communication.

Retrograde signalling:
CB_1R is localized in pre-synaptic neurons and modulates neurotransmitter release into the synaptic cleft. When intracellular Ca^{2+} levels increase in the post-synaptic neuron, endocannabinoid synthetizing enzymes DAGL and NAPE generate endocannabinoids that are released into the synaptic cleft and activate pre-synaptic CB_1R. Thus, depending on the neural type where CB_1R is localized, CB_1R activation modulates the release of GABA (Hoffman and Lupica, 2000), and glutamate (Robbe et al., 2001) and other neurotransmitters. This modulation of excitatory and inhibitory terminals regulates the release of different neurotransmitters in the brain. To protect the brain from over-activation of the CB_1R, a novel negative feedback was recently discovered. The binding of Δ^9-THC to CB_1R, triggers the synthesis of the neurosteroid pregnenolone, that binds to a specific site blocking the CB_1R (Vallee et al., 2014).

Non-retrograde signalling:
Endocannabinoids synthetized in post-synaptic neurons signals through CB_1R and TRPV1 channels located in post-synaptic neurons. 2-AG mediates a slow self-inhibition through CB_1R in neocortical GABAergic interneurons and pyramidal neurons (Bacci et al., 2004; Marinelli et al., 2008; 2009). In medial prefrontal cortex an intracellular CB_2R activation in pyramidal neurons produces a self-inhibition (Boon et al., 2012) but wether this autocrine signalling is generalized needs further investigation. Post-synaptic mGluR5 activation is linked to AEA production that through TRPV1 channels (see box___) mediates AMPA endocytosis and LTD in different brain areas (Chávez et al., 2010; Grueter et al., 2010; Puente et al., 2011).

Neuron-Astrocyte communication:
Endocannabinoids mediate neuron-astrocyte communication in tripartite synapses. CB_1R are located in astrocytes in brain and spinal cord. When CB_1R is activated, there is an increase of intracellular Ca^{2+} through PLC activation that leads to glial glutamate release that act on neurons (Navarrete and Araque, 2008; 2010). The importance of astrocytic CB_1R and not CB_1R in GABAergic or glutamatergic terminals explains some of the harmful effect of cannabis as memory impairment (Han et al., 2012).

ECS Physiology:

Through the previous mechanisms and others, the ECS is involved in several physiological proceses in the CNS. As this Thesis focus on motor control and neuroprotection, these two functions will be more deeply developed further in the manuscript.

Memory:
The presence of CB_1R in the hippocampus explains the short-term working memory deficits caused by cannabinoid activity (Lichtman and Martin, 1996). For instance, the glutamate release in the hippocampus required for LTP related to synaptic plasticity can be inhibited in endocannabinoid-mediated LTD (Heifets et al., 2008). The underlying events explaining the CB_1R activation can be explained through different mechanisms, including mTOR signaling (Puighermanal et al., 2009)

Box 4

The ECS outside the CNS:

Outside the central nervous system, cannabinoids are implied in different physiological processes:

-Blood pressure:

Cannabinoids produce a well-known hypotensive response in blood vessels. This effect is produced by Δ^9-THC (Dewey et al., 1970) and by AEA (Lake et al., 1997b). Initially the effect was though to be mediated by CB_1R (Lake et al., 1997a), but due to the persistence of cannabinoid vasorelaxation in $CB_1R^{-/-}$ and $CB_2R^{-/-}$ mice, it was found that transient receptor potential TRPV1 and TRPA1 where also responsive of this cannabinoid effect. The discovery of an endothelial cannabinoid receptor, called abnormal cannabidiol receptor, explains partially the non-CB_1R, non-CB_2R hypotensive effects of cannabinoids (Járai et al., 1999).

-Cancer:

Most of the protective effects due to cannabinoids in non-tumoral cells have been seen in neural cells (see Neuroprotection). In tumoral cells, the ECS is implied in proliferation/cell cycle arrest, induction of apoptosis, angiogenesis and metastatic decisions of tumoral cells. Through CB_1R and CB_2R activation, cannabinoids that protect non-malignant cells can reduce cell viability and have anti-tumoral effects in transformed cells. Cannabinoids limit cell viability and proliferation both *in vitro* and in xenografts implants from glioma, melanoma, linfoma, pancreas, breast or lung cancer cells (Blázquez et al., 2006; Guzmán, 2003; Ligresti et al., 2006). This antitumoral action is exerted through different pathways: activation of ceramide and overactivation of ERK (Galve-Roperh et al., 2000; Melck et al., 1999; Sánchez et al., 2001), inhibition of PI3K/Akt (Sánchez et al., 2003), arresting cell cycle by modulating cdc2 (Caffarel et al., 2006) or activating reticulum stress through mTORC1/Akt axis (Salazar et al., 2009a; 2009b)

and COX-2 activation (Chen et al., 2013).

Anxiety:

CB_1R are found in the central amygdala and the paraventricular nucleus of the hypothalamus, areas involved in emotional and stress responses. In addition, cannabinoids can activate the hypothalamic-pituitary-adrenal axis (HPA), a neuroendocrine system involved in emotional stress. Thus the ECS participates regulating emotional responses. The lack of CB_1R in CB_1 knockout mice has anxiogenic-like behaviour (Martin et al., 2002). This anxiogenic effect was also seen in humans when blocking the CB_1R with rimonabant, a CB_1R antagonist first used as an antiobesity drug under the trade mark of Acomplia®, which had to be withdrawn from market because of its undesired psychiatric effects (Christensen et al., 2007). However, the increase of AEA and 2-AG by the use of specific inhibitors of FAAH and MAGL degrading enzymes are related with anxiolytic effect in mice (Busquets-Garcia et al., 2011; Kathuria et al., 2002). Interestingly, there seems to be a differential role in the action of the two endocannabinoids. The AEA effect seems to be mediated by CB_1R but the 2-AG action seems to be regulated by CB_2R (Busquets-Garcia et al., 2011).

Nociception:

In physiological conditions, nociceptors in primary afferent fibers detect painful stimuli and send noxious inputs to the spinal cord that may be also detected in supraspinal regions. In the brainstem, spinal and supraspinal regions, activation of CB_1R regulate anti-nociceptive effects by inhibiting presynaptic GABA release in pain suppression areas (Vaughan et al., 1999). CB_2R are involved also in spinal antinociceptive reaction (Malan et al., 2001) modulating the release of pro and anti-inflammatory molecules in cells nearby nociceptive neurons (Nackley et al., 2003; 2004). Finally, in paradigms of chronic pain there are increased endocannabinoids levels (Sagar et al., 2010) in spinal cord which supports the idea of a tonic activity of the ECS controlling the nociceptive response after painful stimuli (Rani Sagar et al., 2012).

Feeding

The CNS integrates signals coming from the liver, gastrointestinal tract and adipose tissue in the anterior hypothalamus, where feeding-related hormones such as neuropeptide Y, leptin, oxytocin, melanocortin exert their effect. Several evidences reveal the involvement of the ECS in feeding. AEA and 2-AG levels in hypothalamus increase with fasting and return to basal levels after feeding (Hanus et al., 2003; Kirkham et al., 2002), and activation of CB_1R by AEA or THC induces feeding even in fed animals, an effect blocked by rimonabant (Jamshidi and Taylor, 2001; Williams and Kirkham, 2002). In the hypothalamus, leptin modulates endocannabinoid levels (Di Marzo et al., 2001b). CB_1R activation however has a biphasic effect as cannabinoids can exert hypofagia as well as hyperfagia, and this effect seems to depend on the localization of the CB_1R in GABAergic terminals or glutamatergic terminals respectively (Bellocchio et al., 2010). Supporting this finding is the fact that obesity is related with a dysregulation in the ECS not only in the CNS, but also in liver, pancreas and adipose tissue (see (Matias and Di Marzo, 2007) for review).

Neurodevelopment:

The ECS drives neural differentiation and proliferation during development (Galve-Roperh et al., 2013). The distribution of the different elements of the ECS shifts during brain development from embryonary stages until its final location in the adult brain. In embryonic stem cells and trophoblast stem cells the ECS is fully functional, and the activation of CB_1R blocks the transition to blastocysts. During embrionary development, the CB_2R is highly expressed in neural precursors and promotes neural cell proliferation (Palazuelos et al., 2006; 2012). Afterwards CB_2R levels are reduced whereas the CB_1R has the opposite fate. CB_1R is involved in neural differentiation and regulates corticospinal motor neuron differentiation (Díaz-Alonso et al., 2012).

ECS in motor control:

Motion involves the fine co-ordination of different CNS regions. While the planning of the movement is produced in the motor cortex, selection of appropriate movement resides in the basal ganglia, the cerebellum contributes to motor learning and fine-tuning of the movement, the spinal cord hosts the motor pathways and the neuromuscular joint connects spinal afferences with muscles to produce muscle contraction. The ECS is present and plays a role in all those regions.

Basal ganglia:

The ECS plays a relevant role in the basal ganglia (striatum, globus pallidus, substantia nigra). The activation or blockade of cannabinoid receptors produce alterations in movement control (Romero et al., 2002) derived from the modulation that the SCE has over GABA, dopamine and glutamate release in the basal ganglia (Giuffrida et al., 2000; González et al., 2005). AEA and 2-AG levels are also elevated in the basal ganglia, specially in the globus pallidus and substantia nigra (Giuffrida et al., 2000). The GABAergic CB_1R modulates *in vivo* GABA release in globus pallidus and substantia nigra (Mailleux et al., 1992; Miller and Walker, 1996). Thus, the release or the inhibition in the GABA reuptake (Romero et al., 1998) could explain the hypokinetic effect of cannabinoids (González et al., 2005). The CB_1R activation in glutamatergic terminals from subthalamic projections modulates the activity of pallidal and nigral neurons (Freiman and Szabo, 2005; Sañudo-Peña et al., 1999; Szabo et al., 2000) as well as the inhibition of glutamate release from corticostriatal neurons (Gerdeman and Lovinger, 2001). Despite the presence of CB_2R in the CNS, in particular in neuronal subpopulations, is a controversial issue, recent evidence points that CB_2R are present

in neurons in the basal ganglia, although restricted to the pallidal complex (e.g. pallidothalamic neurons (Lanciego et al., 2011). This neuronal presence of CB_2R in the basal ganglia may occur only in primates (humans included) and it would be independent of the location of CB_2R in activated glial elements present in basal ganglia disorders (reviewed in Fernández-Ruiz et al., 2011).

Cerebellum:

Cerebellum is involved in motor coordination, motor learning and fine-tuning of the movement. Endocannabinoids mediate suppression of inhibitory and excitatory synaptic transmission to Purkinje cells (Diana et al., 2002; Kreitzer and Regehr, 2000; 2001; Yoshida et al., 2002). In the cerebellum there is a high density of CB_1R, specially in the parallel fibers that innervate Purkinje cells (Kawamura et al., 2006) although recent evidence also situates CB_1R in Purkinje cells (Rodríguez-Cueto et al., 2014). These CB_1R regulate synaptic plasticity in the parallel fibers of the cerebellum (Carey et al., 2011; Re et al., 2014). As in the case of the basal ganglia, CB_2R may also be located in cerebellar neurons, into the granular layer and in Purkinje cells (Rodríguez-Cueto et al., 2014).

Spinal cord

The spinal cord plays a role in the execution of the movement. The CB_1R is present in circuits involved in motor control (i.e. ventral horn motor neurons, interneurons and primary afferent cells) as seen in rodents (Hegyi et al., 2009) and primates (Ong and Mackie, 1999). In the spinal cord Δ^9-THC produces changes in motor neuron synaptic transmission (Turkanis and Karler, 1983). In lamprey motor circuitry, mGluR1 activation induces DAGL activation that synthetizes 2-AG. This endocannabinoid activates CB_1R located in pre-synaptic excitatory and inhibitory terminals, producing long term potentiation of excitatory signals and long term depression of inhibitory signals, thus accelerating locomotive behaviour (Kettunen et al., 2005; Kyriakatos and Manira, 2007). In humans the blockade of the CB_1R with rimonabant increases motor system excitability (Oliviero et al., 2012).

Neuromuscular junction:

The ECS is present in rodents and human muscles (Cavuoto et al., 2007; Devane et al., 1988) but little is know about the role of cannabinoids in the neuromuscular junction. In muscular junctions of frogs, cannabinoids inhibit neurotransmitter release through CB_1R (Sánchez-Pastor et al., 2007), and this effect is opposed by TRPV1 channels (Silveira et al., 2010). CB_1R activation also reduces muscular tension in both slow and fast muscle fibres (Huerta et al., 2009; Trujillo et al., 2014).

Neuroprotection:

The ECS is involved in neural cell survival pathways against chronic and acute brain damage. This role turns cannabinoids into multi-target molecules that can act controlling the damage caused by different mechanisms involved both in MS and ALS such as anti-glutamatergic effects, antioxidant effects and modulating glial activation and inflammation. Preclinical studies have demonstrated these properties in ischemia (Sinor et al., 2000), head trauma (Panikashvili et al., 2001), oxidative stress (Marsicano et al., 2002) and excitotoxic models (Marsicano et al., 2003), all present in acute brain injury. Moreover, the manipulation of the ECS has provided successful therapeutic approaches in different models of chronic neurodegenerative diseases e.g. Alzheimer's disease (Bisogno and Di Marzo, 2008), Parkinson's disease (García-Arencibia et al., 2009), Huntington's disease (Pazos et al., 2008), multiple sclerosis (Baker and Pryce, 2008; Pertwee, 2007) and amyotrophic lateral sclerosis (Bilsland and Greensmith, 2008; Carter et al., 2010).

Cannabinoids and excitotoxicity:

The endocannabinoid synthesis is triggered on demand by the increase of intracellular Ca^{2+} due to excessive glutamate activation of NMDA and AMPA channels. The release of cannabinoids activates CB_1R located presynaptically in glutamatergic terminals inhibiting glutamate release and thus reducing excitotoxicity. In the post-synaptic neuron, cannabinoid agonists can block the Ca^{2+} permeability by closing voltage-sensitive channels. The role of the CB_2R is also important, as in certain AMPA activation paradigm, the anti-excitotoxic effect of HU-210, a non selective cannabinoid was blocked both by CB_1R and CB_2R antagonists (Docagne et al., 2007). Finally there are cannabinoids such as the synthetic cannabinoid HU-211 that antagonize the NMDA receptors (Feigenbaum et al., 1989).

Cannabinoids and oxidative stress

The structures of several cannabinoids contain phenolic groups that enable these compounds to act as potent antioxidants. These compounds can act as ROS scavengers, limiting the damage produced by these molecules. CBD is a more potent antioxidant than classic antioxidants such as vitamin-C or tocopherol. Other cannabinoids with antioxidant structures are CBG, Δ^9-THC and synthetic derivates nabilone, levonantradol and dexanabinol. It is generally accepted that these antioxidant effects are CBR-independent and likely related to the particular structure of phytocannabidois, but recent evidence suggests that signalling through PPAR (Sun et al., 2007) and transcription factors such as Nrf-2 (Juknat et al., 2013) may be also involved in those effects.

Cannabinoids and neuroinflammation

Glial cells are the most numerous cells in the CNS. They give metabolic and trophic support to neurons and help maintaining intact the blood-brain-barrier properties. Microglial cells are the resident macrophages in the CNS. In pathological conditions the glial response has two faces as glial cells can release anti-inflammatory factors and trophic factors as well as pro-inflammatory factors and toxic factors to the neurons entourage. Some cannabinoid agonists may be active on those two sides. On one side, they can reduce the production of pro-inflammatory cytokynes (e.g. TNF-α, IL-1β, IL-12, IL-6) and other inflammatory mediators such as NO. On the

Box 5

Cannabis sativa in medicine

Cannabis has been known for millennia for its mind-altering effect, but also for its medicinal use. 2000 years BC, Assyrians called it *ganzi-gun-nu* ("drug that takes away the mind") or *azzalu*. In China it was named *Dà Má* and appears in the classic medical pharmacopeia Ben T'Sao. It was brought to Europe by the Napoleonic troops from Egypt and Middle East. An Irish physician, William Brooke O'Shaughnessy is the first credited MD that introduced cannabis in western medicine. O'Shaughnessy joined the British East India Company and worked in Calcutta in the mid XIX. There he applied the cannabis used in traditional medicine and published some works treating spasticity with cannabis in patients affected with tetanus. Until its prohibition in the USA in 1937, cannabis preparations in tinctures, pills or cigars were sold in pharmacies in Europe and the USA for various purposes such as insomnia, headaches or as a painkiller. With the cannabinoid research era came some drugs that are used in medicine nowadays: Nabilone (Cesamet®) and dronabinol (Marinol®) are synthetic cannabinoids derived from Δ^9-THC structure that are used as anti-emetic agents in treating nausea derived from chemotherapy. Dronabinol is also used to treat anorexia derived from HIV weight-loss. Nabiximols or Sativex® is a 1:1 equimolar THC:CBD mixture used to treat spasticity and neurophatic pain associated with multiple sclerosis or cancer pain. Recently Epidiolex®, a CBD-based cannabis extract, has been developed by GW Pharma to treat infant epileptic syndromes and approved by the FDA in the US for clinical research as an investigational new drug.

other side cannabinoids may also stimulate the release of anti-inflammatory cytokynes and pro-survival molecules (TGF-β, IL-10). This effect is mediated by CB_2R but CB_1R are also involved. The role of CB_2R is particularly important as the expression of this receptor in healthy brain is very low but in pathological conditions it is overexpressed in glial cells enabling this receptor to play a key role mediating the inflammatory response (Fernández-Ruiz et al., 2007).

THE ECS AND MS

The implications of the ECS in MS have been deeply investigated. Clinical evidence supports the use of cannabinoids to treat some MS symptoms such as pain, spasticity, sleep disturbances that can ameliorate the quality of life of MS patients and their caregivers. The ECS suffers changes also in MS, possibly due to adaptative responses aimed at controlling the inflammation and neurodegeneration due to excitotoxic events. Both active and silent MS patients have increased levels of AEA in spinal cord (Eljaschewitsch et al., 2006), in peripheral lymphocytes and cerebrospinal fluid (Centonze et al., 2007). The OEA and PEA levels are also increased as well as AEA in plasma from MS patients, but not 2-AG (Jean-Gilles et al., 2009). Other studies conducted with post-mortem tissue from MS patients have shown that CB1R, CB2R and FAAH immunoreactivity is increased in demyelinating plaques (Benito et al., 2007). The CB_2R was seen in microglial cells, macrophages and astrocytes. In addition increased levels of CB_1R were found in neurons, olygodendrocytes and olygodendrocyte precursor cells (OPCs), macrophages and lymphocytes whereas FAAH was seen in neurons and astrocytes. Some of these changes in the ECS are seen too in experimental MS models, e.g. increased AEA and PEA levels in EAE mice (Baker et al., 2001) and PEA and CB_2R expression levels in TMEV-IDD (Loría et al., 2008). However, some data in MS models is opposite as seen in human samples, for example decreased encodannabinoid levels in rat EAE (Cabranes et al., 2005) although this is a model of acute inflammation rather than a model of chronic damage.

The changes observed in ECS components is MS patients and experimental MS models strongly support that pharmacological manipulation of specific targets within this system may have therapeutic value in MS. In fact, this suspicion was initiated long before the identification of the changes in ECS. Accumulated anecdotal experience showed that patients use marihuana to ameliorate their symptoms. Thus several cannabinoid compounds have shown a beneficial effect in preclinical investigations, from non-selective cannabinoids Δ^9-THC, WIN55,212-2 (Baker et al., 2000; de Lago et al., 2012; Pryce and Baker, 2007), CB_1R selective agonist methanandamide to CB_2R selective HU-308 (Palazuelos et al., 2008). Phytocannabinoids such as CBG are also effective delaying the progression of TMEV in mice (Granja et al., 2012).

The positive effects found in preclinical studies led to the development of clinical trials (Rog, 2010). Although the massive study Cannabinoids in Multiple Sclerosis (CAMS) showed promising results for Δ^9-THC (Zajicek et al., 2005), the more recent Cannabinoids Use in Progressive Inflammatory brain Disease (CUPID) study showed no effect in delaying the progressive forms of MS versus a placebo (Zajicek et al., 2013), although some individuals with reduced EDSS scores could benefit from Δ^9-THC use.

THE ECS AND ALS

As mentioned before, the pathogenic events linked to ALS include excitotoxicity, oxidati-

ve stress, microglial activation, neuroinflammation and protein aggregation. The ECS modulates all these processes. The endocannabinoids 2-AG and AEA are increased in the SOD1^{G93A} transgenic mice (Witting et al., 2004). The CB_2R expression is also increased when disease progresses in transgenic SOD1^{G93A} mice (Shoemaker et al., 2007). The CB2R is also increased in microglial cells as seen in post-mortem tissue from ALS patients (Yiangou et al., 2006). Not only expression levels of cannabinoid receptors seem to play a role in ALS. It has been shown that the sensitivity of the CB_1R could be potentiated in the SOD1^{G93A} model (Rossi et al., 2010). Besides, the CB_1R may play a dual role in ALS, as the deletion of CB_1R increase survival in SOD1^{G93A} transgenic mice (Bilsland et al., 2006). Different cannabinoids have revealed effectiveness delaying the progression of the disease in SOD1^{G93A} transgenic mice. The phytocannabinoids CBN and Δ^9-THC delayed symptom onset when injected before the first symptoms appear (Raman et al., 2004; Weydt et al., 2005). Cannabinoid agonist Δ^9-THC (Raman et al., 2004), WIN55,212-2 (Bilsland et al., 2006; Shoemaker et al., 2007) and the selective CB_2R agonist AM1241 (Kim et al., 2006; Shoemaker et al., 2007) also delayed disease progression, administered once the first symptoms of the disease appear, which reflects its use in clinics as ALS patients are treated once they are diagnosed. Other parameters e.g. motor neuron survival also improved with either endocannabinoid tone enhancing in SOD-1 FAAH$^{-/-}$ mice or WIN 55,212-2 treatment (Bilsland et al., 2006). The depletion of CB_1R, although not delaying symptom onset, also increased survival in SOD1^{G93A} mice (Bilsland et al., 2006).

There are trials in ALS patients testing the pharmacokinetics of a single dose of Δ^9-THC (Joerger et al., 2012) and also the use of Δ^9-THC to treat muscle cramps (Weber et al., 2010). Although Δ^9-THC was well tolerated, the results showed no improvement, albeit the authors do not discard that higher doses might have a beneficial effect (Weber et al., 2010).

AIMS
Objetivos

This Thesis aims to study the potential applicability of cannabinoids in the treatment of MS and ALS. As it has been described in the Introduction, the ECS has been studied in MS and ALS in relation with the contribution that a possible dysregulation of this system in the pathogenesis of both diseases, as well as its relation with its potential to develop novel disease-modifying therapies. Despite significant advances reviewed in the Introduction, different aspects are still sensible of additional investigation. An open question in MS is to elucidate the involvement of CB_1R and/or CB_2R in disease-modifying effects of cannabinoids, as well as the evaluation of phytocannabinoids combination as disease-modifying agents. In the case of ALS, it is necessary to identify the changes that the disease causes in different ECS elements, especially for the endocannabinoid synthesis and degradation enzymes as well as to evaluate phytocannabinoid combinations as potential novel disease-modifying therapies.
To achieve these aims we have used different *in vitro* and *in vivo* approaches. Thus, the following specific objectives are proposed:

1- Study of the mechanisms underlying the amelioration in disease progression caused by WIN 55,212-2 and the contribution of CB_1R and CB_2R in EAE in mice.

2- Study of the use of the Δ^9-THC-BDS, CBD-BDS and the Sativex-like combination of both phytocannabinoids as disease modifying agents in two models of MS, the TMEV-IDD and EAE.

3- Characterization of the ECS both in the motor neuron cell line NSC-34 and in $SOD1^{G93A}$ transgenic mice and study of the use of a Sativex-like combination of Δ^9-THC-BDS and CBD-BDS as a disease modifying agent.

4- Study of the blockade of cannabinoid receptors in a zebrafish model of ALS.

RESULTS
Resultados

Objective #1

Study of the mechanisms underlying the amelioration in disease progression caused by WIN 55,212-2 and the contribution of CB_1R and CB_2R in EAE in mice.

In this chapter we evaluate the effect of a chronic treatment with 5 mg/kg WIN 55,212-2 in the EAE mouse model. As it was previously described, WIN 55,212-2 improves the clinical decline of the affected animals, but now we focused on the mechanisms underlying the activity of WIN 55,212-2. In particular we paid emphasis in the possible role of CB_1R and CB_2R as well as in the relevance of these receptors in controlling excitotoxic and inflammatory responses. Thus, in different sets of experiments we treated EAE animals with 5 mg/kg WIN 55,212-2 or both 5 mg/kg WIN 55,212-2 and 5 mg/kg of the CB_1R antagonist SR141716 or 5 mg/kg of the CB_2R antagonist AM630 to check whether the effects of WIN 55,212-2 were mediated by CB_1R or CB_2R. We also tried to reproduce the same effect as WIN 55,212-2 with the selective CB_1R agonist ACEA and the selective CB_2R HU-308.

To determine the effect of WIN 55,212-2 on excitotoxic events we used HPLC measurements of GABA and glutamate in spinal cord and brainstem. We measured by RT-PCR the expression of the glutamate transporters GLAST and GLT-1. To determine the effects of WIN 55,212-2 on inflammatory responses we analysed spinal cord section with Nissl's stainings and glial markers immunohistochemistry i.e. IBA-1 and CD11b.

WIN 55,212-2 treated animals had better neurological scored than vehicle treated animals. This effect was reflected in spinal cord sections, with reduced cell infiltrates and less microglial activation. Our results showed no changes in GABA or glutamate levels in spinal cord or brainstem after WIN 55,212-2 treatment. On the other hand we found increased expression levels of the glutamate transporters GLT-1 and GLAST in spinal cord and reduced expression of COX-2, iNOS and TNFα in WIN 55,212-2 treated animals. In our hands, this effect is mediated by the CB_1R, since WIN 55,212-2 and SR141716 treated animals showed a worse neurological score and the same infiltration level and microglial activation than vehicle treated animals. In our hands we could not reproduce the same effect with the selective CB_1R agonist ACEA or the selective CB_2R agonist HU-308.

CONCLUSION:

WIN 55,212-2 exerts anti-glutamatergic and
anti-inflammatory effects through CB_1R in the initial stages of EAE in mice.

Papers in this chapter:

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Cannabinoids ameliorate disease progression in a model of multiple sclerosis in mice, acting preferentially through CB_1 receptor-mediated anti-inflammatory effects

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ABSTRACT

Multiple sclerosis (MS) is an autoimmune disease that affects the CNS and it is characterized by inflammation, demyelination, remyelination, gliosis and axonal damage that occur mainly in the spinal cord. Cannabinoids have been proposed as promising therapeutic agents in MS given their capability to alleviate specific MS symptoms (e.g., spasticity, pain). Although MS has been considered mainly an inflammatory disorder, recent evidence, however, revealed the importance of neurodegenerative events, opening the possibility that cannabinoid agonists, given their cytoprotective properties, may also serve to reduce oligodendrocyte death and axonal damage in MS. Thus, the treatment with WIN55,512-2, a potent CB_1 and CB_2 agonist, was reported to be effective to ameliorate tremor and spasticity in mice with chronic relapsing experimental autoimmune encephalomyelitis, a murine model of MS, but also to delay disease progression in this and other murine models of MS. The purpose of this investigation was to further explore the mechanism(s) underlying the amelioration in disease progression caused by WIN55,212-2. We have particularly focused on anti-glutamatergic and anti-inflammatory effects of this cannabinoid agonist. In this study, we used mice treated with myelin oligodendrocyte glycoprotein (MOG) that induces a progressive pattern of EAE and conducted the pharmacological experiments in early stages of the disease. As expected, the administration of WIN55,512-2 (5 mg/kg, i.p) had a positive effect in reducing neurological disability and improving motor coordination of EAE mice. Levels of glutamate and GABA in the spinal cord and also in the brainstem of EAE mice were similar to control animals, and, accordingly, they were not altered by the treatment with WIN55,212-2. However, EAE mice showed some subtle alterations in mRNA levels for the glutamate transporter GLT1 and, to a lesser extent, GLAST too, changes that were altered by the treatment with WIN55,212-2 in the spinal cord, but not in the brainstem. Regarding to inflammatory responses, EAE mice showed a marked up-regulation in mRNA levels for COX-2, inducible NOS and TNF-α in the spinal cord and the brainstem, these responses being attenuated after the treatment with WIN55,212-2. We also observed the presence of cell aggregates in the spinal cord of EAE mice that were significantly attenuated by the treatment with WIN55,212-2. Immunohistochemical analysis (with Iba-1 and Cd11b) of these aggregates indicated that they corresponded to microglia (resident macrophages) and peripheral macrophages. Lastly, experiments conducted with selective antagonists for the CB_1 (e.g. rimonabant) or CB_2 (e.g. AM-630) receptors revealed that WIN55,212-2 effects in EAE mice were mediated by the activation of CB_1 but not CB_2 receptors, as reflected the reversion of positive effects of this cannabinoid on neurological decline, TNF-α generation and accumulation of cell aggregates in the spinal cord with rimonabant, but not with AM-630. This was

Abbreviations: CFA, complete Freund's adjuvant; CNS, Central Nervous System; COX-2, cyclooxygenase-2; CREAE, chronic relapsing experimental autoimmune encephalomyelitis; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; GLAST, glutamate/asparte transporter; GLT1, glutamate transporter-1; iNOS, inducible nitric oxide synthase; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PBS, phosphate-buffered saline; TMEV, Theiler's murine encephalomyelitis virus; TNF-α, tumor necrosis factor-α.

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[1] *In memoriam* of Ana Cabranes who dreamed the results obtained in these studies, but who unexpectedly died before being all her work finished and published.

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concordant with the lack of positive effects on neurological decline observed in EAE mice when they received HU-308, a selective CB_2 receptor agonist, instead WIN55,212-2. In summary, the treatment of EAE mice with the cannabinoid agonist WIN55,512-2 reduced their neurological disability and the progression of the disease. This effect was exerted through the activation of CB_1 receptors, which would exert a positive influence in the reduction of inflammatory events linked to the pathogenesis of this disease.

1. Introduction

After many years of continuous research, it is becoming more evident that cannabinoid compounds and the endocannabinoid signaling system offer a promising novel form of therapy for multiple sclerosis (MS) that already covers the treatment of specific symptoms (e.g. spasticity, pain; see Rog, 2010, for review), but that might be extended in the future to the control of disease progression (reviewed in de Lago et al., 2010). The first link between MS and cannabinoids derives from anecdotal, uncontrolled or preclinical data generated in the early 90s, which indicated that some MS patients frequently self-medicated with cannabis to alleviate spasticity, dystonia, tremor, ataxia, pain and other MS symptoms (for review, see Consroe et al., 1997; British Medical Association Report, 1997; Pertwee, 2002). Trying to reproduce these beneficial effects in experimental models of MS, Baker and coworkers developed during the present decade a series of pharmacological studies with different types of cannabinoid agonists that always resulted highly effective in reducing spasticity in the chronic relapsing form of experimental autoimmune encephalomyelitis (CREAE) in mice (Baker et al., 2000, 2001; Brooks et al., 2002; de Lago et al., 2004, 2006; Pryce and Baker, 2007; reviewed in Baker and Pryce, 2008). Parallel studies by other authors in this and other MS models also demonstrated efficacy of cannabinoid agonists on other symptoms (reviewed in Pertwee, 2007) or proved immunomodulatory/anti-inflammatory properties for these compounds in MS (Cabranes et al., 2005; Baker et al., 2007; Kubajewska and Constantinescu, 2010). This solid pharmacological evidence has sustained the development of various clinical studies aimed at determining the efficacy of different cannabinoid-based medicines for the treatment of MS symptoms (e.g. spasticity, pain, sleep disturbances) in patients, in general with positive results (Zajicek et al., 2003, 2005; Vaney et al., 2004; Rog et al., 2005; Barnes, 2006; Wade et al., 2006; Collin et al., 2007, 2010; reviewed in Lakhan and Rowland, 2009; Rog, 2010), facilitating the recent approval of the oromucosal spray Sativex®, which contains equimolecular combinations of botanical extracts enriched in Δ^9-THC and cannabidiol (Wright, 2007), for the treatment of spasticity in MS in different countries (Kmietowicz, 2010).

Relief of symptoms in MS by cannabinoids has been reported to be mostly mediated by the activation of CB_1 receptors (Pryce and Baker, 2007), which have been reported to be altered in some CNS structures in CREAE mice (Cabranes et al., 2006) or in post-mortem tissue from MS patients (Benito et al., 2007). Anandamide has been also found to be elevated in active MS lesions (Eljaschewitsch et al., 2006), lymphocytes and CSF samples (Centonze et al., 2007), or in plasma (Jean-Gilles et al., 2009) of MS patients, in concordance with the increase found in the spinal cord and, to a lesser extent, in the brain, of CREAE mice during the spastic phase of disease (Baker et al., 2001). In contrast, other authors found lowered endocannabinoid levels in the CSF of MS patients compared to control subjects, although elevations were found during relapsing periods (Di Filippo et al., 2008). In general, changes reported for the cannabinoid signaling system in patients and different MS models have been interpreted as adaptative responses aimed at limiting neuronal damage (reviewed by de Lago et al., 2010). On one hand, the evidence of higher levels of endocannabinoids or cannabinoid receptors suggests a contribution in limiting excessive excitatory neurotransmission and the ongoing inflammatory process that have potential neuroprotective implications (Centonze et al., 2007). On the other hand, lower endocannabinoid levels, mainly 2-arachidonoylglycerol, occurring during a neuroimmunological attack have been related to the molecular mechanisms that control endocannabinoid generation by microglia (Witting et al., 2006). Irrespective of this interpretation, the changes elicited in the cannabinoid signaling system in CNS structures during the progression of MS support the idea that the pharmacological management of this system may be beneficial in this disease, not only for specific symptoms, but also for the control of disease progression, a fact based on the well-recognized properties of cannabinoid compounds as cytoprotective agents (reviewed in Fernández-Ruiz et al., 2007, 2010). In this respect, a new clinical trial, named Cannabinoid Use in Progressive Inflammatory Brain Disease (CUPID; see http://sites.pcmd.ac.uk/cnrg/cupid.php) is presently being developed with 493 patients with progressive MS to validate the possibility that cannabinoids may be also used as a disease-modifying agent to slowdown MS progression. The marked anti-inflammatory properties of cannabinoid compounds demonstrated in preclinical studies support this possibility (reviewed in Fernández-Ruiz et al., 2005, 2010). These properties have been related to those compounds that selectively target the CB_2 receptor located in glial cells that are recruited and activated in response to inflammatory but also infectious, traumatic or excitotoxic stimuli and that, upon activation, express CB_2 receptors (reviewed in Fernández-Ruiz et al., 2005, 2007 and 2010). Anti-inflammatory/neuroprotective effects can be also reached with inhibitors of the cellular uptake of endocannabinoids, for example UCM707, OMDM1 and OMDM2, which diminished neuroinflammation in parallel to decreased microglial reactivity in the model of MS generated by infection with Theiler's murine encephalomyelitis virus (TMEV; Ortega-Gutiérrez et al., 2005; Mestre et al., 2005). It appears reasonable that these uptake inhibitors are enhancing the action of endocannabinoids at the CB_2 receptor, so that this receptor might represent a new and promising therapeutic target in MS (reviewed in Docagne et al., 2008) able to control inflammation and then providing protection against glial-derived cytotoxic mediators.

However, cannabinoids may also exert significant neuroprotective effects in MS independently from the CB_2 receptor-mediated immunomodulatory and/or anti-inflammatory actions (Croxford et al., 2008). This possibility was described for the first time in 2003 by Baker's group who found that induction of CREAE was more intense in CB_1 knockout mice compared to wild-type animals (Pryce et al., 2003). In a further study (Jackson et al., 2005), the same authors showed greater neuronal/axonal loss and demyelination when CREAE was induced in CB_1 knockout animals and how these responses were accompanied by an intense activation of caspase 3. *In vitro* experiments have also confirmed that neuronal damage is greater in absence of the CB_1 receptor (Jackson et al., 2004). These data suggest that the CB_1 receptor

represents another key target for providing neuroprotection with cannabinoids in the CREAE model of MS. Other authors also described benefits for CB_1 receptor agonists against the neurological deficits in other MS models (Arévalo-Martin et al., 2003; Croxford and Miller, 2003). It is important to remark that this neuroprotective effect appears to be exerted independently of the other properties of cannabinoids, since low doses of WIN55,512, which have no anti-inflammatory potential, were able to reduce axonal loss in the spinal cord and improve the clinical score in the EAE model (Croxford et al., 2008). In this context, the greater vulnerability of CB_1 knockout mice to the induction of CREAE (Pryce et al., 2003; Jackson et al., 2005) has been related to the role played by this receptor in regulating glutamate homeostasis, as suggest the fact that CREAE-CB_1 knockout mice were more vulnerable to excitotoxic insults (Pryce et al., 2003). It is well-known that glutamate is a key mediator in neuronal and oligodendrocyte damage in MS, as has been found in studies with patients and also in experimental models (reviewed in Gonsette, 2008), and that CB_1 receptor agonists exert direct neuroprotective effects by limiting glutamate release and excitotoxic damage in several neurodegenerative disorders (reviewed in Fernández-Ruiz et al., 2010). Therefore, CB_1 receptor agonists might also be useful against excitotoxic death of oligodendrocytes and, subsequently, neurons in MS, as it has been shown in various *in vitro* and *in vivo* studies (Docagne et al., 2007; Loría et al., 2010; reviewed in Fernández-Ruiz et al., 2010). This means that, in addition to the potential of CB_2 receptors in MS described before, CB_1 receptors may also represent an important protective target able to improve glutamate homeostasis and its influence in the progression of MS. These antiexcitotoxic properties of cannabinoids in MS should be investigated more extensively in years to come.

In the present study, we wanted to explore whether a chronic treatment with the non-selective cannabinoid agonist WIN55,212-2, which already showed neuroprotective effects in MS models (see Croxford et al., 2008; de Lago et al., 2010, for recent reviews), may be acting through a CB_1 receptor-mediated improvement of glutamate anomalies described in various CNS structures in MS. We used the model of chronic progressive EAE induced in mice by inoculation of myelin oligodendrocyte glycoprotein (MOG). However, our experiments proved that beneficial effects of WIN55,212-2 treatment on neurological decline in these animals were not related, in general, to changes in glutamate transmission, so that we conducted additional experimentation centered in the anti-inflammatory/neuroprotective effects of WIN55,212-2 that proved positive results. However, contrarily to the expectations, these effects were mediated by CB_1 but not by CB_2 receptors, as reflected in experiments with selective antagonists for both receptor types, or with a selective CB_2 receptor agonist.

2. Materials and methods

2.1. Animals, treatments and sampling

Female C57BL/6 mice were purchased from Charles River Laboratories (Barcelona, Spain) and housed in our animal facilities in a room with controlled photoperiod (12 h light/dark cycle), temperature (20 ± 1 °C) and relative humidity (40–60%). They had free access to standard food and water. They were used at adult age (6–8 week old) for all the experiments, which were performed according to European regulations for experimental work with animals (directive 86/609/EEC). EAE was induced using the method published by Mendel et al. (1995). This method consists of one subcutaneous injection in each flank of an emulsion containing 200 μg of the 35-55 portion of MOG (Advanced Biotechnology Centre, Imperial College, London, UK) and 4 mg/ml of *Mycobacterium tuberculosis* (H37RA DIFCO Lab, Detroit, MI, USA) in a 1:1 mix with incomplete Freund's adjuvant (Sigma/Aldrich, Madrid, Spain) and phosphate-buffered saline (PBS). This injection was repeated after 7 days. For exacerbating the inflammatory response, mice also received 1.5 μg/ml of Pertussis toxin (Sigma/Aldrich, Madrid, Spain) prepared in saline and administered i.p. on days 0 and 2. Control animals were obtained by inoculation with the same emulsion (complete Freund's adjuvant, CFA) without MOG and without injections of Pertussis toxin. After inoculation, mice were examined daily for the presence of neurological signs, using the following scale: 0, no clinical signs; 1, limp tail; 2, hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; and 5, moribundity or death. Daily weight loss was also recorded. Following this method of EAE induction, we noticed that first clinical signs appeared around day 11–12 after inoculation showing a worsening pattern compared to control animals inoculated with CFA (see Fig. 1) that progresses up to day 16 post-inoculation. The magnitude of the neurological decline found at this post-inoculation time in our animals reached values similar to those described in previous studies by other authors (Balabanov et al., 2007; Basso et al., 2008; Aharonowiz et al., 2008). Therefore, pharmacological experiments were initiated at day 11 post-inoculation and consisted of daily injections of WIN55,212-2 (5 mg/kg weight; purchased from Tocris Cookson Ltd. Bristol, UK) or vehicle (experiment I), or combinations of WIN55,212-2 (5 mg/kg weight) with the selective CB_1 receptor antagonist SR141716 (5 mg/kg weight; kindly provided by Sanofi-Aventis, Montpellier, France) or the CB_2 receptor antagonist AM-630 (5 mg/kg weight; purchased from Tocris Cookson Ltd. Bristol, UK) (experiment II). In an additional experiment aimed at further exploring the role of CB_2 receptors, EAE mice were treated with HU-308 (5 mg/kg weight; purchased from Tocris Cookson Ltd. Bristol, UK), a selective agonist for this receptor, or vehicle, following the same schedule than in the previous experiments. On the other hand, as previous studies (Shibaki and Katz, 2002) described adjuvant-induced activation of inflammation, naïve animals not inoculated with CFA (results in the remaining treatments were always expressed as % over this group for all measures), as well as CFA-inoculated animals injected with WIN55,212-2, were used as controls for most of the experiments but their results were not shown in figures. The neurological status of all animals was evaluated by researchers blinded to the treatment corresponding to each animal. Immediately after neurological evaluation at the day 16, animals were perfused with cold PBS and

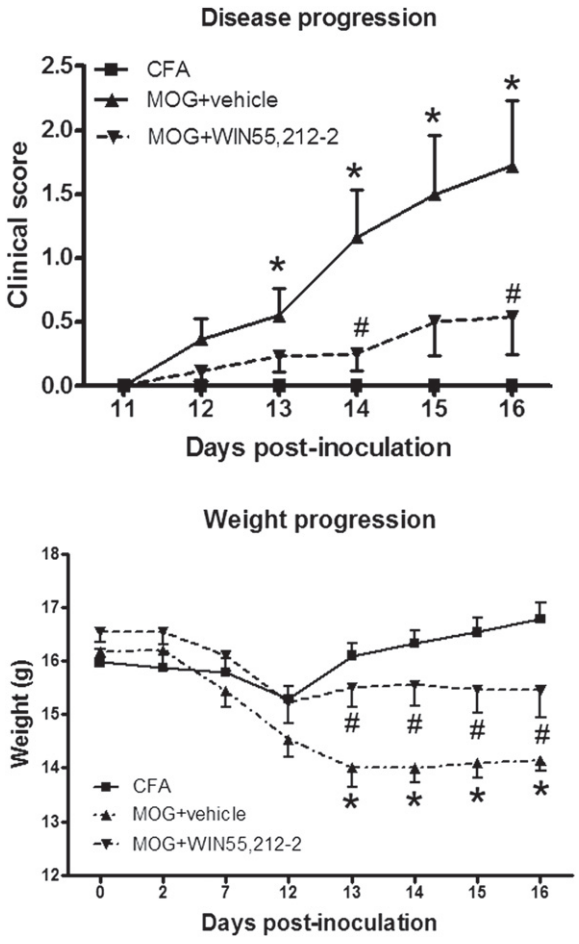

Fig. 1. Effects of a chronic treatment with WIN55,212-2 or vehicle (initiated at day 11 post-inoculation) on neurological status and weight of mice with EAE induced by inoculation of MOG (day 0) and the corresponding controls (exposed to CFA). Details in the text. Values are means ± SEM of 7–8 subjects *per* group. Data were assessed by two-way analysis of variance followed by the Student-Newman-Keuls test (*$p < 0.05$ *versus* CFA, #$p < 0.05$ *versus* MOG + vehicle).

their brains and spinal cords were removed and rapidly frozen by immersion in 2-methylbutane cooled in dry-ice. Samples were stored at −80 °C until used for HPLC or qRT-PCR analysis ($n = 7–8$ subjects *per* experimental group). In a few cases, spinal cords were fixed in 4% paraformaldehyde overnight followed by a cryoprotective treatment with 20% sucrose, and they were used for histological analyses ($n = 4–5$ *per* experimental group).

2.2. HPLC determination of GABA and glutamate

Dissected brain structures (spinal cord and brainstem) were homogenized in 20–40 vol of cold 150 mM potassium phosphate buffer (pH 6.8) and each homogenate was used to analyze GABA and glutamate contents, as described below. An aliquot of each homogenate was used to determine the protein concentration (Lowry et al., 1951). The GABA and glutamate content was analyzed by HPLC with electrochemical detection according to methods previously published (Cabranes et al., 2005; de Lago et al., 2006). Homogenates were diluted with 0.4 N perchloric acid containing 0.4 mM sodium disulfite, 0.90 mM EDTA and 5 μg/ml β-aminobutyrate (BABA) as an internal standard. Afterward, samples were centrifuged for 3 min (15,000 *g*) and 50 μl of each supernatant was removed and neutralized with 100 μl of 0.1 N NaOH. The samples were then stored at 4 °C until they were analyzed. The analysis included the derivatization of glutamate, GABA and BABA by adding 15 μl of o-phthaldehyde (OPA)-sulfite (14.9 mM OPA, 45.4 mM sodium sulfite and 4.5% ethanol in 327 mM borate buffer, pH 10.4) and allowing the samples to react at room temperature for 10 min. Then, 20 μl of each reaction mixture (including derivatizated calibration standards composed of known concentrations of glutamate, GABA and BABA) were injected into the HPLC system. This system consisted of a Spectra-Physics 8810 pump and the column was a RP-18 (Tracer Excel 120 ODSB; 150 mm, 4.6 mm, 5 μm particle size; Teknokroma, Barcelona, Spain). The mobile phase, previously filtered and degassed, consisted of 0.06 M sodium dihydrogen phosphate, 0.06 mM EDTA and methanol (30% for the analysis of GABA and 5% for the analysis of glutamate, pH 4.4), at a flow rate of 0.8 ml/min. The effluent was monitored with a Metrohm bioanalytical amperometric detector, using a glass carbon electrode with a 0.85 V potential relative to an Ag/AgCl reference electrode with a sensitivity of 50 nA (approx. 2 ng *per* sample). The signal was recorded on a Spectra-Physics 4290 integrator and the results obtained from the peaks were calculated by comparison with the area under the corresponding internal standard peak. Values were expressed as μg/mg of protein.

2.3. Histological analyses

Spinal cords fixed in 4% paraformaldehyde and cryoprotected in 20% sucrose were sliced (20 μm thick) with a cryostat and collected on TESPA-coated slides. Slides were used to determine the presence of cell aggregates using Nissl staining (see details in Alvarez et al., 2008), as well as the characteristics of these cells using immunohistochemical analysis with Iba-1, a marker of resident microglia, and with Cd11b, a marker of reactive macrophages. In this case, sections were incubated overnight at 4 °C with: (i) monoclonal anti-rabbit Iba-1 (Wako Pure Chemical Industries Ltd., Osaka, Japan) used at 1/300, or (ii) monoclonal anti-mouse Cd11b antibody (AbD Serotec, Oxford, UK) used at 1:150. After incubation with the corresponding primary antibody, sections were washed in 0.1 M PBS and incubated for 2 h at room temperature with the appropriate biotin-conjugated anti-rat (1:500; Millipore, Temecula, CA, USA) or biotin-conjugated anti-rabbit (1:300; Sigma/Aldrich, Madrid, Spain) secondary antibodies. Reaction was revealed with the Vectastain® Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Negative control sections were obtained using the same protocol with omission of the primary antibody. All sections for each immunohistochemical procedure were processed at the same time and under the same conditions. A Nikon Eclipse 90i microscope and a Nikon DXM 1200F camera were used for slide observation and photography, and all image processing was done using ImageJ, the software developed and freely distributed by the US National Institutes of Health (Bethesda. MD, USA).

2.4. Real-time qRT-PCR analysis

After PBS perfusion, total RNA was isolated from spinal cords, brainstem and other structures using TRI Reagent® RNA isolation reagent (Sigma-Aldrich) with modification in the protocol for structures with high contents of lipids. The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity from the ratio between the absorbance values at 260 and 280 nm. Its integrity was confirmed in agarose gels. After genomic DNA was removed (to eliminate DNA contamination), single-stranded complementary DNA was synthesized from 1 μg of total RNA using a commercial kit (Rneasy Mini Quantitect Reverse Transcription, Qiagen, Izasa, Madrid, Spain). Quantitative real-time PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) to quantify mRNA levels for inducible nitric oxide synthase (iNOS; NM_010927.3), cyclooxygenase-2 (COX-2; NM_011198.3), tumor necrosis factor-α (TNF-α; NM_013693.2), or SyberGreen for glutamate transporter-1 (GLT1; sense: 5′-GGAAGATGGGTGAACAGGC-3′ and antisense: 5′-TTCCCACAAATCAAGCAGG-3′) and glutamate/aspartate transporter (GLAST; sense: 5′-ACGGTCACTGCTGTC ATTG-3′ and antisense: 5′-TGTGACGAGACTGGAGATGA-3′) (Pawlak et al., 2005; Loría et al., 2010) using GAPDH expression with Taqman (NM_008084.2) or SyberGreen (sense: 5′-TGTGATGGGTGTGAACCACGAGAA-3′ and antisense: 5′-GAGCCCTTCCACAATGCCAAAGTT-3′; Schroder et al., 2009), respectively, as an endogenous control gene for normalization. Each sample was assayed in duplicate and suitable control samples were run for each set of RNA extractions. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the threshold cycle was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA, USA).

2.5. Statistics

All data were subjected to one- or two-way (treatment × time) analysis of variance followed by the Student–Newman–Keuls test.

3. Results

3.1. Effects of WIN55,212-2 treatment on neurological status and neuropathological markers of EAE mice

In this study, we used mice treated with MOG that generates a progressive pattern of EAE induction with neurological disabilities that start at 12 days post-inoculation and progress during the following days (time: $F(5,188) = 7.21$, $p < 0.0001$; see Fig. 1). Control animals (CFA-treated) exhibited no neurological decline at all days examined (Fig. 1). As mentioned above, the magnitude of this neurological decline had the same extent than the data reported by other authors in previous studies (Balabanov et al., 2007; Basso et al., 2008; Aharonowiz et al., 2008). A similar pattern was found for weight loss that also starts at 12 days post-inoculation, although it becomes statistically significant only after day 13 (time: $F(7,279) = 6.872$, $p < 0.0001$; see Fig. 1). We conducted the pharmacological experiments with WIN55,212-2 in an early stage of the disease, administering the first dose at 11 days post-inoculation. As expected, the administration of WIN55,512-2 had a positive effect in reducing neurological disability and improving motor coordination of EAE mice (treatment: $F(2,188) = 31.38$, $p < 0.0001$; see Fig. 1). The same positive effects occurred in relation with the weight loss after the treatment of EAE mice with WIN55,212-2 (treatment: $F(2,279) = 33.66$, $p < 0.0001$; see Fig. 1).

Levels of glutamate and GABA in the spinal cord and also in the brainstem of EAE mice were similar to control animals, and, accordingly, they were not altered by the treatment with WIN55,212-2 (Fig. 2). However, EAE mice showed some subtle alterations in mRNA levels for the glutamate transporter GLT1 and, to a lesser extent, GLAST too. For example, GLT1 showed trends toward a decrease in the spinal cords and the brainstem of EAE mice, whereas the opposite was found for GLAST (Fig. 2). In any case, these changes did not reach statistical significance, but the treatment with WIN55,212-2 strongly elevated the low levels of GLT1 observed after MOG inoculation in the spinal cord ($F(2,17) = 12.10$, $p < 0.001$; see Fig. 2) and enhanced the effects of MOG on GLAST in the same structure ($F(2,14) = 9.06$, $p < 0.005$; see Fig. 2). These responses were not visible in the brainstem (Fig. 2). On the other hand, we did not find any significant differences between CFA-treated (used as controls in the figures) and naïve (data not shown, but used to determine 100% level in the figures) animals, thus indicating that adjuvant alone has no relevant effect by itself. Other areas studied for both GABA and glutamate concentrations and GLT1- and GLAST-mRNA levels did not show, as expected, any differences by MOG inoculation and/or WIN55,212-2 treatment (data not shown).

As regards to inflammatory responses, EAE mice showed a marked up-regulation in mRNA levels for various proinflammatory markers, such as COX-2 ($F(2,15) = 226.1$, $p < 0.0001$; see Fig. 3), inducible NOS ($F(2,17) = 517.5$, $p < 0.0001$; see Fig. 3) and TNF-α ($F(2,16) = 611$, $p < 0.0001$; see Fig. 3) in the spinal cord, responses that were all attenuated after the treatment with

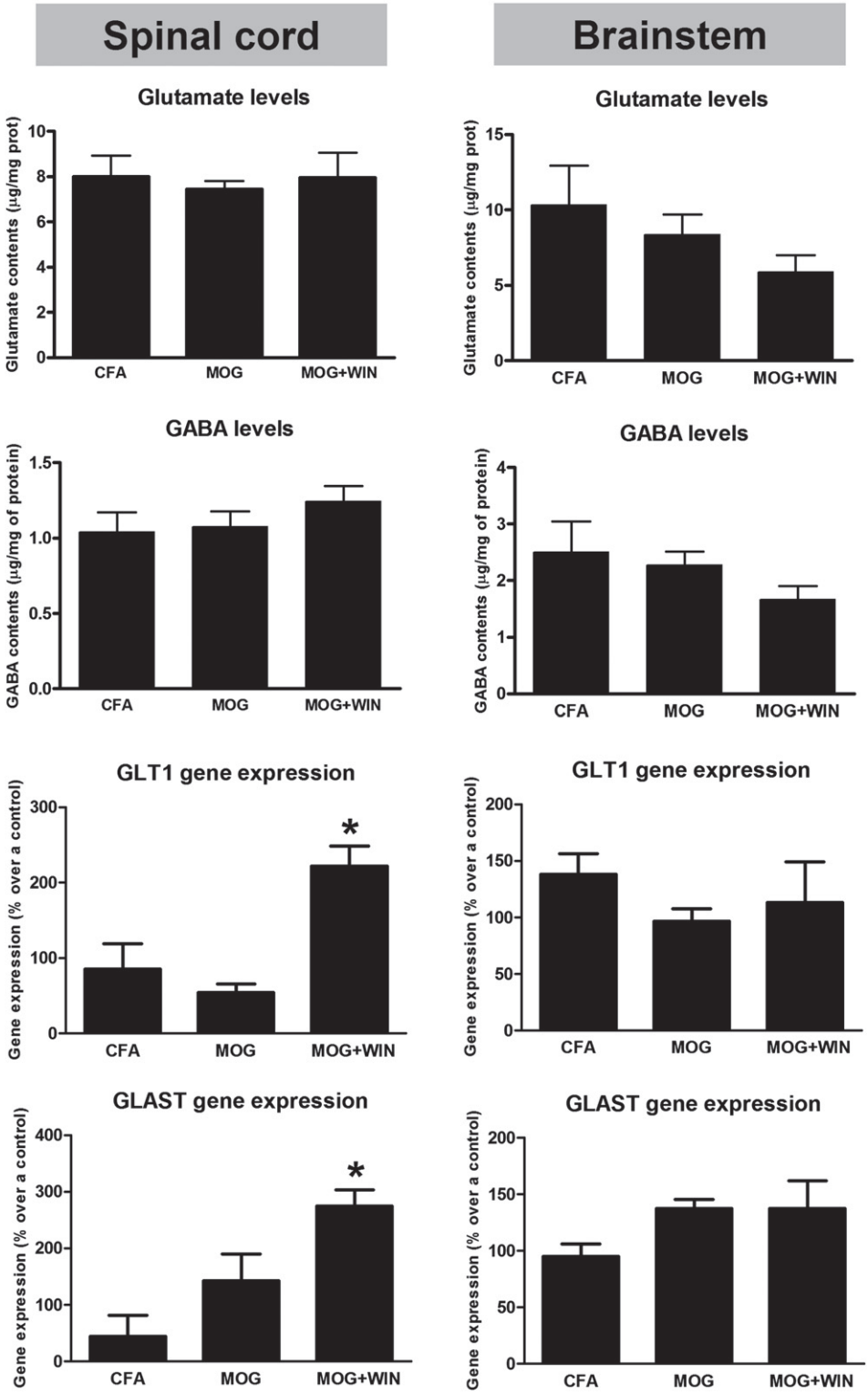

Fig. 2. Effects of a chronic treatment with WIN55,212-2 or vehicle (initiated at day 11 post-inoculation) on glutamate and GABA contents and GLT1 and GLAST mRNA levels in the spinal cord and the brainstem of mice with EAE induced by inoculation of MOG and the corresponding controls (exposed to CFA). Details in the text. Values correspond to animals at the day 16 post-inoculation and are µg/mg of protein for glutamate and GABA contents and % over control (naïve) animals for GLT1 and GLAST mRNA levels. They are expressed as means ± SEM of 7–8 subjects *per* group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (*$p < 0.05$ *versus* the other two groups).

WIN55,212-2, particularly in the case of iNOS and, to a lesser extent, TNF-α. Equivalent responses were found in the brainstem, although statistical significance was only reached in the case of iNOS ($F(2,14) = 316.6$, $p < 0.0001$; see Fig. 3) and TNF-α ($F(2,14) = 857.8$, $p < 0.0001$; see Fig. 3), whereas the pattern of COX-2, although relatively similar to the case of the spinal cord, did not reach statistical significance. It should be also remarked that, as for GABA and glutamate parameters, we did not find any significant

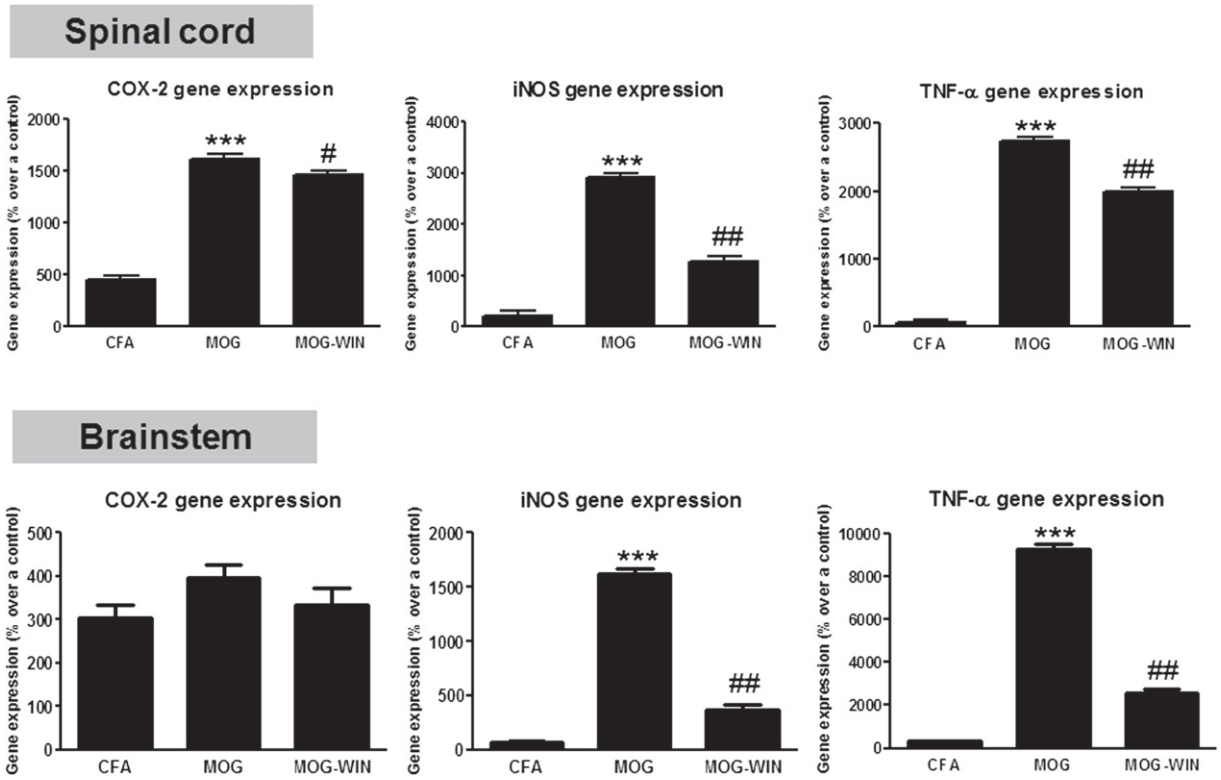

Fig. 3. Effects of a chronic treatment with WIN55,212-2 or vehicle (initiated at day 11 post-inoculation) on COX-2, inducible NOS and TNF-α mRNA levels in the spinal cord and the brainstem of mice with EAE induced by inoculation of MOG and the corresponding controls (exposed to CFA). Details in the text. Values correspond to animals at the day 16 post-inoculation and are % over control (naïve) animals expressed as means ± SEM of 7–8 subjects *per* group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (***$p < 0.0005$ *versus* CFA, #$p < 0.05$, ##$p < 0.005$ *versus* MOG + vehicle).

differences between CFA-treated (used as controls in the figures) and naïve (data not shown, but used to determine 100% level in the figures) animals, except in the case of COX-2, for which CFA-treated animals showed elevations up to 500% in the spinal cord and 300% in the brainstem caused by the administration of adjuvant alone (see Fig. 3). Similar responses have been already described in previous studies (Shibaki and Katz, 2002). We also observed the presence of cell aggregates/infiltrates, stained with Nissl, in the spinal cord of EAE mice that were significantly attenuated by the treatment with WIN55,212-2 (Fig. 4). Immunohistochemical analysis (with Iba-1 and Cd11b) of these aggregates indicated that they corresponded to microglia (resident macrophages labeled with Iba-1; see Fig. 4) and peripheral macrophages (labeled with Cd11b; see Fig. 4).

3.2. Identification of the type of cannabinoid (CB_1 or CB_2) receptor involved in the beneficial effects of WIN55,212-2 in EAE mice

In a second experiment, we wanted to determine the type of cannabinoid receptor underlying positive effects of WIN55,212-2 in EAE mice. To this end, we conducted some experiments with selective antagonists for the CB_1 (e.g. SR141716) or CB_2 (e.g. AM-630) antagonists administered in combination with WIN55,212-2. We observed that the effects of this cannabinoid on neurological decline of EAE mice ($F(3,90) = 7.898$, $p < 0.0001$; see Fig. 5), as well as on TNF-α generation ($F(4,18) = 967.3$, $p < 0.0001$; see Fig. 6) and accumulation of cell aggregates (see Fig. 4) in the spinal cord were attenuated by the combination of WIN55,212-2 and SR141716, thus indicating that WIN55,212-2 effects in EAE mice were mediated by the activation of CB_1 receptors, which is concordant with data reported by other authors (Pryce et al., 2003; Maresz et al., 2007). By contrast, AM-630 did not reverse WIN55,212-2 effects on neurological decline (Fig. 5), TNF-α mRNA levels (Fig. 6) and presence of cell aggregates (Fig. 4). Even, AM-630, when combined with WIN55,212-2, produced an enhancement of positive effects on neurological decline caused by the cannabinoid agonist administered alone ($F(3,100) = 13.22$, $p < 0.0001$; see Fig. 5). This surprising response may be interpreted as the consequence of a higher activity of WIN55,212-2 at the CB_1 receptors when CB_2 receptors are blocked. The fact that CB_2 receptors are not involved in WIN55,212-2 effects was concordant with the fact that the selective CB_2 agonist HU-308, when used instead WIN55,212-2, was unable to modify the neurological decline observed in EAE mice (see Fig. 7).

4. Discussion

Multiple sclerosis is the neurological disease that represents the most frequent cause of chronic disability in young adults (for review, see Frohman, 2003). As yet, no treatment can completely halt the accumulation of disability and substantial efforts are being made to find new therapeutic molecules in this disease. This includes several immunomodulatory and anti-inflammatory agents, but also cytoprotective molecules given the progressive assumption that oligodendrocyte death and axonal loss are key events in the disease (Trapp and Nave, 2008). Cannabinoid-based compounds have been proposed as potentially useful in the treatment of some symptoms of this disease but also as a disease-modifying agent given their anti-inflammatory/neuroprotective properties (for review, see Pryce and Baker, 2005; de Lago et al., 2010). In this study, we have contributed to this last aspect by exploring the mechanisms involved in beneficial effects of the non-selective cannabinoid agonist WIN55,212-2 in MOG-induced EAE mice. Similar beneficial effects of WIN55,212-2 or other similar cannabinoid agonists have been already described in different

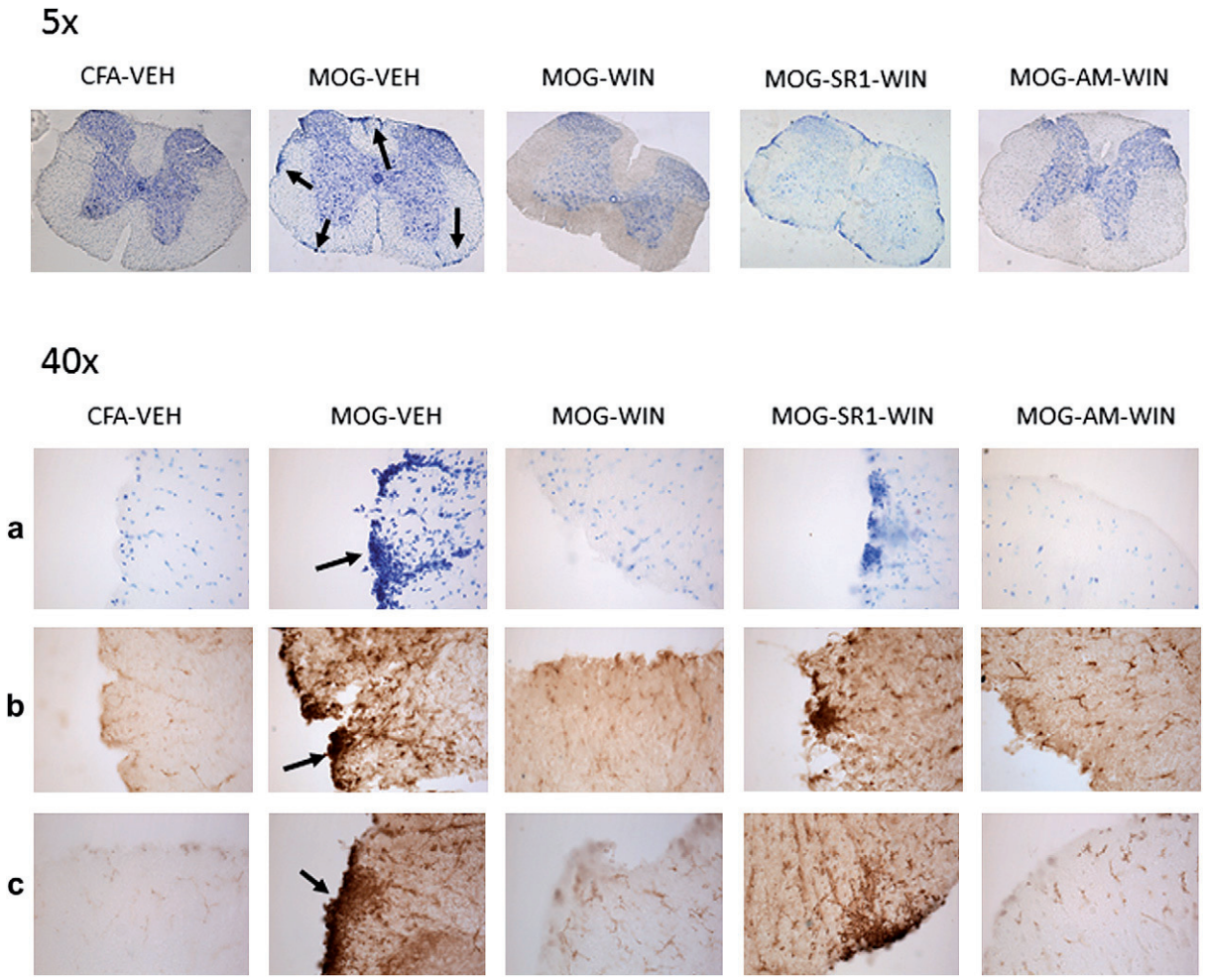

Fig. 4. Representative microphotographs corresponding to Nissl staining (5× images and those in panel a), and immunostaining for IBA-1 (panel b) or Cd11b (panel c) in the spinal cord of mice with EAE induced by inoculation of MOG (day 0) and treated with WIN55,212-2 (initiated at day 11 post-inoculation) combined with SR141716 or AM-630, and the corresponding controls (exposed to CFA). They always correspond to animals at the day 16 post-inoculation. Details in the text. Cell aggregates are indicated by arrows.

models of MS (Arévalo-Martin et al., 2003; Pryce et al., 2003; Mestre et al., 2009; Hasseldam and Johansen, 2010; Downer et al., 2011). The interest of our present study was to correlate neurological recovery with attenuation of different neurobiological mechanisms that are exacerbated during MS pathogenesis, for example, we examined possible changes in glutamate concentrations (and also GABA) in key structures, e.g. spinal cord and brainstem, of mice after induction of EAE and also after the

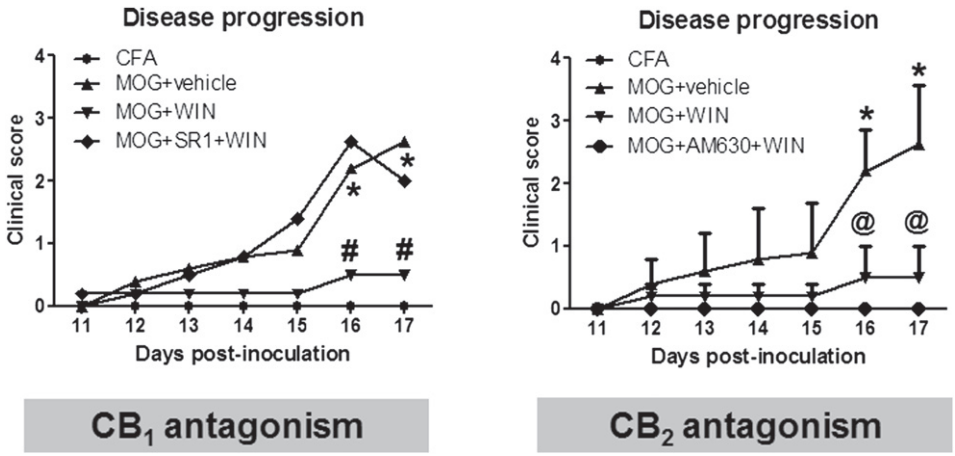

Fig. 5. Effects of a chronic treatment with WIN55,212-2 (initiated at day 11 post-inoculation) administered in the presence or absence of SR141716 (left panel) or AM-630 (right panel), or vehicle on neurological status of mice with EAE induced by inoculation of MOG and the corresponding controls (exposed to CFA). Details in the text. Values are means ± SEM of 7–8 subjects *per* group (in some cases, error bars are omitted but only for presentation). Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (*$p < 0.05$ *versus* CFA, and also *versus* MOG + AM-630 + WIN in right panel; #$p < 0.05$ *versus* MOG + vehicle and MOG + SR1 + WIN; @$p < 0.05$ *versus* MOG + vehicle).

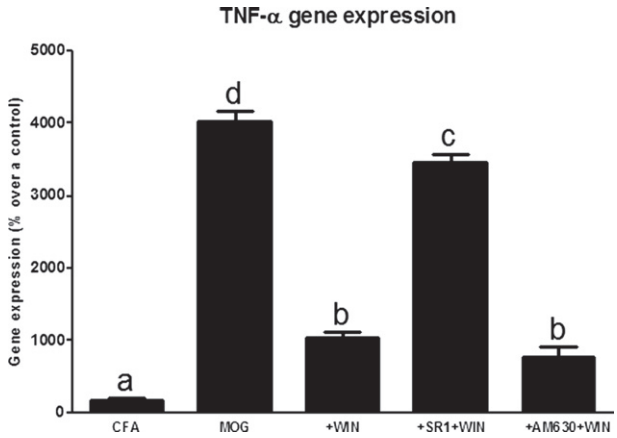

Fig. 6. Effects of a chronic treatment with WIN55,212-2 (initiated at day 11 post-inoculation) administered in the presence or absence of SR141716 or AM-630, or vehicle on TNF-α mRNA levels in the spinal cord of mice with EAE induced by inoculation of MOG and the corresponding controls (exposed to CFA). Details in the text. Values correspond to animals at the day 16 post-inoculation and are % over control (naïve) animals expressed as means ± SEM of 7–8 subjects *per* group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (values with a different letter are statistically different).

treatment with WIN55,212-2. We also paid attention to possible changes in the expression of glutamate transporters, particularly those located preferentially in glial cells (e.g. GLT1 and GLAST), whose malfunctioning or deficiency have been related to excitotoxic neuronal damage in various neurodegenerative disorders including MS (reviewed in Tilleux and Hermans, 2007). Our hypothesis in the case of glutamate transporters was that their expression should be reduced in EAE mice and that WIN55,212-2 treatment should lead to a partial or complete recovery, then allowing a reduction of elevated extracellular glutamate levels responsible of excitotoxic damage to oligodendrocytes and neurons. However, in our hands, neither glutamate and GABA levels nor GLT1 and GLAST expression are significantly altered by EAE induction and/or after WIN55,212-2 treatment, or, when they showed some alterations (the case of GLT1 and GLAST in the spinal cord), they are difficult to interpret in relation with the beneficial effects of WIN55,212-2 in this experimental model of MS. For example, the increased expression of GLT1 and GLAST found in the spinal cord of EAE mice after treatment with WIN55,212-2 may indicate a greater capability to control extracellular levels of glutamate in comparison with the situation during EAE pathogenesis, which would agree with the beneficial effects of this cannabinoid agonist on neurological decline. Supporting in part this idea, Vallejo-Illarramendi et al. (2006) found an increase in gene expression for GLAST, and also for GLT1, in optic nerve post-mortem samples of MS patients, a response that these authors interpreted as a way of protection against excess of glutamate, as our data might also indicate for GLAST during EAE and, particularly, after WIN55,212-2 treatment. Similar results were described by Mitosek-Szewczyk et al. (2008) in different brain structures of EAE rats, although protein levels did not mirror mRNA changes in this case, something that is also unknown in our study. However, making any interpretation extremely complex, there are other studies that show an opposite situation, a notable reduction in both glial transporters in MS patients that was evident in cortical structures in correlation with the presence of activated microglia infiltration (Vercellino et al., 2007). Similar reductions were found by Ohgoh et al. (2002) in the spinal cord of EAE rats.

Given that our data do not appear to support the idea that normalization of glutamate homeostasis is linked with the positive effects of WIN55,212-2 in EAE mice, we concentrated in inflammatory and cell infiltration events that have been described to play a key role in inducing degenerative episodes in MS (reviewed in Stadelmann et al., 2011). We found that the spinal cord and the brainstem of EAE mice experienced a notable increase in various proinflammatory factors, for example, COX-2 and iNOS enzymes, and the cytokine TNF-α, responses that are likely linked to activation of glial elements during EAE. These responses are concordant with data published in this and other MS models (Farias et al., 2007; Centonze et al., 2009; Hasseldam and Johansen, 2010), as well as in patients (Baraczka et al., 2003; Yiangou et al., 2006; Tumani et al., 2009). As in our study, some of these authors and others used analysis of mRNA levels for these factors that they related to the disease progression or to the effects of potential therapies (Tanuma et al., 1997; Villarroya et al., 1997; Wildbaum and Karin, 1999), whereas other studies described that these changes in mRNA levels were always accompanied by equivalent changes in protein levels and/or function (Martino et al., 1997; Villarroya et al., 1997; Molina-Holgado et al., 1999; Ortega-Gutiérrez et al., 2005; Melanson et al., 2009). In our study, the treatment with WIN55,212-2 attenuated these responses, particularly in the case of iNOS, thus reducing inflammatory events and allowing a partial recovery of animals in the neurological examination. As anti-inflammatory effects of cannabinoid agonists are normally mediated by the activation of CB_2 receptors, we expected that the blockade of this receptor would attenuate the positive effects shown by WIN55,212-2. However, we failed to reverse WIN55,212-2 effects with AM-630, a selective CB_2 receptor antagonist, whereas the use of a selective agonist for this receptor did not produce any positive effects in EAE mice, thus discarding that CB_2 receptors have a role in our experimental paradigm. By contrast, we found reversion when we used a selective CB_1 receptor antagonist, thus supporting the participation of this receptor in WIN55,212-2 effects. Paradoxically, the blockade of CB_2 receptors improved the effect of WIN55,212-2 on neurological disabilities of EAE mice, which may be explained as originated by a greater activity of WIN55,212-2 at the CB_1 receptors when CB_2 are closed for this agonist, and not by a possible worsening effect of CB_2 receptors. In support of this, the administration of the CB_2 blocker AM-630 alone to EAE mice did not produce any beneficial effect by itself (data not shown), a fact expected in the case of a negative role of CB_2 receptors. The fact that CB_1 receptors are involved in a reduction of inflammatory factors in EAE mice may appear a paradoxical effect.

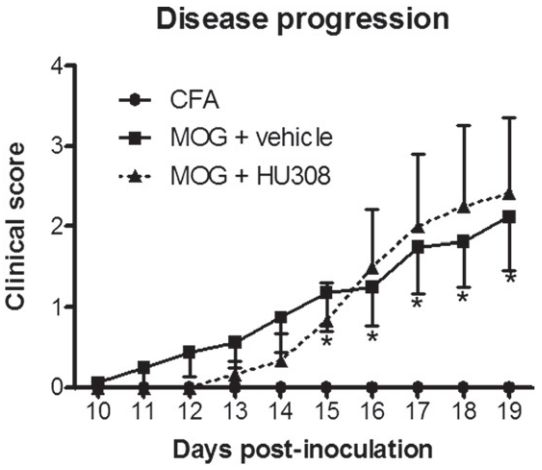

Fig. 7. Effects of a chronic treatment with HU-308 or vehicle (initiated at day 11 post-inoculation) on neurological status of mice with EAE induced by inoculation of MOG (day 0) and the corresponding controls (exposed to CFA). Details in the text. Values are means ± SEM of 7–8 subjects *per* group. Data were assessed by two-way analysis of variance followed by the Student–Newman–Keuls test (*$p < 0.05$ *versus* CFA).

However, it is concordant with data described by other authors that induced EAE in CB_1 receptor-deficient mice and found stronger inflammatory responses (Pryce et al., 2003), or that needed to activate this receptor located in neurons, although also CB_2 receptors in autoreactive lymphocytes, for the suppression of EAE (Maresz et al., 2007).

On the other hand, the inflammatory events (elevated COX-2, iNOS and TNF-α) found in the spinal cord of EAE mice are associated with the presence of cell infiltrates in some parts of this structure, as extensively demonstrated in previous studies (reviewed in Brück, 2005). Here, we have also observed these infiltrates in the form of cell aggregates located peripherally in white matter of the spinal cord of EAE mice. Immunostaining with specific antibodies revealed that these aggregates contain cells labeled with Iba-1, indicating the presence of resident microglial cells, and also with Cd11b, indicating the infiltration of peripheral macrophages, in concordance with data published previously (reviewed in Brück, 2005). The most important observation in relation with these aggregates is that they were significantly reduced by the treatment with WIN55,212-2, in parallel to the rest of the anti-inflammatory effects of this cannabinoid, and that only the blockade of CB_1 receptors abolished the effect of WIN55,212-2 on aggregates, supporting again that the CB_1 receptor is the key target in relation with cannabinoid effects in EAE mice.

In summary, the treatment of EAE mice with the cannabinoid agonist WIN55,512-2 reduced their neurological disability and the progression of the disease. This effect was exerted through the activation of CB_1 receptors, which would exert a positive influence in the reduction of inflammatory events linked to the pathogenesis of this disease.

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Objective #2

Study of the use of the Δ^9-THC-BDS, CBD-BDS and the Sativex-like combination of both phytocannabinoids as a disease modifying agents in two models of MS, the TMEV-IDD and EAE.

In this chapter we evaluated the effect of the phytocannabinoids Δ^9-THC-BDS, CBD-BDS and the Sativex-like combination of both phytocannabinoids in two models of MS in mice: TMEV-IDD and EAE. Our purpose was to confirm the efficacy of Sativex®, a cannabis-based medicine approved for the treatment of spasticity in MS, as a disease-modifying therapy for MS. To this end, we combined two experimental models of MS that are complementary and reproduce specific characteristics of MS. In both models mice were chronically treated with a Sativex-like combination of Δ^9-THC and CBD botanical drug substance (BDS) (10 mg/kg in TMEV-IDD and 20 mg/kg in the EAE) or only with Δ^9-THC-BDS or CBD-BDS (5 mg/kg in TMEV-IDD and 3 mg/kg and 20 mg/kg in EAE). The efficacy of these treatments was determined by recording the neurological decline (horizontal activity in TMEV-IDD and neurological score in EAE) and by analysing several biochemical and histological parameters in the spinal cord of these animals.

In the TMEV-IDD model, Sativex-like combination and CBD-BDS showed higher efficacy than Δ^9-THC-BDS improving the motor disturbances. Mice treated with Sativex-like combination had a reduction of CNS cellular infiltrates, decreased microglial activity, reduced axonal damage and restored myelin morphology. In the spinal cord of Sativex-treated mice there was a decreased expression of IL-1β. The alleviation of symptoms caused by Δ^9-THC-BDS was mediated by CB_1R and in less degree, CB_2R, while the effects of CBD-BDS are mediated by PPARγ receptors.

In the EAE model, Δ^9-THC-BDS and Sativex-like combination improved the neurological decline of EAE, while CBD-BDS treated mice, albeit having a delayed course of the disease where not statistically different from vehicle treated animals. Accordingly we observed a reduction in the cell aggregates infiltrated in the spinal cord of Δ^9-THC-BDS and Sativex-like combination treated mice. Rimonabant blocked the Δ^9-THC-BDS effects indicating that these were mediated by CB_1R and not by alternative anti-inflammatory target such as the nuclear receptor PPARγ. Δ^9-THC-BDS inhibited the activation of COX2 observed in EAE mice.

CONCLUSION:

Our data support the therapeutic potential of Sativex® as a pharmacotherapy able to slow MS progression as we observed in TMEV-IDD and EAE.

Papers in this chapter:

Feliú, A., Moreno-Martet, M., Mecha, M., Carrillo-Salinas, F. J., Fernández-Ruiz J., de Lago, E., Guaza C.
SATIVEX-LIKE COMBINATION OF PHYTOCANNABINOIDS AS A DISEASE-MODIFYING THERAPY IN A VIRAL AUTOIMMUNE MODEL OF MULTIPLE SCLEROSIS. (Preliminary data not published).

Moreno-Martet, M., Feliú, A., Guaza, C., Fernández-Ruiz J., de Lago, E.
PHYTOCANNABINOID SATIVEX-LIKE COMBINATION AS A DISEASE-MODIFYING THERAPY IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS. (Preliminary data not published).

SATIVEX-LIKE COMBINATION OF PHYTOCANNABINOIDS AS A DISEASE-MODIFYING THERAPY IN A VIRAL AUTOIMMUNE MODEL OF MULTIPLE SCLEROSIS

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ABSTRACT

Sativex®, a medicine constituted by an equimolecular combination of Δ⁹-tetrahydrocannabinol (Δ⁹-THC) and cannabidiol (CBD), has been approved for the treatment of spasticity and pain associated to multiple sclerosis (MS) in different countries. In this study, we investigated the therapeutic potential of a Sativex-like phytocannabinoid combination (Sativex-like combination) of Δ⁹-tetrahydrocannabinol (Δ⁹-THC-BDS) and cannabidiol (CBD-BDS) botanical drug substances (BDS) as a disease-modifying therapy in a viral model of MS. In the Theiler's virus induced demyelinating disease (TMEV-IDD) model, Sativex-like combination and individually Δ⁹-THC-BDS and CBD-BDS were administered intraperitoneal (i.p.) for 10 consecutive days to susceptible mice (SJL/J strain) once established symptomatology. The results show that each independent treatment was significantly effective improving the motor disturbances associated with the disease. Nevertheless, Sativex-like combination and CBD-BDS showed higher efficacy than Δ⁹-THC-BDS in alleviating symptomatology. The most significant findings with Sativex-like combination included a reduction of CNS cellular infiltrates, decreased microglial activity, reduced axonal damage and restored myelin morphology. Decreased gene expression of IL-1β was also observed in the spinal cord of Sativex-like combination-treated mice. CB_1 and in less degree, CB_2 receptors were involved in the alleviation of symptomatology induced by Δ⁹-THC-BDS, while PPARγ receptors mediated the effects of CBD-BDS. Collectively, our data support the therapeutic potential of Sativex-like combination as a compound capable to slow MS progression as we observed in TMEV-IDD. Our results might be relevant for the potential of by Sativex® in CNS reparative mechanisms.

INTRODUCTION

Multiple sclerosis (MS), the most common cause of neurological disability in young adults, is a complex autoimmune disease characterized by inflammation, demyelination and axonal damage (Compston and Coles, 2008). Most patients with MS initially have a relapsing-remitting disease course that convert to a secondary progressive clinical form. Another 10-15% of patients with MS have a primary progressive form from onset. Effective treatments for patients with primary or secondary MS remain elusive. Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) is a model of MS (Lipton and Dal Canto 1976), unique in reproducing the putative viral etiology of MS and to study virus-induced autoimmunity (Miller et al., 1997). The TMEV model features a chronic-progressive disease course that lasts the entire lifespan of susceptible strain of mice. Several features of MS such as the role and significance of axonal injury and repair, the partial independence of disability from demyelination, or the significance of re-myelination has been demonstrated in this model (reviewed by Denic et al.,2011). TMEV based MS models also feature several MRI findings of the human disease (Pirko et al.,2011). TMEV is a single-stranded RNA virus, and its intracranial inoculation in susceptible mouse strains (SJL/J) leads to the development of a chronic-progressive $CD4^+$T cell-mediated demyelinating disease. Innate immune responses against the virus influence the immune respon-

ses during TMEV induced demyelinating disease (Bowen and Olson 2009). During TMEV-IDD progression, epitope spreading produces $CD4^+$ T cell responses against myelin peptides.

The cannabinoid system consists of cannabinoid receptors and exogenous and endogenous receptor ligands. Most cannabinoid actions are mediated through the classical cannabinoid receptor type 1 (CB_1R) and cannabinoid receptor type 2 (CB_2R). However, cannabinoids (CBs) also act via mechanisms that do not involve CB_1 or CB_2 receptors (reviewed by Pertwee 2012). The exogenous ligands include natural (plant derived) ligands and synthetic CB_1R/CB_2R agonists and inverse agonist/antagonists. The best characterized endocannabinoids include anandamide (AEA) and 2-arachydonoylglycerol (2-AG), both partial CB_1R/CB_2R agonists. Interestingly, CB_1R, CB_2R and fatty acid amide hydrolase (FAAH) are specific markers of plaque cell subtypes in human multiple sclerosis (Benito et al., 2007)

Cannabinoids are being studied for the treatment of MS and other neurodegenerative diseases (Velayudhan et al., 2013; Pryce and Baker 2012). Preclinical studies have demonstrated that cannabinoids can alleviate MS associated symptoms such as spasticity (Baker et al., 2000) and exhibited anti-inflammatory (Arévalo-Martín et al., 2003), antioxidant, anti-excitotoxic and neuroprotective properties (Pryce et al.,2003; Loría et al., 2010 Fernández Ruiz et al., 2010). Cannabinoids also protect oligodendrocytes *in vitro* and *in vivo* (Molina-Holgado et al., 2002; Mecha et al., 2012; Gómez et al., 2011; Solbrig et al.,2010). The oromucosal spray Sativex® (1:1 ratio of Δ^9-THC/CBD) has been approved in a number of EU countries and elsewhere for use in patients with MS-related spasticity and neuropathic pain. In clinical trials, Sativex® provided relief of spasticity symptoms showing clinically significant improvement in spasticity (30% or higher reduction from baseline). Sativex® also improves associated MS symptoms such as sleep disturbances, bladder problems and loss of motility (Pozzilli 2013). Safety studies indicate a low risk for serious adverse drug reactions. Both components of Sativex® have been described to independently modify immune responses during neuroinflammatory processes as shown in experimental models of MS. However, there is a lack of studies investigating the potential of Sativex® as a treatment for progressive MS. Experimental evidence for a neuroprotective effect of Sativex® has been recently reported in inflammatory models of Huntington disease (Valdeolivas et al., 2012). Zajicek at al., (2013) investigated the CBs use in progressive inflammatory brain disease (CUPID) trial, by testing dronabinol (Δ^9-THC) against placebo in a cohort of patients with progressive MS. The results of this study showed that dronabinol has no overall effect on the progression of MS in its progressive phase, but, it encouraged the use of experimental models that better represent progressive MS to continue investigating whether CBs might change the disease course of progressive MS.

In the present study we investigate the therapeutic potential of a Sativex-like combination of phytocannabinoids as a disease-modifying therapy in a model of primary progressive MS. The treatment with Sativex-like combination or individually Δ^9-THC-BDS or CBD-BDS to TMEV-infected mice, once established symptomatology, improved the motor disturbances associated with the disease. The treatment with Sativex-like combination reduced cellular infiltrates, decreased microglial activity and diminished axonal damage in TMEV-infected mice. In addition, myelin morphology was restored and cytokines were reduced by Sativex-like combination administration. Our results suggest the involvement of CB_1 and CB_2 receptors in the beneficial effects of Δ^9-THC-BDS and the PPARγ receptors in the effects mediated CBD-BDS in TMEV-IDD.

MATERIALS AND METHODS

Animals and Theiler´s virus infection

TMEV-IDD-susceptible female SJL/J mice from Harlan (Barcelona, Spain) were maintained our in-house colony (Cajal Institute, Madrid) on a 12 h light/dark cycle with ad libitum access to food and water. Four-week-old mice were inoculated intracerebrally in the right hemisphere with 2 x 106 plaque forming units (pfu) of the Daniels (DA) strain of TMEV in 30 µl of Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with 10% fetal calf serum (FCS), as described previously (Lledó et al., 1999), or with 30 µl of DMEM + 10% FCS in the case of Sham (non-infected) operated mice. All experiments were performed in strict accordance with EU and governmental regulations (Real Decreto 53/2013 BOE nº34 and Comunidad de Madrid: ES 280790000184). The Ethics Committee on Animal Experimentation of the Instituto Cajal, CSIC, approved all proce-

dures described in this study (protocol number: 2013/03 CEEA-IC). Measures to improve welfare assistance and clinical status as well as endpoint criteria were established to minimise suffering and ensure animal welfare. Briefly, wet food pellets are placed on the bed-cage when the animals begin to develop clinical signs to facilitate access to food and hydration.

Sativex-like combination, CBD-BDS, Δ9-THC-BDS and antagonist treatments

Sham or TMEV-IDD mice were administered daily with vehicle or combinations of botanical extracts enriched with either Δ^9-THC-BDS, kindly provided by GW Pharmaceuticals Ltd., Cambridgeshire, U.K. [Δ^9-THC botanical drug substance (Δ^9-THC-BDS) contains 67.1% Δ9-THC, 0.3% CBD, 0.9% cannabigerol, 0.9% cannabichromene, and 1.9% other phytocannabinoids] or CBD-BDS, also provided by GW Pharmaceuticals [CBD botanicaldrug substance (CBD-BDS) contains 64.8% CBD, 2.3% Δ^9-THC, 1.1% cannabigerol, 3.0% cannabichromene, and 1.5% other phytocannabinoids]. The dose of Sativex-like combination administered was 10 mg/kg [(Δ9-T^HC-BDS(5 mg/kg): CBD-BDS (5 mg/kg)]. Cannabinoids were prepared in Tween-80: saline (1:16) gassing it previously with N2 to avoid its oxidation, and administered intraperitoneally (i.p.), once daily from days 70 to 80 post-infection (p.i.) when the signs of disease were evident in the TMEV-infected animals. The duration of the treatment was chosen based on previous studies using the TMEV-IDD model (Docagne et al., 2007; Ortega-Gutiérrez et al., 2005). Sativex-like combination compounds, Δ^9-THC-BDS (5 mg/kg) and CBD-BDS (5 mg/kg), were also administered separately to identify their effects alone. To identify the implication of CB_1, CB_2 and PPARγ receptors, the antagonists AM251 (2 mg/kg) and AM630 (2 mg/kg) (Tocris Bioscience; Bristol, UK) were administered 30 min before Δ9-T^HC-BDStreatment and T0070907 (5 mg/kg) (Cayman Chem; Ann Arbor, MI, USA), before CBD-BDS treatment. After 10 days of treatment, the motor activity was evaluated and the animals were sacrificed with an overdose of anesthetic (pentobarbital) for tissue collection.

Behavioral analysis: spontaneous motor activity

Locomotor activity was evaluated in mice using an activity cage (Activity Monitor System, Omnitech Electronics Inc., Columbus, OH, USA) coupled to a Digiscan Analyser. The number of times that the animals broke the horizontal sensor beams was measured in two 5-min sessions.

Tissue processing

Mice were anesthetized with pentobarbital (Dolethal, 50 mg/kg body weight, i.p.) and transcardially perfused with saline. The spinal cords were fixed overnight in 4% paraformaldehyde prepared in 0.1 M phosphate buffer (PB), and cryoprotected in sucrose solution in 0.1 M PB (15% followed by a 30%). Coronal and longitudinal cryostat spinal cord sections (15 and 30 µm thick) were cut (cryostat; Leica Microsystems CM1900, Barcelona, Spain) and then processed for immunohistochemistry.

Immunohistochemistry

Free-floating spinal cord sections (15/30 µm thick) were washed three times for 10 min with 0.1 M phosphate buffer (PB) and with PB + 0.2% Triton X-100 (PBT) and blocked for 1 h at room temperature in blocking buffer (PBT and 5% normal goat serum: Vector Laboratories, Burlingame, CA, USA) after inhibiting the endogenous peroxidase with 50% methanol and 1.66% hydrogen peroxide in the case of immunostaining with DAB. The sections were then incubated overnight at 4°C with the primary antibody. For IHC, microglial cells were stained with a rabbit anti-mouse Iba-1 antibody in blocking buffer (1:1000; Wako, Osaka, Japan) on spinal cord sections (30 µm thick). For immunofluorescence, axons were stained with a rabbit anti-mouse Neurofilament H antibody (1:1,000; Millipore; Billerica, MA, USA) (longitudinal and transversal sections of 30 µm thick). The following day, after primary antibody incubation, sections were rinsed three times for 10 min with PBT and they were then incubated for 1 h in the case of inmunohistochemistry with a biotinylated goat anti rabbit (for Iba-1) (Vector Laboratories, Inc., CA, USA). For immunofluorescence staining, Alexa 488 Fluor-conjugated goat anti-rabbit antibody (for Neurofilament-H). For immunofluorescence, sections were then rinsed three times for 10 min with PB and mounted. After staining, the sections were dehydrated, cleared with xylene and coverslipped. In all cases, the specificity of staining was confirmed by omitting the primary antibody.

Microscopy and image analysis

Images were acquired for immunofluorescence on a Leica TCS SP5 confocal microscope, and for immunohistochemistry with a Zeiss Axiocam high resolution digital color camera. Individual optical sections were acquired and 5-6 sections from at least 5-6 animals per group were analyzed. Quantification of staining was performed using Image J software (designed by NIH, Bethesda, MD, USA) maintaining the threshold intensity constant during the comparison and measurement of all experimental and control images within the experiments. The data are presented as the percentage of the total area that was stained with respect to Sham animals.

Inflammatory infiltrates analysis and Luxol fast blue staining

The slices were stained with hematoxylin and eosin (H&E) to analyse the number of infiltrates in the parenchyma and with Luxol fast blue (LFB) to see myelin integrity.
For LFB staining free-floating spinal cord sections (15 µm thick) were washed with 0.1 M PB three times for 10 min. Tissue samples were dehydrated in ethanol successively from 70% to 95%. Then, sections were incubated in LFB solution at 56oC oven overnight. The following day, sections were rinsed off excess stain with 95% ethyl alcohol, and differentiated the slides in the lithium carbonate solution for 30 seconds. Sections were dehydrated, cleared with xylene and cover slipped.

Reverse transcription (RT) and real-time polymerase chain reaction (PCR)

Total RNA was extracted from nervous tissue (spinal cords) or primary rat astrocytes cultures using RNeasy mini columns (Qiagen; Manchester, UK). Genomic DNA contamination was avoided by DNase I degradation (DNase I; Sigma-Aldrich; Saint Louis, Missouri, USA) and the RNA yield was determined using a Nanodrop spectrophotometer (Thermo Scientific; Wilmington, DE, USA). Total RNA (1 µg

Sativex in TMEV-IDD

in 20 µL) was reverse transcribed into cDNA using poly-dT primers and a reverse transcription kit (Promega Biotech Ibérica, S.L., Madrid, Spain). Primers were the following; TNFα: sense (GACTCCCCCCTCCGTCTAAG), antisense (CGCAGTAAAGCCCACGTTGT); IL-1β: sense (TGGTGTG-TGACGTTCCCATT), antisense (TCCATTGAGGTGGAGA-GCTTTC); IFNγ: sense (GGCCATCAGCAACAACATAAGCGT), antisense (TGGGTTGTTGACCTCAAACTTGGC); 18S: sense (ATGCTCTTAGCTGAGTGTCCCG), antisense (ATTCCTA-GCTGCGGTATCCAGG) (Applied Biosystems, Warrington, UK) and SYBR® (Applied Biosystems, Warrington, UK) PCR was performed. Cycling conditions were as follows: 50°C for 2 min; 95°C for 10 min; and 40 amplification cycles of 95°C for 15 sec. and 60°C for 1 min. Samples were assayed using the Applied Biosystems PRISM 7500 Sequence detection system, assaying each sample in triplicate. To ensure the absence of contamination with genomic DNA, a control sample using RNA as the template was run for each set of extractions. Relative quantification was obtained by calculating the ratio between the values obtained for each gene of interest and those of the 18S house-keeping gene. Expression levels were calculated using the $2^{-\Delta\Delta}$Ct method. The results are expressed as a percentage with respect to the Sham animals for each time point.

Data analysis

All the data are expressed as the mean ± SEM. One-way ANOVA followed by the Bonferroni post-hoc test, or Kruskal-Wallis ANOVA followed by Mann-Whitney U test was used to determine the statistical significance in all cases. The level of significance was set at $p \leq 0.05$. $p \leq 0.05$, significant (represented as *), $p \leq 0.01$, very significant (represented as **) and $p \leq 0.001$, highly significant (represented as ***).

RESULTS

Sativex-like combination of phytocannabinoids or individual Δ⁹-THC-BDS and CBD-BDS treatment significantly improves motor function in TMEV-IDD: Involvement of CB_1, CB_2 and PPARγ receptors.

We studied the effect of the Sativex-like combination and Δ⁹-THC-BDS and CBD-BDS individually in the model of murine primary progressive MS TMEV-IDD. Hence, 70 days after TMEV infection the mice were treated with Sativex-like combination (10 mg/kg) (Δ⁹-THC-BDS 5 mg/kg: CBD-BDS 5 mg/kg) or received separately CBD-BDS (5 mg/kg) and Δ9-THC-BDS (5mg/kg), or an equivalent amount of vehicle, for 10 consecutive days. Then, mice were examined and the motor activity was assessed using the activity cage. As expected in the chronic phase of the model, TMEV infection dramatically reduced both horizontal and vertical activities (data not shown) while Sativex-like combination treatment significantly improved the motor deficits (Fig1A). From a comparative point of view Sativex-like combination and individually CBD-BDS were the most effective treatments as they completely abrogated the motor deficits. These data indicate that Δ⁹-THC-BDS and CBD-BDS independently or combined in Sativex-like combination

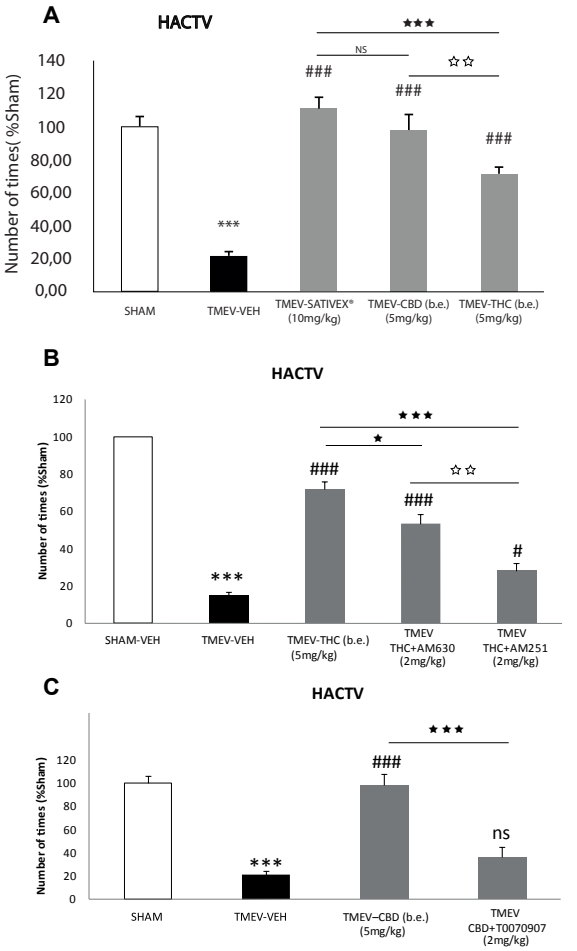

Fig.1. Sativex-like combination treatment significantly improves motor deficits in the chronic phase of TMEV infection. A) Sativex-like combination significantly attenuates motor deficits in horizontal activity in the TMEV-IDD model. Regarding the comparative analysis, Sativex-like combination and individually CBD-BDS were the most effective treatments as they completely abrogated the decreased motor activity, recovering motor activities to normal levels measured by horizontal activity. B) We observed that the positive effect of Δ⁹-THC-BDS was significantly blocked mainly by the administration of CB_1 antagonist AM251. C) The effect of CBD-BDS treatment was significantly attenuated by the antagonist of PPARγ receptor T0070907. Activity parameters were recorded for 10 min and the results represent the mean ± SEM: A) n=9, $^{***}p \leq 0.001$ vs. sham; $^{\#\#}p \leq 0.01$; $^{\#\#\#}p \leq 0.001$ vs. TMEV-VEH animals; $^{***}p \leq 0.001$ vs. TMEV-Sativex-like combination; $^{**}p \leq 0.01$ TMEV-CBD-BDS vs. TMEV-THC-BDS; B,C) n=6, $^{***}p \leq 0.001$ vs. sham; $^{\#}p \leq 0.05$ $^{\#\#\#}p \leq 0.001$ vs. TMEV-VEH animals;$^{*}p \leq 0.05$ vs. TMEV-BS-THC; $^{***}p \leq 0.001$ vs. TMEV-BS-THC; vs. TMEV-CBD-BDS; $^{**}p \leq 0.01$ TMEV-THC-AM630 vs. TMEV-THC-AM251; Statistical analysis: one-way ANOVA followed by Tukey's test.

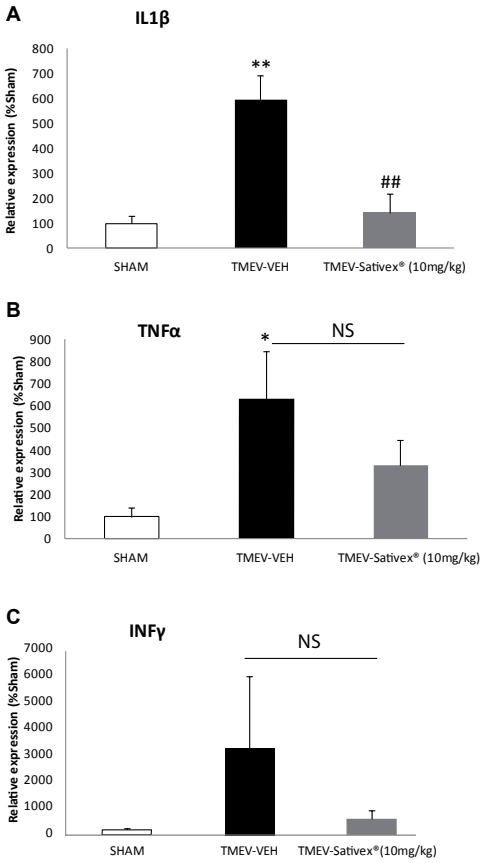

Fig.2. Sativex-like combination treatment downregulates proinflammatory cytokines in the chronic phase of TMEV-IDD. Sativex-like combination treatment decreased IL-1β (A) TNFα (B) and IFN-γ (C) mRNA expression as determined by RT-PCR, in the spinal cord of TMEV-infected animals. mRNA expression normalized to that of the 18S gene. Data represent the mean ± SEM. n=6. *p ≤ 0.05 **p ≤ 0.01 ***p ≤ 0.001 vs. sham; ##p ≤ 0.01 ###p ≤ 0.001 vs. TMEV-Veh animals; Statistical analysis: Kruskal-Wallis ANOVA followed by Tukey's -test.

treatment recover motor activity function in TMEV-infected mice. Treatments had no effect in Sham animals (data not shown).

To determine the receptors involved in the beneficial effects of Sativex-like combination of Δ^9-THC-BDS and CBD-BDS we administered a selective antagonist for the CB_2R (AM630) or an antagonist for the CB_1R (AM251) 30 min before Δ^9-THC-BDS treatment. Because previous studies showed that CBD displays very low CB_1/CB_2 receptor affinity (Izzo et al.,2009; Mecha et al.,2013) we did not use CB_1R nor CB_2R antagonists but the PPARγ receptor antagonist, T0070907, that was administered 30 min before CBD-BDS treatment. We observed that the positive effect of Δ^9-THC-BDS was significantly blocked mainly by the administration of the antagonist of CB_1R although the CB_2R antagonist was able to induce a partial blockade of these effects. Moreover, the antagonist of PPARγ receptors significantly blocked the beneficial effect of CBD-BDS treatment (Fig1C). These data suggest the involvement of CB_1R and in a minor degree of CB_2R in the ameliorating effect of Δ^9-THC-BDS in TMEV-IDD motor disturbances. Our data also indicate the involvement of PPARγ receptors in the ability of CBD-BDS for improving TMEV-IDD symptomatology.

Sativex-like combination reduces leukocyte infiltration in the spinal cord of TMEV-infected animals

As revealed by H&E staining (Fig.2), the infection with TMEV caused the infiltration of immune cells into the spinal cord. In this line, Sativex-like combination treatment partially decreased the number of infiltrates in the spinal cord. These results suggest that one of the mechanisms of action of Sativex-like combination may include the restriction of the permissiveness of immune cells into the CNS parenchyma. No infiltrates were detected in sham mice administered with Sativex-like combination.

Sativex-like combination decreases microglial activity and downregulates the expression of proinflammatory cytokines in the spinal cord of TMEV-infected mice

Microglial/macrophage activation plays a critical role in TMEV-IDD. Thus, we analysed the effect of Sativex-like combination in the immunohistochemical expression of Iba1, a marker of microglial cells, in the spinal cord of TMEV-infected mice (Fig. 2) The staining with Iba-1 revealed that TMEV-infection increased microglial reactivity in the spinal cord at the chronic phases of the disease. Following Sativex-like combination treatment significant reduction of the intensity of labelling of microglia was observed. No microglia reactivity was detected in sham mice administered only with Sativex-like combination.

Moreover, the reduction of microglia reactivity was accompanied by a significant reduction of gene expression of IL-1β (Fig. 3A), and a tendency towards reduction in the case of TNFα and IFNγ (Fig3B and 3C). These data suggest that Sativex-like combination could be acting as an inmunomodulator agent limiting the inflammatory processes that occur in

Sativex in TMEV-IDD

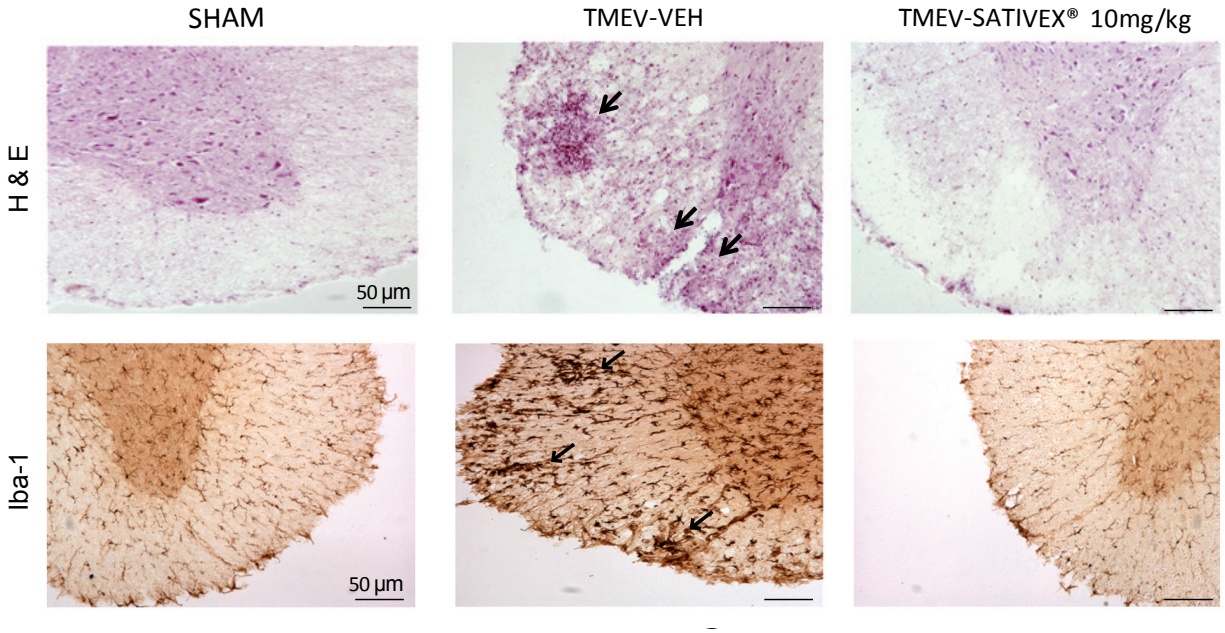

Fig.3. Sativex-like combination treatment decreases leukocyte infiltration and attenuates the microglial response Transversal cervical spinal cord sections (30 µm thick) were obtained at day 80 d.p.i. and stained with hematoxylin-eosin or Iba-1. A) TMEV infection induced increases in leukocyte infiltration. This effect was attenuated by Sativex-like combination treatment (5 mg/kg) B) Representative microphotographs of Iba1 immunostaining showing morphological changes in the microglial cells of infected animals that were reverted after Sativex-like combination treatment (5 mg/kg). For histology analysis; 5-6 spinal cord slices per animal; n=6 animals per group. Scale bar = 50 µm.

TMEV-IDD disease. No effect was observed in sham animals treated with Sativex-like combination (data not shown).

Sativex-like combination restores myelin morphology and prevents the axonal damage in TMEV-infected mice.

Demyelinated areas, detected by luxol fast blue staining, were observed in vehicle-treated infected mice at the chronic phases of TMEV-IDD. However this pathologic hallmark diminished in TMEV infected mice treated with Sativex-like combination as it promoted a pronounced restructuring of myelin sheaths (Fig 4A).

To further assess the potential effect of Sativex-like combination as a neuroprotective agent, we evaluated the axonal damage in TMEV infected mice subjected to vehicle or Sativex treatment using Neurofilament-H staining. Vehicle-treated TMEV-infected animals showed clear axonal damage in the white matter tracts that was absent in sham mice. The treatment with Sativex-like combination restored Neurofilament-H staining (Fig. 4B) preventing the disintegration of axonal package that occurred in TMEV-infected mice (Fig. 4C).

DISCUSSION

No treatments have shown clinical efficacy treating progressive MS. Cannabinoid compounds are effective ameliorating the severity of symptomatology in different models of experimental MS. The approval of the oromucosal spray Sativex® (1:1 ratio of Δ^9-THC/CBD) in several countries for the treatment of spasticity and neuropathic pain in MS patients favors the investigation about the therapeutic potential of cannabinoids as modifying disease agents. Here, we studied the efficacy of Sativex-like combination in ameliorating the symptoms severity in a murine model of primary progressive MS, the TMEV-IDD model. The results of our study show an overall treatment effect of Sativex-like combination of phytocannabinoids in improving the motor deficits associated with the disease. Histopathological analysis of TMEV-infected mice that received Sativex-like combination reveals that this combination of Δ^9-THC-BDS/CBD-BDS exerted immunomodulatory (decreased infiltrates, reduced microglia activity, reduced gene expression of pro-inflammatory cytokines) and neuroprotective

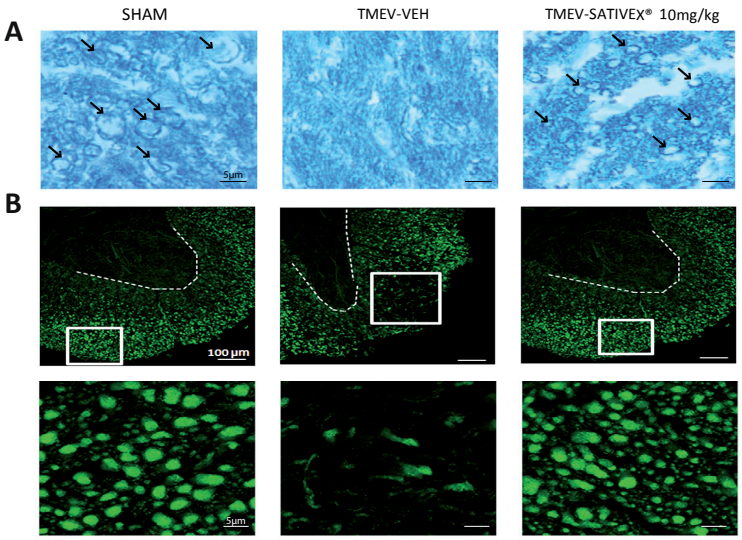

Fig. 4 Sativex-like combination treatment restores myelin morphology and prevented the axonal damage in TMEV infected mice. Transversal cervical spinal cord sections were obtained at day 80 d.p.i. and stained with Luxol fast blue (15 µm thick) and Neurofilament-H (30 µm thick). The white matter cervical spinal cord sections from infected animals treated with vehicle show a clear disruption of myelin, and Sativex-like combination (5mg/kg) treatment contributed to maintain the myelin structure (A) arrows shows myelin sheaths. (B) Representative images of Neurofilament-H staining showing that there is prominent axonal damage in the white matter of the spinal cord of vehicle-treated infected animals that was significantly diminished by Sativex-like combination treatment. Scale Bar=5 µm or 100 µm.

effects (reorganization of myelin and diminished axonal damage) that together may be underlying, at least in part, its benefits on mice motor skills.

Data from clinical trials support the efficacy and safety of Sativex® in patients with MS-related spasticity (reviewed by Koehler 2014; Novotna et al., 2011; Wade et al., 2010; Collin et al.,2007; Sastre-Garriga et al.,2011; García-Merino et al.,2013). However, there is a lack of studies evaluating the Sativex® use in progressive MS as a disease modifying drug. The CUPID trial results recently published (Zajicek et al., 2013) which tested dronabinol (Δ^9-THC) administration in patients with progressive MS do not show efficacy in terms of slowing the course of the disease. Potential causes for the failure of the above therapy include the high attrition rate and a lower rate of disease progression than expected. Our study is the first to address the effect of Sativex-like combination of phytocannabinoids in primary progressive MS and the results obtained are highly promising in terms of reduction of axonal damage, suggesting a neuroprotective profile of Sativex-like combination. Previous studies demonstrate the antispasticity effect of Sativex® in mice using a chronic relapsing experimental allergic encephalomyelitis model (Hilliard et al.,2012).

If we considered studies using only Δ^9-THC or CBD in animal models of MS, there are several articles showing the beneficial effects of one or both compounds in EAE, CREAE and in TMEV-IDD. The first study that described that Δ9-THC inhibited both clinical and histological EAE in rats reducing inflammation was published in 1989 by Lyman et al. Δ^9-THC was also shown to ameliorate tremor and spasticity in mice using the chronic relapsing experimental allergic encephalomyelitis model (Baker et al.,2000). In our study Δ^9-THC-BD-Sameliorates TMEV-IDD symptomatology with a main participation of CB_1R, but the blockade of CB_2R partially prevent the benefits elicited by this phytocannabinoid. CB_1R likely mediated the neuroprotective effects of Δ^9-THC against excitotoxicity as pioneer studies pointed out the importance of CB_1R (Marsicano et al.,2003) and excitotoxicity mechanisms are involved in TMEV-IDD (Docagne et al.,2007). In addition, our findings are in agreement with a previous study in which Δ^9-THC suppress CNS autoimmune inflammation through the participation of CB_1R and CB_2R (Maresz et al.,2007).

Diminished symptomatology severity by CBD treatment have been demonstrated in both experimental models of MS, EAE and TMEV-IDD (Kozela et al,2011; Mecha et al.,2013). The attenuation of disease progression in both models involved the reduction of immune cell infiltrates and the decrease in microglial activation following systemic 5mg/kg CBD treatment. Our findings differ from the results of one study (Maresz et al., 2007) that showed a lack of effect of CBD in the CREAE model of MS. Differences in the strain of mice (C57BL/6 and ABH mice) and or the antigen used (MOG35-55 peptide vs spinal cord homogenate) may account for the discrepancies

among studies. Accordingly, recent evidence have shown the influence of genetic background on the induction of EAE in endocannabinoid system-related knockout mice (Sisay et al.,2013). In our study we found the involvement of PPARγ receptors as mediators of CBD-BDS positive effects in attenuating TMEV-induced motor deficits. CBD has anti-inflammatory properties (Malfait et al.,2000; Kozela et al., Mecha et al.,2013) and is also an antioxidant agent showing an attractive neuroprotective profile (Sagredo et al.,2007; rev by Fernández-Ruiz et al.,2013) however, its pharmacology is not well defined. Most evidence show that CBD is not acting directly through cannabinoid receptors, while targets for this phytocannabinoid are diverse including the endocannabinoid system by inhibiting processes involved in the inactivation of endocannabinoids (Bisogno et al.,2001) and therefore, increasing the endocannabinoid tone. Other targets for CBD actions include 5HT-1A receptors (Magen et al., 2011; Espejo-Porras et al., 2013), adenosin A2 receptors (Castillo et al., 2010; Carrier et al.,2006; Mecha et al 2013), the endoplasmic reticulum stress in its ability to protect oligodendrocyte progenitors (OPCs) from inflammatory damage (Mecha et al.,2012), or nuclear receptors of the PPAR-γ family, such as PPARγ (O'Sullivan and Kendall 2010; Esposito et al., 2011) as we have observed in the present study. In this case, the inhibition of PPARγ receptors led to a complete blockade of CBD-BDS induced motor amelioration in TMEV-IDD clearly indicating the participation of the nuclear receptors in the benefits of CBD-BDS in this model of primary progressive MS. This is in agreement with studies showing that activation of PPARγ receptors agonists ameliorates MS symptomatology (Feinstein et al., 2002)

CONCLUSION

Sativex-like combination, Δ^9-THC-BDS and CBD-BDS improve neurological deficits at the chronic phases of the Theiler's virus model. One of the potential explanations for the beneficial effects of Sativex-like combination could be the reduced transmigration of cell infiltrates into the parenchyma. Sativex-like combination also acts as an immunomodulator by decreasing microglial reactivity and the gene expression of proinflammatory cytokines. Moreover, Sativex-like combination diminishes axonal damage and restores myelin morphology suggesting that Sativex-like combination exerts neuroprotective effects. The present findings may help to develop clinical therapeutics in progressive MS.

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PHYTOCANNABINOID COMBINATION AS A DISEASE-MODIFYING THERAPY IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Abstract

Sativex®, an equimolecular combination of Δ⁹-tetrahydrocannabinol (Δ⁹-THC) and cannabidiol (CBD), is a licensed drug approved for the treatment of spasticity and pain associated to multiple sclerosis (MS). In this study, we investigated the therapeutic potential of a Sativex-like combination enriched with Δ⁹-tetrahydrocannabinol botanical drug substance (Δ⁹-THC-BDS) and cannabidiol botanical drug substance (CBD-BDS) as a disease-modifying therapy in experimental autoimmune encephalomyelitis (EAE) in mice, a model of MS. 20 mg/kg Sativex (10 mg/kg Δ⁹-THC-BDS and 10 mg/kg CBD-BDS) or individually 20 mg/kg Δ⁹-THC-BDS or 20 mg/kg CBD-BDS were administered intraperitoneal (i.p.) to EAE induced mice from symptom onset until the first relapse of the disease. The results show that the Sativex-like combination and the Δ⁹-THC-BDS treatments significantly improved the neurological decline associated with the disease. The most significant findings with Sativex-like combination included a reduction of CNS cellular infiltrates. CB_1R were involved in the alleviation of symptomatology induced by Δ⁹-THC-BDS. Collectively, our data support the therapeutic potential of Sativex® as a compound capable to slow MS progression as we observed in EAE. Our results might be relevant for the potential implication of Sativex® in CNS reparative mechanisms.

INTRODUCTION

Multiple sclerosis (MS) is the first cause of neurological decline in young adults (Noseworthy et al., 2000). The inflammation, demyelination and neurodegeneration that occurs in MS produces symptoms that range from spasticity, dystonia, tremor, ataxia and pain (Compston and Coles, 2008). Effective treatment for MS patients remains elusive. However, MS patients frequently self-medicate with cannabis to alleviate pain, spasticity, sleep disturbance and other MS symptoms (for review, see (Consroe et al., 1997; Pertwee, 2002)). This anecdotal evidence has been extensively investigated in the last 15 years and has generated solid data supporting that cannabinoid compounds and the endocannabinoid signaling system (ECS) offer a promising form of therapy for MS that covers the treatment of specific symptoms (e.g. spasticity, pain; see (Rog, 2010) for review), but that might be extended in the future to the control of disease progression (reviewed in (de Lago et al., 2009)).

Numerous studies have investigated the relation of the ECS with MS. For example, several studies have demonstrated that the CB_1R, CB_2R and the fatty acid amide hydrolase (FAAH) (i.e. enzyme that degrades the endocannabinoid anandamide (AEA)) are specific

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markers of plaque cell subtypes in human MS (Benito et al., 2007). There are elevated AEA levels in active MS lesions (Eljaschewitsch et al., 2006), lymphocytes and CSF samples (Centonze et al., 2007) or in plasma of MS patients (Jean-Gilles et al., 2009), in concordance with the increased levels of AEA found in the spinal cord and brain of CREAE mice during the spastic phase of disease (Baker et al., 2001). In contrast, other authors found lowered endocannabinoid levels in the CSF of MS patients during relapsing periods of the disease, compared to control subjects (Di Filippo et al., 2008). These changes reported for the ECS in patients and different MS models have been interpreted as adaptive responses aimed at limiting neuronal damage (reviewed by de Lago et al., 2009).

Preclinical studies have demonstrated that cannabinoids can alleviate MS associated symptoms such as spasticity (Baker et al., 2000) and exhibited anti-inflammatory, antioxidant, anti-excitotoxic and neuroprotective properties (Arévalo-Martín et al., 2003; de Lago et al., 2012; Loría et al., 2010; Pryce et al., 2003). Sativex®, an oromucosal spray (1:1 ratio of Δ^9-THC/ CBD) is a licensed drug in a number of EU countries and elsewhere for use in patients with MS-related spasticity and neuropathic pain. In clinical trials, Sativex® provide relief of spasticity symptoms showing clinically significant improvement in spasticity (30% or higher reduction from baseline) (Collin et al., 2007). Associated MS symptoms such as sleep disturbances, bladder problems and loss of motility show clear improvements (Russo et al., 2007). Safety studies indicate a low risk for serious adverse drug reactions. Both components of Sativex® have been described to independently modify immune responses during neuroinflammatory processes as occurs in MS and its experimental models. However, there is a lack of studies investigating the potential of Sativex® as a disease-modifying therapy for progressive MS. Experimental evidence for a neuroprotective effect of Sativex® has been recently reported in inflammatory models of Huntington disease (Valdeolivas et al., 2012). Recently a trial to investigate the use of cannabinoids in progressive inflammatory brain disease (CUPID) tested dronabinol (Δ^9-THC) in a cohort of patients with progressive MS (Zajicek et al., 2013). The results of this study showed that dronabinol has no overall effect on the progression of MS in its progressive phase, but it encouraged the use of experimental models that better represent progressive MS to continue investigating whether cannabinoids might change the disease course of progressive MS.

The experimental autoimmune encephalomyelitis (EAE) provides an excellent model to reproduce MS progression and the underlying pathological mechanisms (Croxford et al., 2011). After immunization with a portion of the myelin oligodendrocyte glycoprotein (MOG), the blood-brain barrier (BBB) becomes permeable to autoreactive lymphocytes and several days later mice exhibit typical MS relapses with symptoms such as weight loss and progressive tail and hindlimb weakness and paralysis.

In the present study we investigate the therapeutic potential of a Sativex-like combination of phytocan-

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nabinoids with a 1:1 combination of Δ^9-THC-BDS (10mg/kg) and CBD-BDS (10 mg/kg) as a disease-modifying therapy in chronic progressive EAE induced mice by inoculation of myelin oligodendrocyte glycoprotein (MOG). The treatment with the Sativex-like combination, Δ^9-THC-BDS or CBD-BDS on MOG immunized mice delayed symptoms onset. In the EAE affected mice, Sativex-like combination and Δ^9-THC-BDS treated animals showed better neurological score whereas CBD treated animals showed no improvement compared to vehicle treated mice. The treatment with Sativex-like combination or Δ^9-THC-BDS reduced cellular infiltrates in the spinal cord of EAE mice. Our results suggest beneficial effects of a Sativex-like combination and that this effect is caused mainly by Δ^9-THC-BDS via the activation of CB_1R.

Methods

Animals, treatments and sampling

The experiments were performed according to European regulations for experimental work with animals (directive 86/609/EEC). 6-8 week old Female C57BL/6 mice were purchased from Harlan Laboratories (Barcelona, Spain) and housed in our animal facilities with controlled photoperiod (12 h light/dark cycle), temperature (20 ± 1ºC) and relative humidity (40-60%). They had free access to standard food and water. EAE was induced using the method published by (Mendel et al., 1995). Briefly, mice were injected in each flank with an emulsion containing 200 mg of the 35-55 portion of MOG (Advanced Biotechnology Centre, Imperial College, London, UK) and 4 mg/ml of Mycobacterium tuberculosis (H37RA DIFCO Lab, Detroit, MI, USA) in a 1:1 mix with incomplete Freund's adjuvant (Sigma/Aldrich, Madrid, Spain) in phosphate-buffered saline (PBS). This injection was repeated after 7 days. To enhance the inflammatory response, mice received 1.5 mg/ml of *Pertussis* toxin (Sigma/Aldrich, Madrid, Spain) prepared in saline and administered i.p. on days 0 and 2. Control animals were obtained by inoculation with the same emulsion (complete Freund's adjuvant, CFA) without MOG. After inoculation, mice were daily scored using the following scale: 0, no clinical signs; 1, limp tail; 2, hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; and 5, moribundity or death. Daily weight loss was also recorded. First clinical signs appeared around day 11-12 after inoculation showing a worsening pattern compared to control animals inoculated with CFA (see Fig. 1) that progress up to day 16 post-inoculation. At the time point were first symptoms appear, mice were daily injected with a Sativex-like combination, 1:1 Δ^9-THC-BDS:CBD-BDS (10 mg/kg weight each; GW Pharmaceuticals Ltd. UK) or vehicle (experiment I). In a second set of experiments aimed to further explore the action of THC, animals were treated with Δ^9-THC-BDS (10 mg/kg weight) or in combination with the selective CB_1R antagonist SR141716 (5 mg/kg weight; kindly provided by Sanofi-Aventis, Mont- pellier, France) or the PPARγ inhibitor T0070907 (5 mg/kg weight; purchased from Tocris Cookson Ltd. Bristol, UK) (experiment II). SR141716 and T0070907 were injected 30 min before Δ^9-THC-BDS, or vehicle, following the same schedule than in the previous experiments. Researchers blinded to the treatment evaluated the neurological status of all animals. Immediately after neurological evaluation at the day 16, animals were perfused with cold PBS and their brains and spinal cords were removed and rapidly frozen by immersion in 2-methylbutane cooled in dry-ice. Samples were stored at -80 ºC until used for analysis (n = 7-8 subjects per experimental group). In a few cases, spinal cords were fixed in 4% paraformaldehyde overnight followed by a cryoprotective treatment with 20% sucrose, and they were used for histological analyses (n = 4-5 per experimental group).

Nissl staining

Frozen spinal cords were sliced with a cryostat at the lumbar level to obtain coronal sections (20 µm thick) that were collected on gelatin-coated slides. Slices were used for Nissl staining using cresyl violet, which permitted to de-

termine the effects of particular treatments on cell aggregates.

Western Blotting

Spinal cords were lysed in Tris-HCl buffer and subjected to centrifugation at 15,000 × g for 30 min at 4°C. Proteins for each extract were electrophoresed in SDS-PAGE. Precision Plus ProteinTM Standards (Bio-Rad Laboratories, Madrid, Spain) were included on each gel. Proteins were then electroblotted onto PVDF mem- branes (GE Healthcare UK Ltd., Buckinghamshire, UK). The resulting blot was incubated with an antibody anti COX2 (1:1000, Santa Cruz, USA) overnight at 4°C in blocking buffer. Finally, blots were incubated with monoclonal anti-rabbit Ig peroxidase conjugate (1:5000; GE Healthcare UK Ltd., Buckinghamshire, UK) for 1h at room temperature and revealed with Amersham ECLTM Western Blotting Detection Reagents (GE Healthcare UK Ltd., Buckinghamshire, UK).

Results

Phytocannabinoids delay and improve the neurological decline in EAE.

In this study, we used mice treated with MOG that generates a progressive pattern of EAE induction with neurological disabilities that start at 11 days post-inoculation and progress during the following days. Control animals (CFA-treated) exhibited no neurological decline at all days examined (data not shown). We conducted the pharmacological experiments with Sativex-like combination, Δ^9-THC-BDS or CBD-BDS in an early stage of the disease, administering the first dose at 11 days post-inoculation when symptoms onset. As expected, the administration of all the treatments had a positive effect in delaying symptoms onset (fig. 1). However, only the Sativex-like combination and the Δ^9-THC-BDS succeed in reducing the neurological disability and improving motor coordination of EAE mice.

Phytocannabinoids reduce the number of cell infiltrates in the spinal cord of EAE mice and Δ^9-THC-BDS diminishes COX2 activation.

Spinal cord sections of MOG-induced EAE show the presence of cell aggregates presumably formed by infiltrated

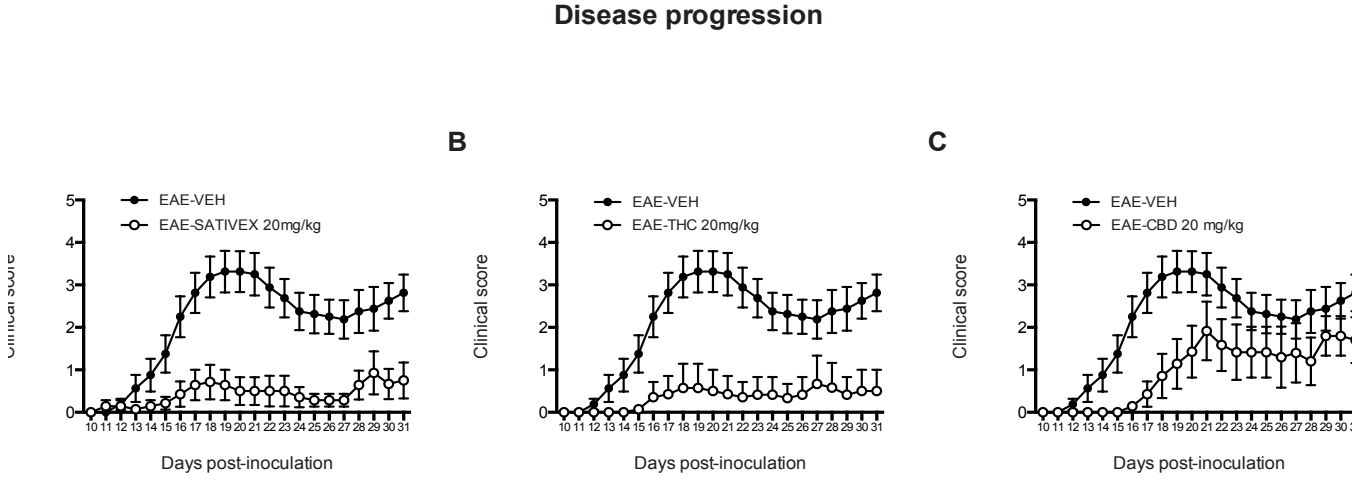

Figure 1: Phytocannabinoids delay and improve the neurological decline in EAE. Sativex-like combination (A), Δ^9-THC-BDS (B) and CBD-BDS (C) delay the first relapse in treated EAE mice. However, Sativex-like combination and Δ^9-THC-BDS are the only treatments that reduce the neurological decline in treated EAE mice.

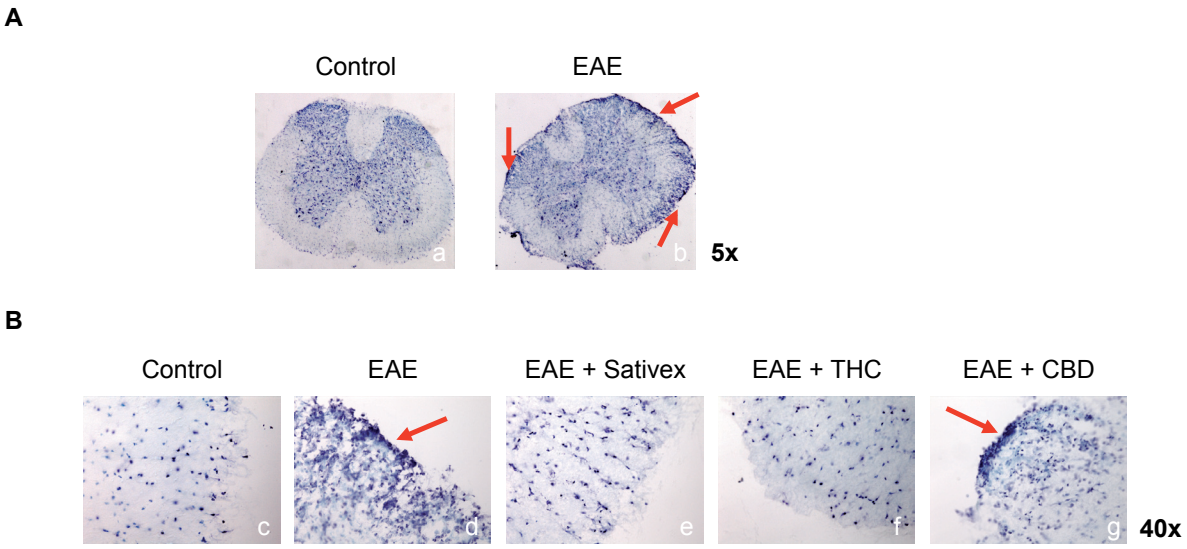

Figure 2. BS-Sativex treatment decreases lymphocyte infiltration and cell aggregation. Nissl stainings of EAE mice spinal cords. In MOG-induced EAE mice, there are cell agreggates (b 5x). Sativex-like combination and Δ^9-THC-BDS treated animals show a marked reduction in cell agreggates (40x e,f). On the other side, CBD-BDS failed reducing the number of aggregates in EAE mice.

lymphocytes and recruited macrophages in the white matter as seen with Nissl stainings. In previous studies (see (de Lago et al., 2012) we demonstrated that these aggregates are mainly formed by activated microglial cells and and recruited peripheral macrophages. These aggregates are reduced in the EAE mice treated with either Δ^9-THC-BDS or Sativex-like combination (fig. 2). However, there is no reduction in the presence of aggregates in the EAE mice treated with BS-CBD. This result suggests that Sativex-like combination treatment restricts the infiltration of activated lymphocytes through the blood-brain barrier.

In addition we analysed the protein levels of COX2 by western blotting (see figure 3). Although our results are preliminary, the data suggest that Δ^9-THC-BDS treatment reduces the activation of COX2 in the spinal cord of EAE mice.

The effect of the Δ^9-THC-BDS is mediated by CB_1R and not by the anti-inflammatory target PPARγ.

To check whether the beneficial effects of the Sativex-like combination were due to Δ^9-THC-BDS activation of CB_1R and not by acting through other anti-inflammatory targets, we performed a second set of experiments using selective antagonists. In this experiment we treated MOG-induced EAE mice with an i.p. injection either with the CB_1R antagonist rimonabant or the PPARγ inhibitor T0070907 30 min prior to Δ^9-THC-BDS injection. Rimonabant completely blocked the effect of Δ^9-THC-BDS treatment in EAE mice. By contrast, PPARγ nuclear receptor does not mediate the effect of Δ^9-THC-BDS treatment, as T0070907 did not reverted the Δ^9-THC-BDS effect.

DISCUSSION

Our results show the effect of the

Sativex in EAE

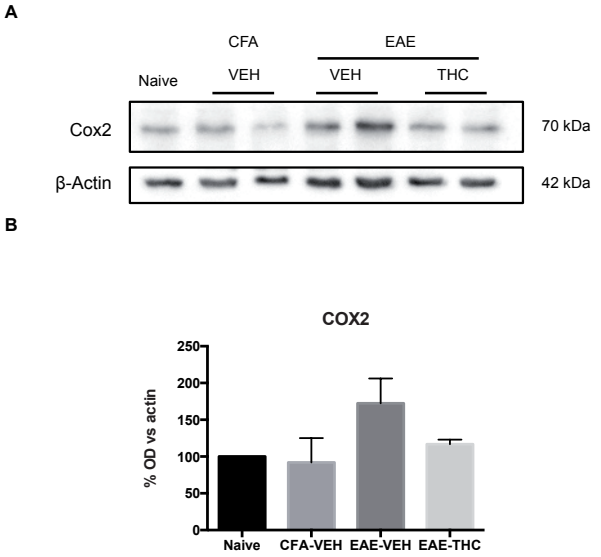

Figure 3. The effect of BS-Δ⁹-THC in EAE mice affects COX2 activation. A) Representative image of COX2 detection by western blotting in spinal cord lysates B) The quantification of the western blott shows that COX2 levels are increased in EAE mice and that the treatment with Δ⁹-THC-BDS decreases COX2 levels.

phytocannabinoids Δ⁹–THC–BDS, CBD–BDS and the combination of both in a Sativex–like combination as a disease–modifying treatment in MOG–induced EAE in mice. Δ⁹–THC–BDS, CBD–BDS and the Sativex–like combination delay the first relapse when EAE mice are treated at symptom onset. Histopathological studies of spinal cord sections showed that the treatment with Δ⁹–THC–BDS and the Sativex–like combination reduce the cell aggregates present in the spinal cord of MOG–induced EAE mice. In addition, BS–Δ⁹–THC reduced COX2 activation present in MOG–induced EAE mice. Rimonabant completely blocked the effect of the treatment with Δ⁹–THC–BDS.

These results confirm the effect of phytocannabinoids in the modulation of the progression of MS. The first evidence was published in 1989, where Δ⁹–THC reduced inflammation clinically and histopathologically in EAE rats (Lyman et al., 1989). Δ⁹–THC reduces spasticity and tremor in chronic relapsing EAE (Baker et al., 2000). In our hands Δ⁹–THC–BDS also delay the neurological decline present in EAE mice. The doses we used confirm the results published by (Maresz et al., 2007), where Δ⁹–THC 10 mg/kg, 20 mg/kg and 25 mg/kg was effective reducing the symptoms of EAE in mice. CBD at different doses (0.5 mg/kg, 5mg/kg, 10 mg/kg or 25 mg/kg) did not have any effect in EAE mice. In our hands low

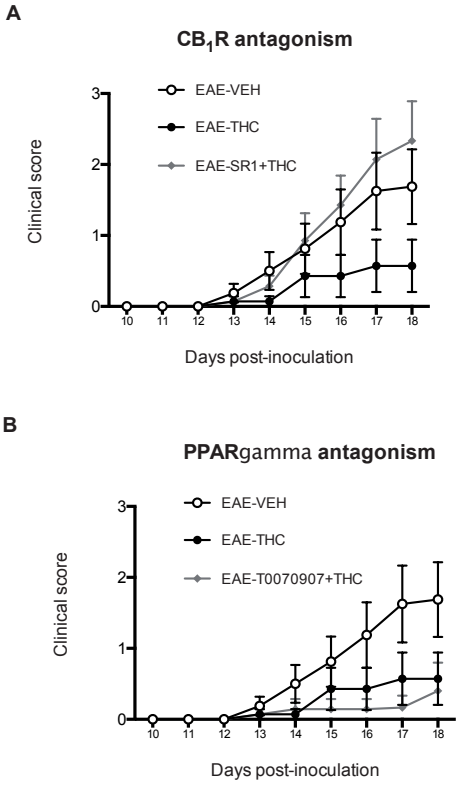

Figure 4: The effect of the Δ⁹-THC-BDS is mediated by CB_1R. Effect of Δ⁹-THC-BDS treatment in EAE mice in the presence of the CB_1R antagonist rimonabant (A) or the PPARγ inhibitor T0070907 (B). Rimonabant fully blocked the effect of Δ⁹-THC-BDS treatment in EAE mice, confirming that CB_1R mediates the response of Δ⁹-THC-BDS.

doses of 3 mg/kg Δ^9-THC-BDS, CBD-BDS and the Sativex-like combination were not effective to delay neurological decline in EAE mice (data not shown). At higher doses CBD-BDS had no effect either. This contradicts data already published where acute treatment with CBD was effective ameliorating EAE in mice (Kozela et al., 2011). In the TMEV-IDD model of MS CBD was also effective diminishing the severity of symptomatology (Mecha et al., 2013). The difference of TMEV-IDD and EAE can be explained by different pathological events that take place in each of the models, thus one model complements the other resuming the symptoms of human MS. Besides, we have seen that Δ^9-THC-BDS reduces the increased levels of COX2 in EAE mice. COX2 produce prostaglandins from arachidonic acid. This inducible enzyme is activated by several pro-inflammatory cytokines and is related with inflammatory response (Goppelt-Struebe and Beiche, 1997). COX2 levels are increased macrophages of MS patients (Yiangou et al., 2006). PPARγ is a nuclear receptor that promotes anti-inflammatory responses and can be activated by cannabinoids (O'Sullivan and Kendall, 2010). In a model of Parkinson's disease, PPARγ mediated neuroprotective effects of Δ^9-THC (Carroll et al., 2012). However in EAE mice, the effect of Δ^9-THC-BDS is not mediated by PPARγ but by CB_1R. The importance of the CB_1R in EAE has been already described (de Lago et al., 2012; Maresz et al., 2007; Pryce et al., 2003). CB_1R activation has psychoactive effects that if too strong, can be problematic for its use as a clinical target. The approval of the oromucosal spray Sativex® (1:1 ratio of Δ^9-THC/ CBD) in several countries for the treatment of spasticity and neuropathic pain in MS patients favors the investigation about the therapeutic potential of cannabinoids as modifying disease agents. Sativex® gives a low-dose of THC in plasma, thus producing none to mild negative effects. The safety of Sativex® has been proved in a number of publications (Collin et al., 2007; Flachenecker et al., 2014; Hilliard et al., 2007). However, there is a lack of studies evaluating the Sativex® use in progressive MS as a disease modifying drug. Although the effect of Sativex in spasticity has already been shown in EAE (Hilliard et al., 2007) our study is the first to address the effect of a Sativex-like combination of phytocannabinoids in primary progressive MS and the results obtained are highly promising suggesting a neuroprotective profile of Sativex®.

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Objective #3

Characterization of the ECS both in the motor neuron cell line NSC-34 and in SOD^{1G93A} transgenic mice and study of the use of a Sativex-like combination of Δ^9-THC-BDS and CBD-BDS as disease modifying agent.

In this chapter we have identified for the first time the components of the ECS i.e. CB_1R, CB_2R and the endocannabinoid synthesis and degrading enzymes NAPE-PLD, DAGL, FAAH and MAGL in the NSC-34 cell line and the ALS model B6SJL-Tg(SOD1^{G93A})1Gur/J mice. The NSC-34 cell line is a motor neuron cell line obtained from the fusion of neuroblastoma and spinal cord motor neurons. Cells were grown in a complete growth medium (i.e. DMEM with 10% fetal bovine serum) or in a differentiation medium (i.e. DMEM F-12 with 1% fetal bovine serum). We used different approaches to measure the endocannabinoid system components: western blotting, RT-PCR and immunocytochemistry for the cannabinoid receptors and RT-PCR for the NAPE-PLD, DAGL, FAAH and MAGL enzymes, both in undifferentiated and differentiated NSC-34 cells.

Our results showed an up-regulation of the CB_1R expression in the differentiated cells versus the non-differentiated cells. FAAH expression was increased in differentiated cells. As expected, there was no expression of the CB^2R.

B6SJL-Tg(SOD1^{G93A})1Gur/J mice are the ALS model used in pre-clinical research. Transgenic mice start to develop tremor at around 70 days old that progresses to hindlimb paralysis and death around 50-60 days later. We characterized the mRNA levels of endocannabinoid receptors and endocannabinoid synthesis and degradation enzymes at late stage of the disease in the spinal cord of both transgenic SOD1^{G93A} males and females and their non-transgenic littermates. The results showed a significant up-regulation of the CB_2R both in transgenic SOD1^{G93A} males and females and an up-regulation of the NAPE-PLD enzyme in transgenic SOD1^{G93A} males.

We also analyzed for the first time the therapeutic potential of the phytocannabinoid based medicine Sativex® both in males and females in transgenic SOD1^{G93A} mice and their non-transgenic littermates. The treatment with a Sativex-like combination of 20 mg/kg Δ^9-THC-BDS and 20 mg/kg of CBD-BDS at symptoms onset showed an improvement in the neurological decline of transgenic animals in the initial steps of the disease, although this effect was lost over time. Our treatment did not show any benefit in the behavioural tests (i.e. rotarod and grid test), or in the muscular volume in the NMR scan of males aged 100 days old. However, Nissl-stained spinal cords from end stage animals treated with the Sativex-like combination had a higher number of motor neurons in the dorsal horn compared to vehicle-treated animals.

CONCLUSION:

The ECS is altered during ALS progression in SOD1^{G93A} transgenic mice and the use of a Sativex-like combination of Δ^9-THC-BDS and CBD-BDS has mild effect in the initial stages of the disease.

Papers in this chapter:

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Identification of receptors and enzymes for endocannabinoids in NSC-34 cells: Relevance for *in vitro* studies with cannabinoids in motor neuron diseases

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ABSTRACT

NSC-34 cells, a hybridoma cell line derived from the fusion of neuroblastoma cells with mice spinal cord cells, have been widely used as an *in vitro* model for the study of motor neuron diseases [*i.e.* amyotrophic lateral sclerosis (ALS)]. In the present study, they were used to characterize different elements of the cannabinoid signaling system, which have been reported to serve as targets for the neuroprotective action of different natural and synthetic cannabinoid compounds. Using RT-PCR, Western blotting and immunocytochemistry, we first identified the presence of the cannabinoid CB_1 receptor in these cells. As expected, CB_2 receptor is not expressed in this neuronal cell line, a result that is concordant with the idea that this receptor type is preferentially expressed in glial elements. Diacylglycerol-lipase (DAGL) and N-arachidonoylphosphatidylethanolamine-phospholipase D (NAPE-PLD), the enzymes that synthesize endocannabinoids, have also been detected in these cells using RT-PCR, and the same happened with the endocannabinoid-degrading enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol-lipase (MAGL). The presence of the CB_1 receptor in these cells supports the idea that this receptor may play a role in the regulation of cellular survival face to excitotoxic injury. Interestingly, the expression of CB_1 receptor (and also the FAAH enzyme) was strongly up-regulated after differentiation of these cells, as previously reported with glutamate receptors. No changes were found for NAPE-PLD, DAGL and MAGL. Assuming that glutamate toxicity is one of the major causes of neuronal damage in ALS and other motor neurons diseases, the differentiated NSC-34 cells might serve as a useful model for studying neuroprotection with cannabinoids in conditions of excitotoxic injury, mitochondrial malfunctioning and oxidative stress.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the selective loss of motor neurons in the spinal cord, brainstem, and motor cortex [19]. ALS exists in two forms, familial ALS (only 5% of cases) and sporadic ALS (most of cases) [4]. Complete pathogenic causes of ALS are presently unknown but several mechanisms have been suggested and these include excitotoxicity, chronic inflammation, oxidative damage and protein aggregation [4,9,17]. For example, several studies have identified changes in the function of glutamate transporters that have been associated with the initiation of the disease (reviewed in [9]). High amounts of activated microglia have been found in those brain regions that are affected in ALS patients [21]. Lastly, genetic studies have identified several mutations in the copper-zinc superoxide dismutase (SOD-1), a key antioxidant enzyme, in approximately 20% cases of familial ALS [16], being pathological through a gain-of-neurotoxic function. However, other mutated genes, *i.e.* TDP-43 and FUS, have also been recently identified and related to the disease and to mechanisms other than oxidative injury, leading to a novel molecular exclusive classification of ALS cases (reviewed in [13]).

Despite the intensive research conducted in the last years, an effective treatment for this disease remains elusive, with Rilutek® as the only licenced medicine [11]. Recent evidence points that

Abbreviations: ALS, amyotrophic lateral sclerosis; CB1 receptor, cannabinoid receptor type 1; CB2 receptor, cannabinoid receptor type 2; DAGL, diacylglycerol-lipase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol-lipase; NAPE-PLD, N-arachidonoylphosphatidylethanolamine-phospholipase D; SOD-1, superoxide dismutase-1.

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cannabinoids may be beneficial as neuroprotectant agents in ALS (reviewed in [3]). Cannabinoids include some active ingredients present in *Cannabis sativa* (termed phytocannabinoids), various intercellular signaling lipids (so-called endocannabinoids) and different synthetic molecules. Their neuroprotective properties have been studied in different neurodegenerative disorders and would be based on their ability to decrease excitotoxicity, microglial activation, neuroinflammation and oxidative stress (see [8] for a recent review), then becoming an interesting therapeutic option in ALS too. In fact, the administration of Δ^9-tetrahydrocannabinol was effective in delaying motor impairment and prolonging survival in the SOD-1 (G93A transgenic mice) mouse model of ALS [14]. Similar results were reported with cannabinol, a less psychotrophic plant-derived cannabinoid [22], with the synthetic cannabinoid WIN55,212-2 [2], or with the selective CB_2 agonist AM1241 [12,18]. In parallel, genetic ablation of fatty acid amide hydrolase (FAAH) enzyme leading to elevated levels of endocannabinoids also prevented the appearance of disease signs in SOD1 mutant mice [2], whereas genetic ablation of the CB_1 receptor had no effect on the onset of the disease in this model [2]. Taken together, these results show that cannabinoids might have neuroprotective effects in ALS mediated by the combination of different mechanisms. In part, these mechanisms might be related to the cannabinoid receptor-independent antioxidant properties of certain cannabinoids, but the data obtained with WIN55,212-2 [2], which is not antioxidant, as well as using FAAH deficient mice [2] or treatments with selective CB_2 receptor agonists [12,18], suggest an additional contribution of this cannabinoid receptor type associated with the important role of glial elements in this disease [17,21]. An important aspect of these previous pharmacological studies is that they were conducted in absence of data on the changes that the development of ALS causes in the receptors and enzymes for endocannabinoids, which may be an important factor to determine the efficacy of potential cannabinoid treatments. Only a couple of studies have explored this issue and described elevated levels of CB_2 receptors in microglia from post-mortem human spinal cords of ALS patients [25] or elevated levels of endocannabinoids in the spinal cord of SOD-1 mutant mice [23].

In the present study, we used NSC-34 cells, a hybridoma cell line derived from the fusion of neuroblastoma cells with mice spinal cord cells [5]. These cells have been widely used as an *in vitro* model for the study of ALS and other disorders affecting motor neurons, in particular after they become differentiated by serum depletion [7]. Both non-differentiated and differentiated cells have been used for the evaluation of the effects of potential neuroprotective compounds against different insults (*i.e.* excitotoxins, mitochondrial toxins, oxidants, *etc.*) affecting cell survival [7,10,15,20,24]. We plan to use these cells for evaluating the effects of various types of cannabinoid compounds that have shown neuroprotective effects in other disorders. However, before these studies, we wanted to identify and analyze, using RT-PCR, Western blotting and immunocytochemistry, the presence in these cells of those elements of the cannabinoid signaling system (receptors and enzymes) that have been reported to serve as targets for the neuroprotective action of different natural and synthetic cannabinoid compounds.

2. Materials and methods

2.1. Cell culture

NSC-34 cells (purchased from Cedarlane Laboratories Ltd., Ontario, Canada) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM ultra-glutamine and 1% penicillin/streptomycin (LONZA, Verviers, Belgium), and under a humidified 5% CO_2 atmosphere at 37 °C. To slow the proliferation of these cells and enhance their maturation towards a differentiated state, they were grown in a medium containing 1:1 DMEM plus Ham's F12, 1% fetal bovine serum, 1% penicillin/streptomycin, and 1% modified Eagle's medium with non-essential amino acids (Sigma–Aldrich, St. Louis, MO, USA), as previously described [7]. For each experiment, cells were seeded at 2×10^4 cells/mm^2.

2.2. Immunocytochemistry

Cells were grown in 24-well laminin-coated plates (200,000 cells per well) overnight, then fixed in cold 4% paraformaldehyde for 30 min at 4 °C and permeabilized in 1:1 methanol–acetone. Cells were incubated overnight at 4 °C with the primary antibodies anti-CB_1 (1:400, Pierce Biotechnology, Rockford, IL, USA) or anti-SMI-312 (1:1000, Covance, Emerville, CA, USA). The secondary antibody was added for 2 h at 37 °C (Alexa Fluor® 488 donkey anti-rabbit IgG; Invitrogen Corp., Life Technologies, Madrid, Spain). Hoechst staining was also used for the identification of cell nuclei. A Leica DM-IL microscope and a Leica DFC-300FX camera were used for well observation and photography.

2.3. Western blotting

Cells were lysed in ice-cold RIPA buffer and subjected to centrifugation at $40{,}000 \times g$ for 30 min at 4 °C. Proteins for each extract were electrophoresed in SDS-PAGE. Precision Plus Protein™ Standards (Bio-Rad Laboratories, Madrid, Spain) were included on each gel. Proteins were then electroblotted onto PVDF membranes (GE Healthcare UK Ltd., Buckinghamshire, UK). The resulting blot was incubated with an antibody anti-CB_1 (1:400, Pierce Biotechnology, Rockford, IL, USA) during 2 h at room temperature in blocking buffer. Finally, blots were incubated with monoclonal anti-rabbit Ig peroxidase conjugate (1:2500; GE Healthcare UK Ltd., Buckinghamshire, UK) for 1 h at room temperature and revealed with Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare UK Ltd., Buckinghamshire, UK).

2.4. Reverse transcription (RT) and real-time polymerase chain reaction (PCR)

mRNA (1 μg) was reverse transcribed into cDNA using the QuantiTect® reverse transcription kit (Qiagen) with poly-dT primers. For the genes used in this study (CB_1, CB_2, DAGL, NAPE, FAAH) we used specific TaqMan gene expression assays (Cnr2, NM_009924.3; Cnr1, NM_007726.3; DAGL,

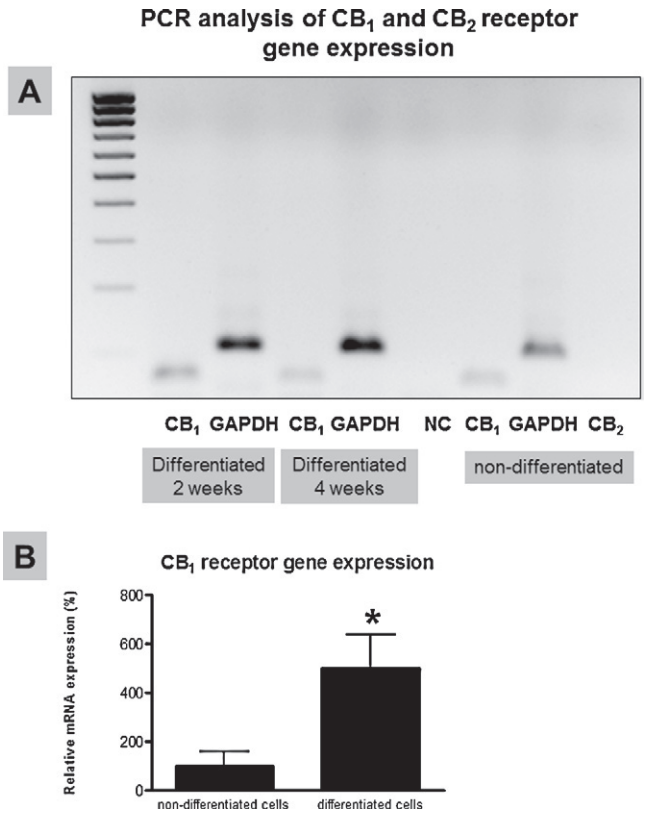

Fig. 1. PCR analysis of CB_1 and CB_2 receptor gene expression in non-differentiated and differentiated (after 2 or 4 weeks) NSC-34 cells (panel A; NC = negative control). Quantitative RT-PCR analysis of gene expression for the CB_1 receptor in non-differentiated *versus* differentiated (after 4 weeks) (panel B). Values represent means ± SEM of more than 5 different groups of cells and are expressed as % over the level of expression in non-differentiated cells. Data were analyzed by the Student's *t*-test (*$p < 0.05$).

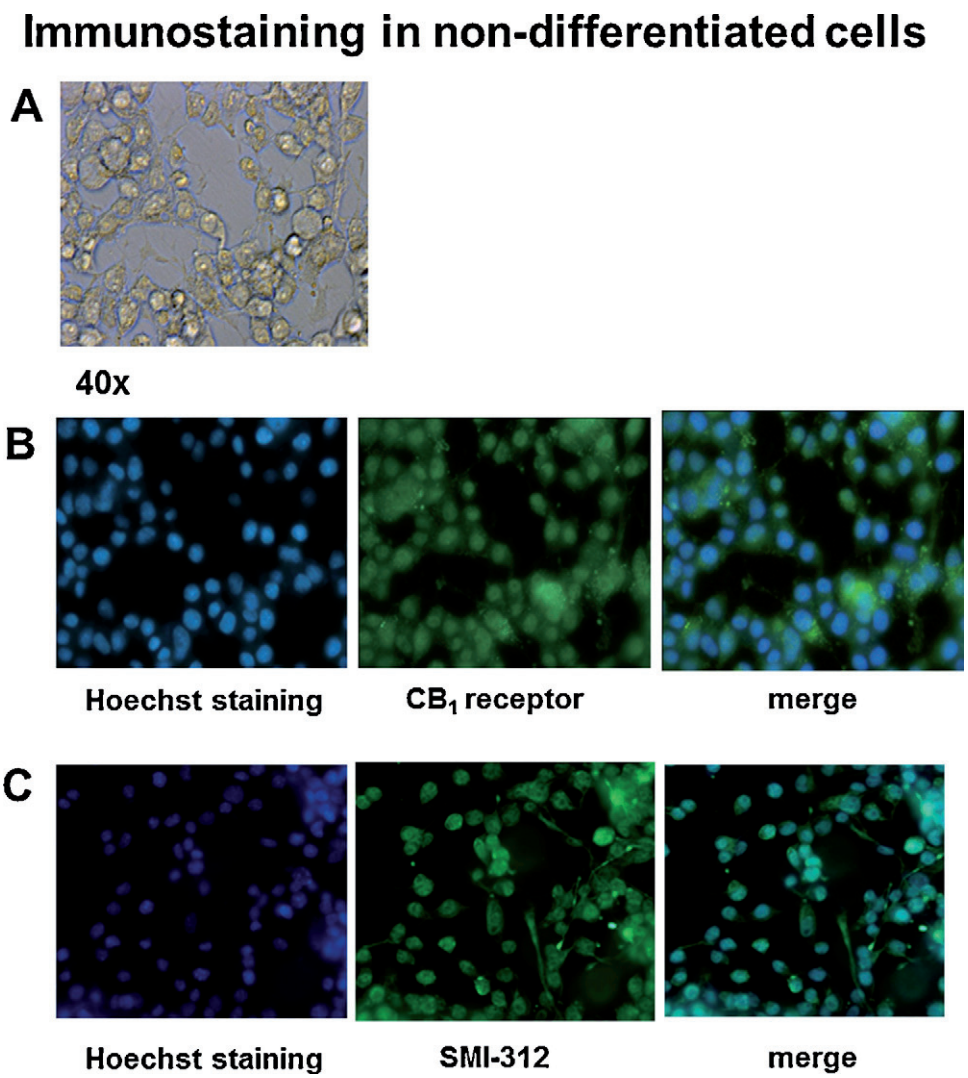

Fig. 2. Immunostaining for CB_1 receptors (panels B) and SMI-312 (panels C) against Hoechst staining in non-differentiated NSC-34 cells. Panel A contains a phase-contrast microphotograph of the area used for immunostainings. Non-differentiated cells present an immature form characterized by a rounded morphology, a small size and a few and short neuronal processes, as reported by Eggett et al. [7]. See details in the text. Magnification was always 40×.

Mm_008138300_m1; NAPE, Mm_00724591_m1; FAAH, Mm_00515684_m1; Applied Biosystems, Life Technologies, Madrid, Spain) or synthetic primers (MAGL, forward: 5′-CAGAGAGGCCAACCTACTTTTC-3′; reverse: 5′-ATGCGCCCCAAGGTCATATTT-3′). Each sample was assayed at least in duplicate and a 6-point standard curve was run in parallel. To ensure the absence of genomic DNA contamination, a control sample of non-reverse-transcribed RNA was run for each set of RNA extractions. Relative quantification was obtained by calculating the ratio between the values obtained for each gene of interest and the housekeeping gene GAPDH or 18 s. Results are expressed as a percentage of control condition.

2.5. Data analysis

All data were assessed by Student's *t*-test using the GraphPad software (version 4.0).

3. Results

We have first identified the presence of the cannabinoid CB_1 receptor in NSC-34 cells. This has been observed by using PCR (Fig. 1) and immunocytochemistry (Fig. 2), and even checked out by western blotting (data not shown). The presence of CB_1 receptors was evident not only in non-differentiated cells (Figs. 1 and 2) but also when these cells become differentiated after 2 (Fig. 1) or 4 weeks (Figs. 1 and 3) of being cultured with an appropriate medium. In both cases, immunostaining with the neurofilament protein SMI-312 was used as control for cell specificity, and, in both cases CB_1 and SMI-312 immunostaining was found in the same cells (Figs. 2 and 3). Details of the morphological characteristics of both types of cells can be found in the legends of Figs. 2 and 3. We also quantified the CB_1 receptor expression in non-differentiated and differentiated cells by using RT-PCR and found a significant up-regulation (5-fold) in differentiated cells (Fig. 1), similar to data described by Eggett et al. [7] with ionotropic and metabotropic glutamate receptors whose expression was strongly promoted after cell differentiation.

In contrast with CB_1 receptor, CB_2 receptor expression was not found in this neuronal cell line (Fig. 1) and it was not affected by cell differentiation (data not shown). This fact is concordant with the idea that this receptor type is preferentially expressed in glial elements [8]. However, other elements of the cannabinoid signaling system, as the enzymes that synthesize endocannabinoids, DAGL and NAPE-PLD, were found in non-differentiated cells using RT-PCR but their expression was not altered after cell differentiation (Fig. 4). Lastly, mRNA levels for the endocannabinoid-degrading enzymes FAAH and MAGL were also measurable in non-differentiated cells. Interestingly, cell differentiation did produce a significant up-regulation (2-fold) in FAAH with no changes in MAGL (Fig. 4).

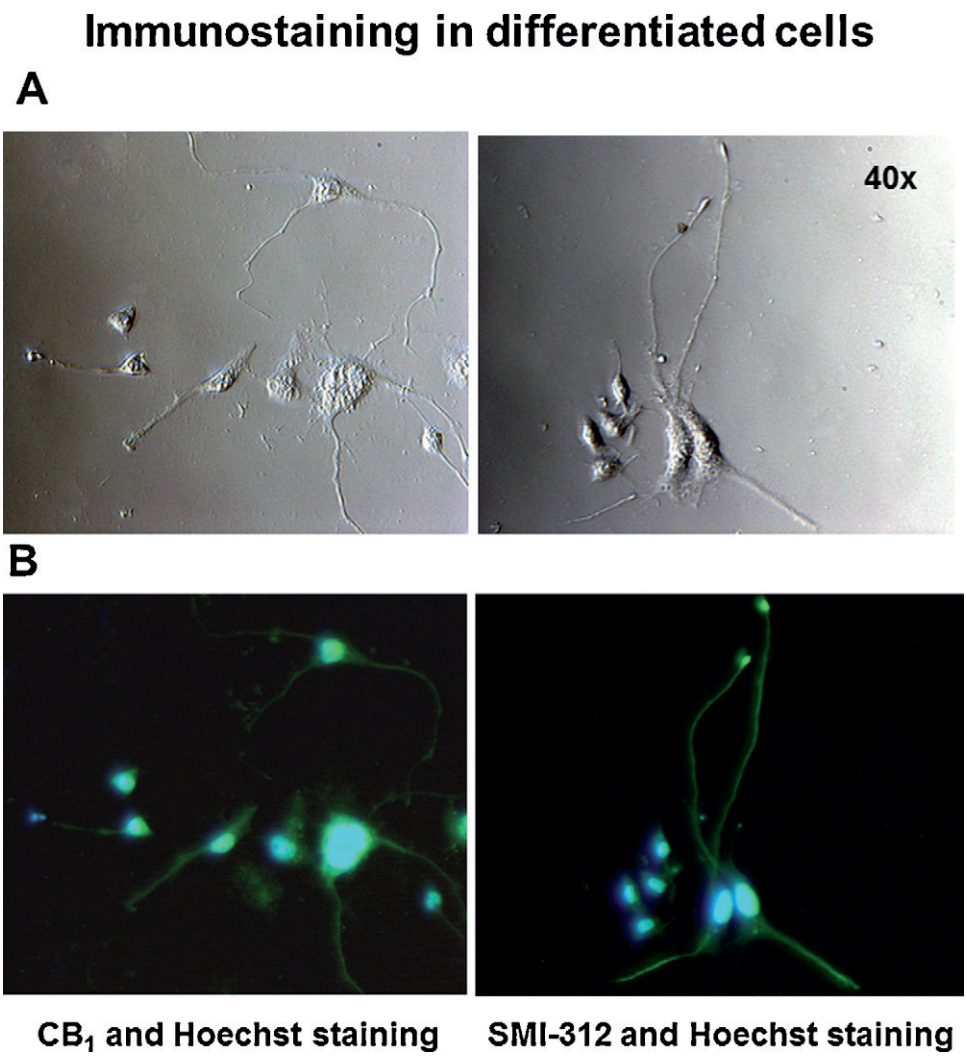

Fig. 3. Immunostaining for CB_1 receptors (left panel B) or SMI-312 (right panel B) against Hoechst staining in differentiated (after 4 weeks) NSC-34 cells. Panels A contain phase-contrast microphotographs of the area used for immunostainings. In contrast with non-differentiated cells, differentiated ones present a different morphology characterized by the formation of numerous and longer neuronal processes. See details in the text. Magnification was always 40×.

4. Discussion

NSC-34 cells were generated as a hybridoma cell line derived from the fusion of neuroblastoma cells with mice spinal cord cells [5]. They express many morphological and physiological characteristics of primary motor neurons, *i.e.* extension of neurites, generation of action potentials, activity of choline acetyltransferase (it allows acetylcholine synthesis), acetylcholine storage and release, expression of neurofilament triplet proteins, and synapse formation with myotubes [5,7], which enable these cells to be used as an *in vitro* model of neurotoxicity [6] useful for the study of those pathologies directly or indirectly affecting the motor neuron, *i.e.* multiple sclerosis [15], spinal injury [10,24] and, in particular, ALS [7,20]. Several compounds that showed capability to enhance cell survival against several chemicals known to be toxic to these cells may be further tested with positive results in *in vivo* models of these diseases (see [1] for review).

We are interested in using these cells for evaluating the effects of various types of cannabinoid compounds that have shown neuroprotective effects in other disorders (reviewed in [8]). However, before that, we wanted to identify whether these cells contain those elements of the cannabinoid signaling system that have been reported to serve as targets for the neuroprotective action of different natural and synthetic cannabinoid compounds, as well as to analyze their levels. Our results demonstrate that these cells contain the CB_1 receptor in concordance with the preferential location of this receptor in neuronal elements. This fact was demonstrated using different methodological strategies, *i.e.* using RT-PCR, immunocytochemistry, and also western blotting. Accordingly, these cells do not contain the other major cannabinoid receptor type, CB_2, which is characteristic of glial rather than neuronal elements [8]. In previous studies aimed at demonstrating the role of both receptors in neuroprotective properties of cannabinoids, CB_1 receptors have been preferentially related to the control of excitotoxic damage given their well-known location in presynaptic terminals of glutamatergic neurons and their inhibitory effects on glutamate release [8]. By contrast, CB_2 receptors have been involved preferentially in the control of glial-related processes as the generation of different types of compounds active on inflammatory states [8]. This difference, as well as the fact that the expression of CB_1 receptor (and also the FAAH enzyme; see below) was strongly up-regulated after differentiation of these cells by modification of growth conditions (*i.e.* serum depletion), as previously reported that happens with different markers of glutamate transmission (*i.e.* ionotropic and metabotropic receptors [7]), support the idea that CB_1 receptors may be beneficial against the damage of these cells when they are subjected to conditions of excitotoxic injury, particularly in the case of differentiated cells.

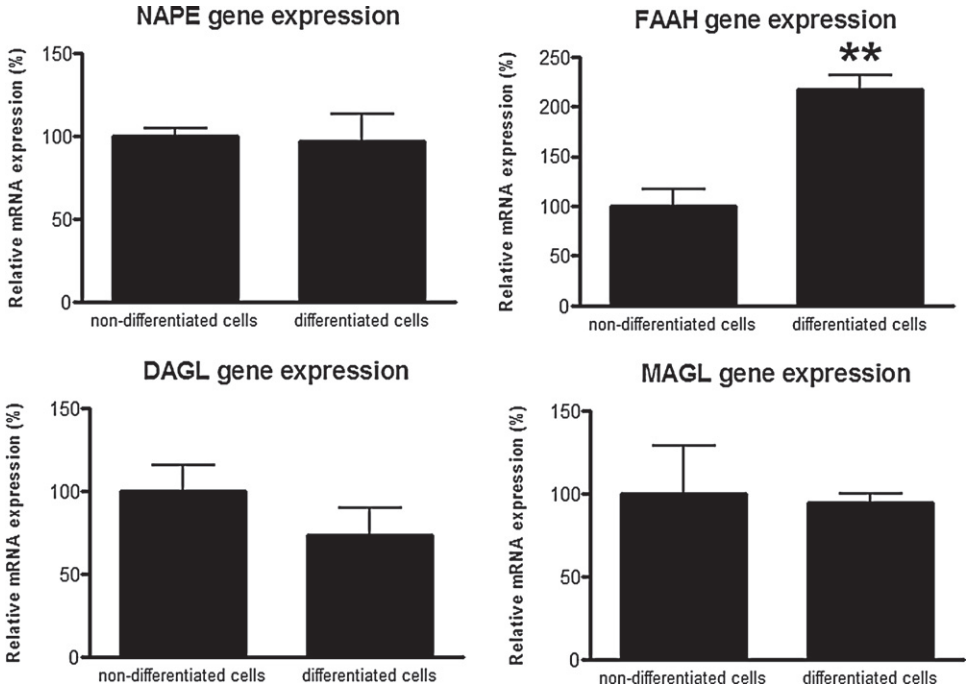

Fig. 4. Quantitative RT-PCR analysis of gene expression for the enzymes FAAH, MAGL, NAPE-PLD and DAGL in non-differentiated *versus* differentiated (after 4 weeks). Values represent means ± SEM of more than 5 different groups of cells and are expressed as % over the level of expression in non-differentiated cells. Data were analyzed by the Student's *t*-test (**$p < 0.01$).

Thus, Eggett and coworkers [7] found that the exposure of differentiated NSC-34 cells to glutamate causes cell death associated with enhanced production of reactive oxygen species and with an increase in intracellular calcium levels. These changes were prevented by anti-excitotoxic compounds such as glutamate receptor antagonists [7]. Given the well-known positive effects of CB_1 receptor agonists on glutamate transmission, calcium homeostasis and toxicity by reactive oxygen species, it is expected that they may also preserve differentiated NSC-34 cells against excitotoxic stimuli. We are presently analyzing this possibility with the idea that, if results are positive, to study this property in *in vivo* models of ALS, in which excitotoxicity may represent a key pathogenic mechanism.

In addition to CB_1 receptors, other elements of the endocannabinoid signaling system were also identified in NSC-34 cells. This includes the two major enzymes synthesizing endocannabinoids, *i.e.* NAPE-PLD and DAGL, as well as the degradative enzymes FAAH and MAGL. These four enzymes were found in both non-differentiated and differentiated cells, with equivalent levels of expression for NAPE-PLD, DAGL and MAGL, but with a significant increase in the case of FAAH after cell differentiation, similar to the one found for the CB_1 receptor. The physiological meaning of this response is still unknown and would need additional experimentation, but it may be of interest given that inhibition of FAAH enzyme has been used as a pharmacological strategy to challenge the endocannabinoid system in pathological conditions.

5. Conclusion

Different elements of the endocannabinoid signaling system have been identify in cultured NSC-34 cells, including the CB_1 receptors and the enzymes responsible of the synthesis and degradation of endocannabinoids. The presence of the CB_1 receptor supports the idea that this receptor may play a role in the regulation of cellular survival face to excitotoxic injury. Interestingly, the expression of CB_1 receptor (and also the FAAH enzyme) was highly up-regulated after differentiation of these cells, as previously reported that happens with ionotropic and metabotropic glutamate receptors [7]. No changes were found for NAPE-PLD, DAGL and MAGL. Assuming that glutamate toxicity is one of the major causes of neuronal damage in ALS and other motor neurons diseases, the differentiated NSC-34 cells might serve as a useful model for studying neuroprotection with cannabinoids in conditions of excitotoxic injury, mitochondrial malfunctioning and oxidative stress.

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ORIGINAL ARTICLE

CNS Neuroscience & Therapeutics

Changes in Endocannabinoid Receptors and Enzymes in the Spinal Cord of SOD1^{G93A} Transgenic Mice and Evaluation of a Sativex®-like Combination of Phytocannabinoids: Interest for Future Therapies in Amyotrophic Lateral Sclerosis

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Keywords
Amyotrophic lateral sclerosis; Cannabinoids; CB_1 and CB_2 receptors; Endocannabinoid enzymes; Sativex-like combination of phytocannabinoids; SOD1 mutant mice.

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SUMMARY

Aims: Cannabinoids afford neuroprotection in SOD1^{G93A} mutant mice, an experimental model of amyotrophic lateral sclerosis (ALS). However, these mice have been poorly studied to identify alterations in those elements of the endocannabinoid system targeted by these treatments. Moreover, we studied the neuroprotective effect of the phytocannabinoid-based medicine Sativex® in these mice. **Methods:** First, we analyzed the endocannabinoid receptors and enzymes in the spinal cord of SOD1^{G93A} transgenic mice at a late stage of the disease. Second, 10-week-old transgenic mice were daily treated with an equimolecular combination of Δ^9-tetrahydrocannabinol- and cannabidiol-enriched botanical extracts (20 mg/kg for each phytocannabinoid). **Results:** We found a significant increase of CB_2 receptors and NAPE-PLD enzyme in SOD1^{G93A} transgenic males and only CB_2 receptors in females. Pharmacological experiments demonstrated that the treatment of these mice with the Sativex®-like combination of phytocannabinoids only produced weak improvements in the progression of neurological deficits and in the animal survival, particularly in females. **Conclusions:** Our results demonstrated changes in endocannabinoid signaling, in particular a marked up-regulation of CB_2 receptors, in SOD1^{G93A} transgenic mice, and provide support that Sativex® may serve as a novel disease-modifying therapy in ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease caused by the selective damage of motor neurons in the spinal cord, brainstem, and motor cortex [1]. ALS exists in two forms, familial ALS (only 5% of cases) and sporadic ALS (most of cases) [1,2]. The pathogenesis of ALS is still pending of complete identification, but some mechanisms have been found to be involved including excitotoxic damage, chronic inflammation, oxidative stress, and protein aggregation [2–4]. For example, several studies have identified changes in the function of glutamate transporters that have been associated with the initial phases of the disease [4]. High amounts of reactive microglia have been found in those brain regions that are affected in ALS patients [5]. Genetic studies have identified several mutations in the copper–zinc superoxide dismutase gene (*SOD1*), which encodes for a key antioxidant enzyme, in approximately 12% cases of familial ALS [6], being pathological through a gain-of-neurotoxic function. In the last years, mutations in other genes, such as *TARDBP* (TAR-DNA binding protein) and *FUS* (fused in sarcoma), which encode proteins involved in pre-mRNA splicing, transport and/or stability [7,8], and, in particular, the CCGGGG hexanucleotide expansion

in the *C9orf72* gene, which appears to account for up to 40% of genetic cases [9], have been also identified and related to the disease. Their pathogenic mechanisms, which differ, in part, from the toxicity associated with mutations in SOD1, led to a novel molecular classification of ALS subtypes [10].

Despite the intensive investigation developed in the last years, the disease still lacks of an effective treatment, with Rilutek® as the only approved medicine [11]. Recent studies support that cannabinoids may be beneficial as neuroprotective agents in ALS [12]. Thus, the motor impairment was delayed, and the animal survival prolonged after the treatment with the phytocannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC) in the SOD1^{G93A} transgenic mouse model of ALS [13]. Other cannabinoids, including the less psychotropic plant-derived cannabinoid cannabinol [14], the nonselective agonist WIN55,212-2 [15], and the selective CB_2 agonist AM1241 [16,17], produced similar effects. The efficacy shown by compounds that target the CB_2 receptor [16,17] correlates with the fact that this receptor is overexpressed in microglial cells in postmortem spinal cords from ALS patients [18] or SOD1^{G93A} transgenic mice [17], becoming a promising target for the development of disease-modifying therapies in ALS, as has been investigated in other disorders [19,20]. This has been also proposed for the fatty acid amide hydrolase (FAAH) enzyme, which plays a key role in the degradation of the endocannabinoids anandamide and, to a lesser extent, 2-arachidonoyl-glycerol. Thus, elevated levels of endocannabinoids, reached by means of genetic ablation or pharmacological inhibition of this enzyme, also caused a delay in the appearance of the disease in SOD1^{G93A} transgenic mice [15]. This was not seen with the genetic ablation of the CB_1 receptor, although these animals showed an increased survival rate [15]. As for the CB_2 receptor, the efficacy of FAAH inhibition/inactivation [15] agrees with the elevated levels of anandamide found in the spinal cord of SOD1^{G93A} mutant mice [21], thus indicating that such response, as in the case of the CB_2 receptor, seems to represent an endogenous protective response against the insults that damage the motor neurons in the spinal cord, which would deserve to be pharmacologically exploited.

Despite the promising evidence supporting that cannabinoids may serve to develop a disease-modifying therapy in ALS, several issues remain to be investigated. For example, it is poorly known how the disease affects other elements of the endocannabinoid signaling system, such as the CB_1 receptor, the key enzyme in anandamide synthesis, N-arachidonoyl-phosphatidylethanolamine-phospholipase D (NAPE-PLD) or its equivalent in 2-arachidonoylglycerol synthesis, diacylglycerol lipase (DAGL), and the key enzyme in the degradation of 2-arachidonoylglycerol, monoacylglycerol lipase (MAGL). We believe extremely important to determine the changes in these elements, as well as in FAAH enzyme and CB_2 receptor, in ALS, as these changes may greatly influence the efficacy of those therapies based on targeting the different elements of this signaling system. Therefore, the first objective of our study was to analyze the status of endocannabinoid receptors (e.g., CB_1 and CB_2) and enzymes (e.g., NAPE-PLD, DAGL, FAAH, and MAGL), using RT-PCR, in the spinal cord of SOD1^{G93A} transgenic mice at a late stage of the disease.

The pharmacological studies conducted so far in experimental ALS appear to indicate that neuroprotective properties of cannabinoids in ALS depend on the combination of different mechanisms. The data obtained in *in vivo* studies using the nonselective cannabinoid receptor agonist WIN55,212-2 [15] or compounds that selectively target the CB_2 receptor [16,17] suggest the participation of cannabinoid receptors and, in particular, the CB_2 receptor type associated with the inflammatory role of glial elements in this disease. However, other mechanisms, such as those involved in cannabinoid receptor-independent antioxidant properties of certain cannabinoids, for example, phytocannabinoids, cannot be excluded. The fact that the neuroprotective effects in experimental models of ALS were reached through the activation of multiple targets and the treatment with different cannabinoid compounds suggests the convenience of using a cannabinoid with a broad-spectrum action or, alternatively, a combination of different cannabinoids with different profiles. This may be the case of Sativex® (GW Pharmaceuticals Ltd, Cambridgeshire, UK), a cannabinoid-based medicine that has been recently approved for the treatment of spasticity and pain in multiple sclerosis patients [22]. Sativex® combines botanical extracts enriched with Δ^9-THC and cannabidiol (CBD), which facilitates the activation of different mechanisms/targets, for example, both phytocannabinoids may act through cannabinoid receptor-independent antioxidant mechanisms, whereas Δ^9-THC may activate CB_1 and CB_2 receptors [23]. It is important to remark that the fact that Sativex® is already licensed may facilitate the development of clinical studies in ALS patients in the case of positive effects. Therefore, the second objective of our study was to evaluate a Sativex®-like combination of phytocannabinoid botanical extracts (administered i.p. vs. the oromucosal form used in patients, which implies some differences in pharmacokinetics), as a disease-modifying therapy in this experimental ALS model.

Materials and methods

Animals, Treatments and Sampling

All experiments were conducted with B6SJL-Tg(SOD1*G93A)1Gur/J transgenic, and nontransgenic littermate sibling mice bred in our animal facilities from initial breeders generously provided by LagenBio-Ingen (University of Zaragoza, Zaragoza, Spain) and subjected to genotyping for identifying the presence or absence of the transgene containing the SOD1^{G93A} mutation (protocol provided by LagenBio-Ingen). All animals were housed in a room with controlled photoperiod (08:00–20:00 light) and temperature (22 ± 1°C) with free access to standard food and water. All experiments were conducted according to local and European rules (directive 2010/63/EU) and approved by the Committee for Animal Experimentation of our university. In a first experiment, we used nontransgenic and B6SJL-Tg(SOD1*G93A)1Gur/J mutant mice of both genders at the age of 120 days after birth, an age at which, according to previous studies [21,24], motor deficits in mutant mice are already evident and strongly disabling. Before being euthanized, animals were subjected to neurological examination to confirm the existence of such motor deficits according to the following scale: 5 = no symptoms; 4 = tremor in hindlimbs when suspended by the tail; 3 = gait anomalies; 2 = paralysis in one hindlimb; 1 = paralysis in both hindlimbs; and 0 = unability to turn when lying on the back for 15 seconds (see details in refs.

21 and 24). Immediately after, animals were euthanized and their spinal cords were rapidly removed, frozen in 2-methylbutane cooled in dry ice, and stored at −80°C for subsequent biochemical analyses (qRT-PCR). In this experiment, at least 6–8 animals were used *per* experimental group. In a second experiment, we conducted pharmacological studies with B6SJL-Tg(SOD1*G93A) 1Gur/J mutant mice and their nontransgenic littermate siblings starting the treatment at ages (9 weeks after birth in the case of males and 10 weeks in the case of females, as the disease initiates earliest in males than females; see Figure 4) at which, according to previous studies [21,24], $SOD1^{G93A}$ mutant mice show the first evidence of motor anomalies. Treatments consisted of a daily i.p. injection of a 1:1 combination of botanical extracts enriched with either Δ^9-THC (Δ^9-THC botanical extract contains 67.1% Δ^9-THC, 0.3% CBD, 0.9% cannabigerol, 0.9% cannabichromene, and 1.9% other phytocannabinoids) or CBD (CBD botanical extract contains 64.8% CBD, 2.3% Δ^9-THC, 1.1% cannabigerol, 3.0% cannabichromene, and 1.5% other phytocannabinoids), both provided by GW Pharmaceuticals Ltd. (Cambridgeshire, UK), or vehicle (Tween 80-saline; 1:16). The total dose of cannabinoid administered was always 40 mg/kg (equivalent to 20 mg/kg of pure CBD + 20 mg/kg of pure Δ^9-THC), a dose within the range of effective doses of phytocannabinoids when they were administered in pure form in experimental models of neurodegenerative disorders including ALS [13,14]. The treatment was repeated every day up to the end stage of the disease, when animals reached a clinical score of 0 (they were euthanized to avoid animal suffering) enabling to evaluate animal survival using Kaplan–Meier curves. Every week, all animals were subjected to neurological examination following the clinical scale described before. In this experiment, at least 8–12 animals were used *per* experimental group.

Real-Time qRT-PCR Analysis

Total RNA was extracted from spinal cord samples using Trizol (Life Technologies, Alcobendas, Spain) and purified using PureLink® RNA Mini Kit RNATidy reagent (Life Technologies, Alcobendas, Spain). The total amount of RNA extracted was quantitated by spectrometry at 260 nm, and its purity was evaluated by the ratio between the absorbance values at 260 and 280 nm, whereas its integrity was confirmed in agarose gels. To prevent genomic DNA contamination, DNA was removed and single-stranded complementary DNA was synthesized from 1 μg of total RNA using a commercial kit (Rneasy Mini Quantitect Reverse Transcription, Qiagen, Izasa, Madrid, Spain). The reaction mixture was kept frozen at −20°C until enzymatic amplification. Quantitative real-time PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) to quantify mRNA levels for CB_1 receptor (ref. Mm00432621_s1), CB_2 receptor (ref. Mm00438286_m1), FAAH (ref. Mm00515684_m1), MAGL (ref. Mm00449274_m1), DAGL (ref. Mm00813830_m1), and NAPE-PLD (ref. Mm00724596_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), and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System; Applied Biosystems).

Statistics

Data were assessed by unpaired Student's *t*-test or two-way ANOVA followed by the Student–Newman–Keuls test, as required.

Results

Experiment I: Analysis of the Endocannabinoid Signaling in $SOD1^{G93A}$ Mutant Mice

In the first objective of this study, the spinal cord of 17-week-old animals was used for biochemical analysis of endocannabinoid receptors and enzymes using RT-PCR. Our analyses proved a significant increase of CB_2 receptor expression in $SOD1^{G93A}$ transgenic females and, in particular, males, with no changes in CB_1 receptors (Figure 1). In addition, the anandamide-synthesizing enzyme NAPE-PLD increased in $SOD1^{G93A}$ transgenic males, although not in females (Figure 2), whereas DAGL, the 2-arachidonoylglycerol-synthesizing enzyme, was not altered in $SOD1^{G93A}$ transgenic animals, although the probability levels were close to reach statistical significance in males ($P = 0.078$; Figure 2). Endocannabinoid degrading enzymes, FAAH and MAGL, were not significantly affected between $SOD1^{G93A}$ transgenic mice compared to their littermate nontransgenic siblings (Figure 3). It is important to remark that $SOD1^{G93A}$ transgenic mice showed, in some cases and for some parameters, a high variability, presumably related to differences in the degree of neurological deterioration (see below). This may explain that some differences do not reach statistical significance and remain as mere trends toward a change.

Experiment II: Investigation of Sativex® as a Disease-Modifying Therapy in $SOD1^{G93A}$ Mutant Mice

In the second objective of this study, we examined potential neuroprotective effects of the Sativex®-like combination of phytocannabinoids in $SOD1^{G93A}$ transgenic mice. To this end, we treated males and females when first motor symptoms have appeared (9 weeks in the case of males and 10 weeks in females; see Figure 4) up to the end stage of the disease (around 130 days). We observed that the treatment with the Sativex®-like combination of phytocannabinoids slightly delayed the progression of neurological deficits in the early stages of the disease, in particular in females (Figure 4). It also tended to increase animal survival, an effect only observed in females (Figure 4), as well as it produced a partial recovery in the weight loss typical of transgenic animals, although this effect was seen only in males, not in females (data not shown). Nontransgenic littermate siblings treated with vehicle or Sativex® did not present any differences in relation to the neurological score (constantly maintained at a value of 5), animal survival data, and weight recording, so their data were omitted from the figures to improve clarity.

Discussion

The first objective of the present study was to identify possible differences in the expression of endocannabinoid receptors and enzymes in the spinal cord between $SOD1^{G93A}$ mutant mice and

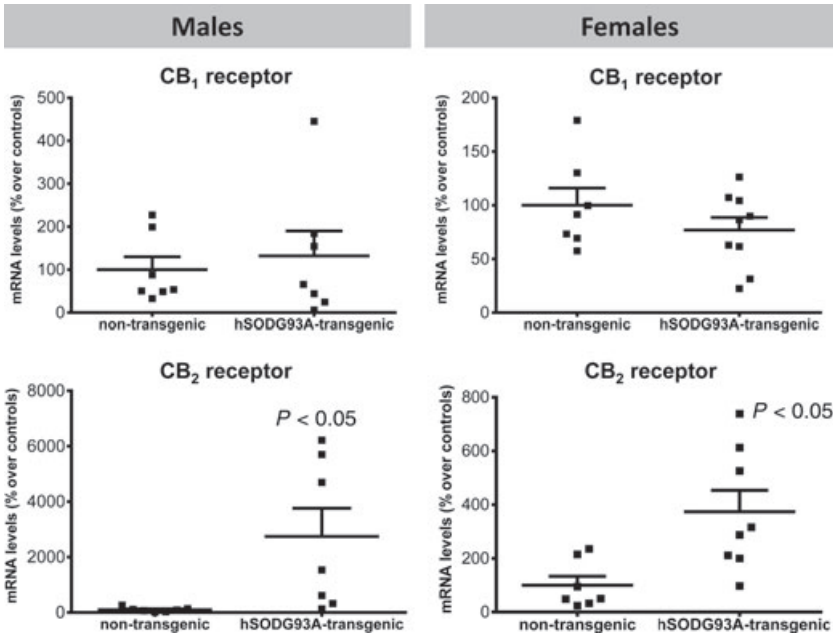

Figure 1 Gene expression for CB_1 and CB_2 receptors measured in the spinal cord of male and female SOD-1 transgenic or wild-type mice (at 120 days after birth). Details in the text. Values correspond to % of change over wild-type animals and are expressed as means ± SEM for 7–8 animals *per* group. Data were analyzed using unpaired Student's *t*-test.

their nontransgenic littermate siblings. These differences may be of interest for a better design of future cannabinoid treatments targeting the altered endocannabinoid elements. To this end, we used $SOD1^{G93A}$ transgenic animals at an end stage of the disease in which the neurological deterioration is evident and highly disabling. According to previous literature [21,24] and also to our own data, the disease initiates in transgenic mice around 9–11 weeks after birth with some subtle differences between genders (males being affected earlier than females; see Figure 4). There is also certain degree of individual variability, with some animals being affected earlier than others, which is intrinsic to the hybrid B6SJL background of animals used here. The disease progresses up to moribundity that occurs at 19–20 weeks after birth [21,24]. For this objective, we used male and female transgenic mice at 17 weeks after birth, which presented a neurological score of 1.83 ± 0.33 (n = 12) in males and of 1.06 ± 0.31 (n = 11) in females, compared to their corresponding wild-type animals of similar age and gender whose clinical score was always 5. These mice showed a marked up-regulation of CB_2 receptors, presumably in glial elements, as has been found in experimental models of other chronic progressive disorders (reviewed in refs. 19 and 20). In fact, this up-regulation was also found in ALS patients in a

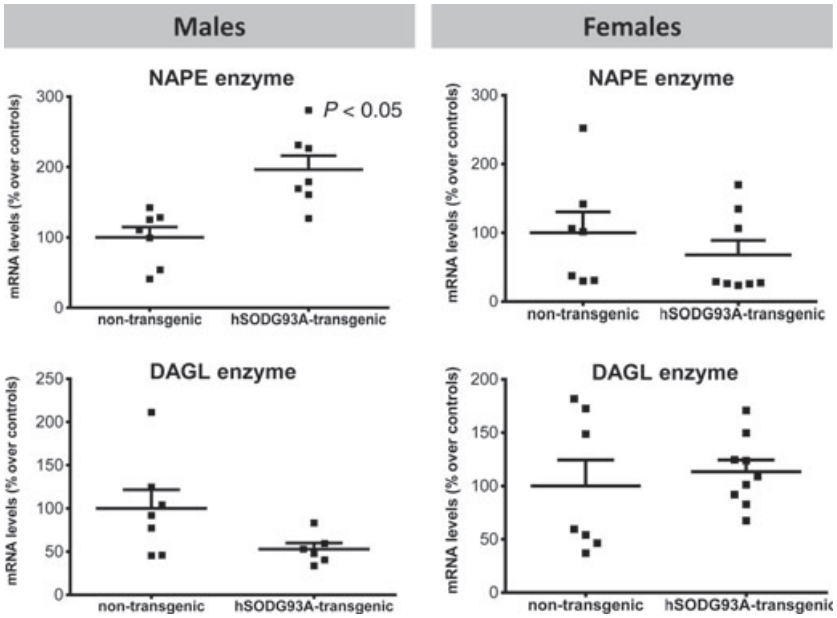

Figure 2 Gene expression for NAPE-PLD and DAGL enzymes measured in the spinal cord of male and female SOD-1 transgenic or wild-type mice (at 120 days after birth). Details in the text. Values correspond to % of change over wild-type animals and are expressed as means ± SEM for 6–8 animals *per* group. Data were analyzed using unpaired Student's *t*-test.

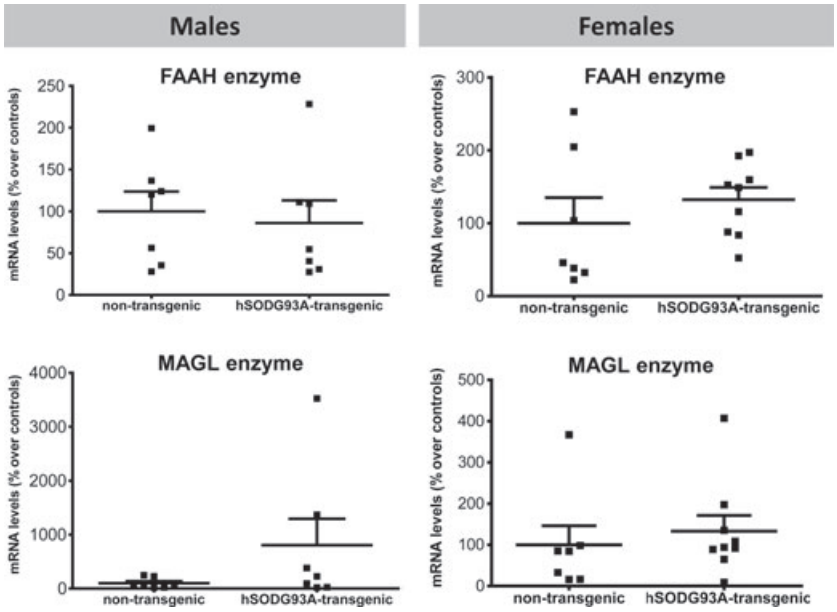

Figure 3 Gene expression for FAAH and MAGL enzymes measured in the spinal cord of male and female SOD-1 transgenic or wild-type mice (at 120 days after birth). Details in the text. Values correspond to % of change over wild-type animals and are expressed as means ± SEM for 7–8 animals *per* group. Data were analyzed using unpaired Student's *t*-test.

previous study conducted in postmortem spinal cord samples [18], as well as in experimental models [17]. This type of response supports the activity of those cannabinoids targeting the CB_2 receptor as neuroprotective and antiinflammatory agents, a fact successfully demonstrated in the $SOD1^{G93A}$ transgenic mouse model of ALS [16,17]. The novel aspect of our observation is that the up-regulation of CB_2 receptors occurred in both genders, although it was more pronounced in the case of $SOD1^{G93A}$ mutant males. These animals also exhibited other notable changes that were not found in females. For example, certain trends in MAGL (increase) and DAGL (decrease) enzymes, which would be compatible with the reduction in 2-arachidonoyl-glycerol levels seen by Witting et al. [21]. In addition, the anandamide-synthesizing enzyme NAPE-PLD was significantly increased in $SOD1^{G93A}$ transgenic males with no changes in the FAAH enzyme that degrades this endocannabinoid, which correlates with the increased levels of anandamide detected in the spinal cords of $SOD1^{G93A}$ transgenic mice in the same study [21].

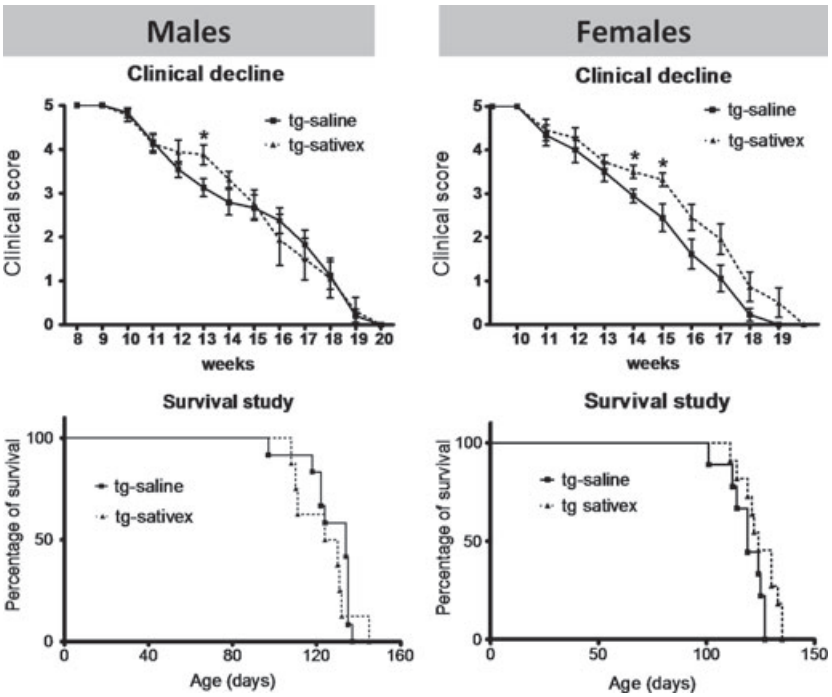

Figure 4 Clinical score and animal survival measured in male and female SOD-1 transgenic mice daily treated, from the age of 9 (in males) or 10 (in females) weeks after birth, with the Sativex®-like combination of phytocannabinoids at a dose of 40 mg/kg (equivalent to 20 mg/kg for each major phytocannabinoid) or vehicle (Tween 80-saline). Details in the text. Values are expressed as means ± SEM for 8–12 animals *per* group. Data were analyzed using two-way analysis of variance followed by the Student–Newman–Keuls test (*$P < 0.05$ compared to animals treated with vehicle).

In addition to these biochemical data, the present study also provides additional evidence in support of cannabinoids as a possible neuroprotective therapy in ALS, as was indicated by some previous studies (reviewed in ref. 12). Given that most of these previous pharmacological studies used individual cannabinoid compounds although identified different potential targets (see ref. 12), we wanted to investigate a phytocannabinoid combination, the cannabis-based medicine Sativex®, with a broad spectrum of pharmacological actions. Sativex® has been recently approved for the treatment of other neurological disorders [22], which may facilitate the clinical projection of the potential effects that may be found in this study. As mentioned above, Sativex® may cover all pharmacological targets that have been found of interest in this experimental ALS model: (1) it contains Δ^9-THC, which was beneficial in previous studies [13] and is active at the CB_2 receptor, whose activation with a selective synthetic cannabinoid was also beneficial [16,17]; and (2) it also contains CBD, which had not been previously investigated in ALS, but it provides important antioxidant properties and also the possibility to inhibit FAAH enzyme (reviewed in ref. 25), which has been found to serve as a potential target in studies using genetic ablation or pharmacological inhibition [15]. Our study demonstrated that Sativex®-like combination of phytocannabinoids was effective to delay disease progression in the initial stages of the disease, in particular in females, although the effects were lost during progression of the disease. We also quantified the animal survival using Kaplan–Meier curves, and although the results did not reach statistical significance, we could appreciate a trend toward an increase in the survival of females after the treatment with a Sativex®-like combination of phytocannabinoids. The fact that females were apparently more responsive to Sativex® is intriguing and may be related to the differences in the hormonal status between both genders. In this sense, a recent study [26] has demonstrated: (1) that the treatment with Δ^9-THC increases pregnenolone synthesis in the brain and, to a lesser extent, in the spinal cord, through stimulation of CB_1 receptors; and (2) that this neurosteroid, which plays a critical role as precursor in the synthesis of different steroid compounds, serves as a negative allosteric modulator of CB_1 receptor signaling [26]. It is well known that males and females present some differences in their requirements for neurosteroid biosynthesis, which may be in part responsible for the different levels of pregnenolone found in male and female brain areas, including the spinal cord [27]. Therefore, it is possible that gender-dependent differences in pregnenolone availability to inhibit CB_1 receptor signaling may explain that transgenic females are more responsive to Sativex® than transgenic males, but this would require additional investigation. However, preliminary data obtained in our laboratory using analysis by Nissl staining of the spinal cord of $SOD1^{G93A}$ transgenic mice euthanized at a late stage of the disease revealed that large motor neurons could be relatively preserved in those animals treated with this phytocannabinoid combination. However, they may not be functional given that this protection does not necessarily translate at the neurological level, as found in the present study, and these effects were similar in both genders. Follow-up studies will have to confirm these preliminary data, but, if confirmed, this will mean that the protective effect of phytocannabinoids could affect primarily the survival of the motor neuron, whose cell body is stained with cresyl-violet, but this level of protection would not be sufficient with the neuron-muscle synapse, which appears to be significantly hampered in view of the weak clinical recovery. Anyway, our data support a relative efficacy of this treatment, although they also suggest the need to optimize it in follow-up studies, for instance: (1) by using increasing doses of Sativex®-like combination of phytocannabinoids (despite that, dose used here is already high and, in the case of Δ^9-THC, has been found to be effective in previous studies [13]); (2) by using a different combination of phytocannabinoids (e.g., a mixture with high Δ^9-THC and low CBD given that CBD may act as an antagonist for certain Δ^9-THC effects; see ref. 28); and (3) by using Sativex®-like combination of phytocannabinoids as an adjunctive therapy with other therapies used or investigated in ALS (e.g., riluzole).

In conclusion, our results demonstrated different changes in endocannabinoid signaling, in particular a marked up-regulation of CB_2 receptors, in $SOD1^{G93A}$ transgenic mice, and provide support that Sativex® (or alternative Sativex®-like combinations of phytocannabinoids) may serve as a novel disease-modifying therapy in ALS, a disorder with a poor therapeutic outcome at present with only one medicine already approved, Rilutek®, but with a modest efficacy on disease progression. Anyway, more preclinical studies in additional models of ALS, i.e., TDP-43 transgenic mice, will be necessary before testing the clinical efficacy of Sativex® in ALS patients.

Acknowledgments

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Conflict of Interest

The authors have formal links with GW Pharmaceuticals that funds some of their research.

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Supplementary methods

Nissl staining

Frozen spinal cords were sliced with a cryostat at the lumbar level to obtain coronal sections (20 µm thick) that were collected on gelatin-coated slides. Slices were used for Nissl staining using cresyl violet, as previously described, which permitted to determine the effects of particular treatments on cell number. Particle analysis from ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012) was used to count large motor neurons (> 400 μm^2) in the anterior horn (3 sections per mouse, n=3).

Magnetic resonance imaging

Magnetic resonance imaging (MRI) was performed in males mices aged 100 days old at the Nuclear Magnetic Resonance Centre of Complutense University (Madrid, Spain) using a Biospec 47/40 (Bruker, Ettlingen, Germany) operating at 4.7T, equipped with a 12 cm gradient set and using a 4 cm radio frequency surface coil. The 3D T2-weighted spin-echo images were acquired using a fast spin-echo sequence. The acquisition parameters were: TR=100 ms, TE=6 ms, FOV = 40 mm*40 mm, Slice thk = 2 mm, Matrix = 128*128.

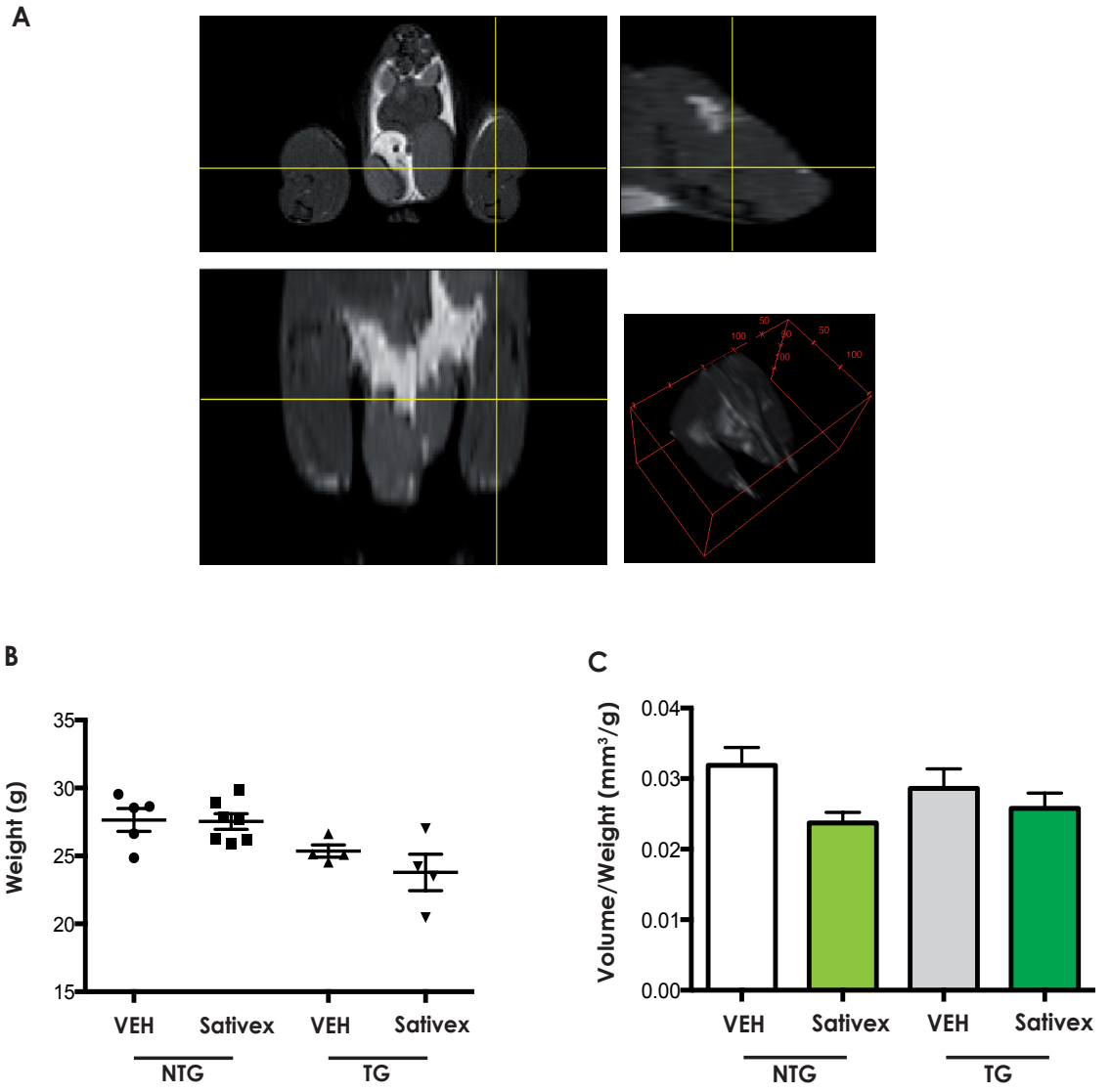

Figure S1 MRI of male mice hindlimb aged 100 days old, treated with either vehicle or a Sativex®-like combination of phytocannabinoids at a dose of 40 mg/kg. A) Representative image of the T2 weighted structural mouse hindlinmb. B) weight distribution of the mice and C) hindlimb muscle volume as a function of weight. The diference between treated and non-treated animals are non significant after a one-way ANOVA with Bonferroni post test analysis.

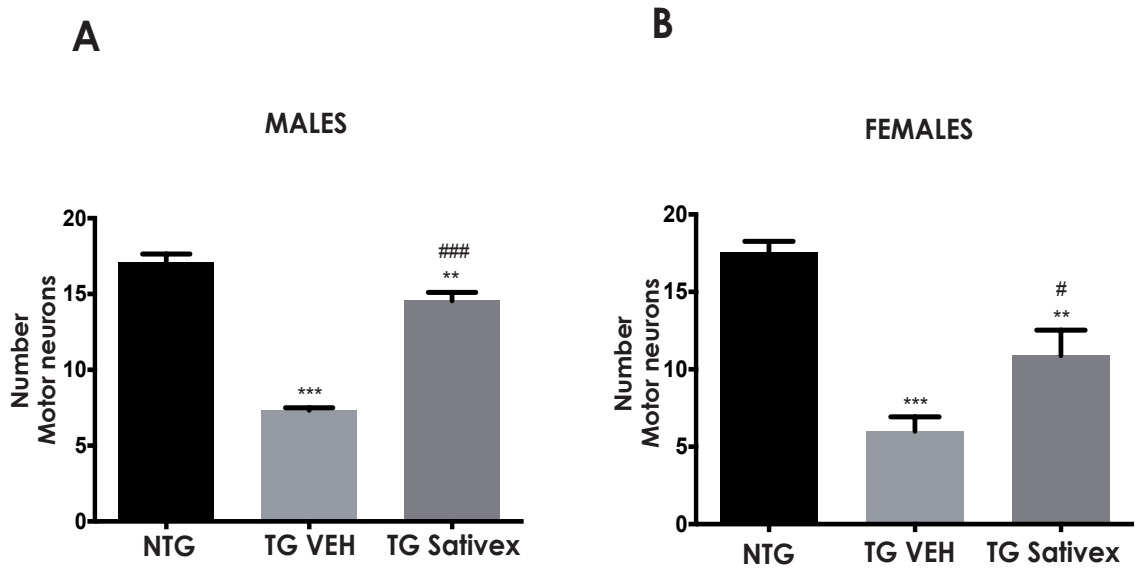

Figure S2 Number of Nissl-stained cells (>400 μm^2) in the spinal cord of male (A) and female (B) SOD-1 transgenic or wild-type mice that were daily treated, from the age of 9 (in males) or 10 (in females) weeks after birth, with the Sativex®-like combination of phytocannabinoids at a dose of 40 mg/kg (equivalent to 20 mg/kg for each major phytocannabinoid) or vehicle (Tween 80-saline), and that were euthanized at the age of 130 days after birth. Details in the text. Values are expressed as means ± SEM for 3 animals per group. Data were subjected to one-way analysis of variance followed by the Student-Newman-Keuls test ($^{**}p<0.01$, $^{***}p<0.005$ compared to non-transgenic mice; $^{\#}p<0.05$, $^{\#\#\#}p<0.005$ compared to vehicle-treated transgenic mice).

Objective #4

Study of the role of the cannabinoid receptors in a zebrafish model of ALS.

In this chapter we have checked how the supression of the expression of cannabinoids receptors affect the axonopathy observed the paradigm of zebrafish (*Danio rerio*) embryos injected with ALS causing genes (i.e. *SOD1* and *C9ORF72*). To this end we have designed morpholino antisense oligonucleotides (MAO) aimed to supress the expression of the *CNR1* and *CNR2* present in the zebrafish.

First we have injected zebrafish embryos with increasing concentrations of MAO to study the toxicity of the MAO and to check if the supression of *CNR1* or *CNR2* caused an aberrant phenotype in the caudal motor neurons. We observed no effect when supressing *CNR1* expression. However, when we supressed *CNR2* expression the caudal motor neuron axons where shortened and had aberrant sprouting.

Finally we co-injected zebrafish embryos with *CNR1* MAO and SOD1A4V mRNA or mRNA containing 75 repeats of the CCGGGG hexanucleotide found in *C9ORF72*. Surprisingly we observed a reversion of the axonopathy caused by $SOD1^{A4V}$ when *CNR1* expression was blocked.

CONCLUSION:

The blockade of *CNR1* in the Zebrafish ALS model may reverse the axonopathy caused by $SOD1^{A4V}$ mutations.

Papers in this chapter:

Moreno-Martet, M., Timmers, M., Robberecht, W., Fernández-Ruiz, J., de Lago, F.
Potential role of cannabinoid CB_1 and CB_2 receptors in a zebrafish model of ALS.
(preliminary data not published).

POTENTIAL ROLE OF CANNABINOID CB_1 AND CB_2 RECEPTORS IN A ZEBRAFISH MODEL OF ALS

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Summary: Neurodegenerative diseases are the challenge for research in the XXIst century as the cure for any of these diseases is yet to be found. Zebrafish (*Danio rerio*) is a novel model for neurodegenerative diseases and has become a very convenient tool for drug research due to its cheap maintenance, rapid outgrowth, transparent development and easy gene manipulation. In this paper, we focus on a study of the role of the cannabinoid receptors CB_1 and CB_2 in a model of amyotrophic lateral sclerosis in zebrafish.

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that affects mainly young adults. The death of upper and lower motor neurons in the primary motor cortex, the brainstem and the spinal cord implies the progressive weakening and atrophy of muscles, leading to paralysis and finally death when the respiratory muscles are affected (Wijesekera and Leigh, 2009). The molecular pathogenic events that produces the motor neuron death includes oxidative stress, excitotoxicity, mitochondrial impairment and inflammation, in which the non-neural cells of the central nervous system (CNS) play an essential role (Boillée et al., 2006). Mutant SOD-1 has been until recent years the main protein that researchers could use for modelling ALS, but it represents only 5-10% of familial ALS (Turner and Talbot, 2008). In the last years, new proteins forming aggregates in familial and sporadic ALS patients, as *TDP-43*, *FUS* and the hexanucleotide expansion GGGGCC in the *C9ORF72* gene have been discovered (DeJesus-Hernandez et al., 2011; Kwiatkowski et al., 2009; Neumann et al., 2006; Renton et al., 2011; Vance et al., 2009). These findings have led to the development of new transgenic models of the disease that will allow researchers to understand key pathogenic events in this neurological disease.

The endocannabinoid system (ECS)

is an intercellular communication system that has a crucial homeostatic role in development, feeding, relaxing, sleeping, memory processes and defence against various toxic stimuli (Scotter et al., 2010). The ECS is formed by two G-protein coupled receptors, the CB_1 and CB_2 receptors (Howlett, 2002) which are activated by various endogenous ligands, known as endocannabinoids, being the most important the N-arachidonoylethanolamine (anandamide or AEA) and the 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1998). The enzymes that synthetize AEA and 2-AG (NAPE-PLD and DAGL, respectively) and the enzymes that degrade them (FAAH and MAGL, respectively) also form part of the ECS (Di Marzo et al., 1998).

It is known that certain cannabinoid compounds may provide neuroprotection against acute or chronic brain damage (Fernández-Ruiz et al., 2007; Galve-Roperh et al., 2008; Sagredo et al., 2007). This is relevant considering the postmitotic characteristics of neuronal cells, which makes repair processes extremely difficult, although cannabinoids have been also involved in the regulation of neurogenic mechanisms in specific structures of the adult brain (Galve-Roperh et al., 2007). In addition, cannabinoids also serve as neuroprotective agents against excitotoxicity, inflammation, oxidative stress, mitochondrial impairment and energy failure, common pathogenic mechanisms to different chronic neurodegenerative diseases such as ALS (Bilsland and Greensmith, 2008), Alzheimer's disease (AD) (Bisogno and Di Marzo, 2008), Huntington's disease (HD) (Pazos et al., 2008), multiple sclerosis (MS) (de Lago et al., 2009)and Parkinson's disease (PD) (García et al., 2011; García-Arencibia et al., 2009).

On the other hand, different elements of the endocannabinoid system are enhanced in response to different cytotoxic insults. This enhancement involves the endocannabinoid ligands, their GPCR receptors, or both. For example, several studies have described an increased accumulation of AEA, 2-AG, or both in specific brain structures in different neurodegenerative disorders including MS, ALS, PD, ischemia, brain trauma and others (Bisogno and Di Marzo, 2010). Up-regulation in the expression of cannabinoid receptors, as the CB_1 receptor, has been shown in brain damage injuries (Hansen et al., 2001; Jin et al., 2000) although the most relevant data is related with the CB_2 receptor, a receptor mostly absent on neural cells of the CNS in healthy conditions but that is significantly induced/up-regulated in many degenerative pathologies(Fernández-Ruiz et al., 2011). This response occurs in astrocytes and reactive microglial cells that in their resident state do not express CB2 receptors. This up-regulation points out that CB2 receptors may play a role in the trophic and/or the cytotoxic regulation of these cells over neurons. This up-regulation of CB2 receptors has been observed in patients or animal models of AD (Benito et al., 2007; Esposito et al., 2007), ALS (Yiangou et al., 2006), HD (Palazuelos et al., 2009; Sagredo et al., 2009), MS neuropathic pain (Zhang et al., 2003), SIV encephalitis (Benito et al., 2005) and stroke (Ashton et al., 2007). Also noteworthy is the fact that the use of selective agonists for the CB2 receptor, which bypasses the undesired psychoactive effects due to the CB1 activation, has proven a delayed progression of brain damage in most of these disorders, thus supporting the promising neuroprotective properties of this type of agonists (Fernández-Ruiz et al., 2008).

Recent evidence points out that cannabinoids may be beneficial as neuroprotectant agents in ALS (Bilsland and Greensmith, 2008). The link between cannabinoids and ALS has been shown in some studies conducted mainly in the transgenic mouse model expressing the hSODG93A protein.

Changes in the endocannabinoid levels of transgenic mice expressing hSODG93A protein have been reported (Witting et al., 2004). Moreover, the administration of the phytocannabinoid Δ^9-THC, the use of the synthetic cannabinoid WIN55-212,2 or the increase in the endocannabinoid tone by genetic FAAH ablation was found to improve muscular functions and motor neuron survival in late stages of the disease (Bilsland et al., 2006; Moore and Abood, 2004). Similar results were also obtained with other cannabinoids, including antioxidant cannabinoids and selective CB_2 agonists (Kim et al., 2006; Weydt et al., 2005). An up-regulation of CB_2 receptors in glial elements in the spinal cord of ALS patients has been described (Yiangou et al., 2006). Therefore, there is preliminary evidence supporting that cannabinoids may be a promising therapy in ALS but this information is still limited, particularly in two aspects: (i) the type of alterations caused by the disease in different elements of the cannabinoid system and their relationship with the benefits found with those cannabinoid compounds targeting these elements and (ii) the type of cellular and molecular mechanisms underlying the potential beneficial effect of certain cannabinoid agonists in ALS.

The endocannabinoid system is well conserved through evolution, and zebrafish (Danio rerio) genome has all the elements as receptors, endogenous ligands and synthesis and degradation enzymes (Klee et al., 2012). However, in the zebrafish genome some of the endocannabinoid system's redundancy is shifted, and some other genes have duplicated paralogs (McPartland et al., 2007). The distribution of the cannabinoid receptors have been already characterized (Lam et al., 2006; Rodriguez-Martin et al., 2007a; 2007b) as well as the functionality of the CB_1 receptor (Watson et al., 2008) and possible roles for the endocannabinoid signalling (Migliarini and Carnevali, 2009). The overexpression of mutated proteins related with ALS in zebrafish such as $hSOD^{G93A}$ and $hTDP43^{A315T}$ reproduce clinical features of the disease, such as motor neuron axonopathy and reduced motility in zebrafish larvae(Laird et al., 2010; Lemmens et al., 2007; Paquet et al., 2009). Zebrafish is thus an optimal model to seek for neuroprotective effects of the endocannabinoid system in a neurodegenerative disease as ALS.

In these experiments we have studied the effect of knocking down the *CNR1* expression or the *CNR2* expression with morpholino antisense oligonucleotides in the caudal motor neurons in zebrafish. We have also measured the effects of knocking down *CNR1* in $hSOD^{G93A}$ injected zebrafish embryos.

Methods

Constructs, mRNA production and morpholinos

Antisense morpholinos (MO) against the start codon (ATG) of CNR1 or 5'UTR of CNR2 were designed and obtained from Gene Tools (Philomath, OR, USA). Standart control morpholinos (CO MO), were also obtained from Gene Tools. The sequences of the morpholino used are as follows:

***CNR1* ATG-MO 5' GAACAGCATGGTCAGAGATGCTCTA 3'**

***CNR2* UTR-MO 5' AGGCCATGAAACAAACAGTACCTGT 3'**

***CONTROL* MO 5' CCTCTTACCTCAGTTACAATTTATA 3'**

SOD1 was cloned in the pBCM vector behind a T_3 promoter. The construct was linearized using Asp718. By using the mMESSAGE mMACHINE T_3 Kit (Ambion, Huntingdon, UK), RNA was transcribed from DNA and purified using the MEGA-clearTM Kit (Ambion). mRNA-concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA).

The construct was linearized by ….. and mRNA was transcribed from the linear transcript using the mMESSAGE mMACHINE T_7 Kit (Ambion, Huntingdon, UK) followed by purification with a MEGAclearTMKit (Ambion, Huntingdon, UK). mRNA-concentration was determined using a NanoDrop 1000

Zebrafish, ALS and cannabinoid receptors

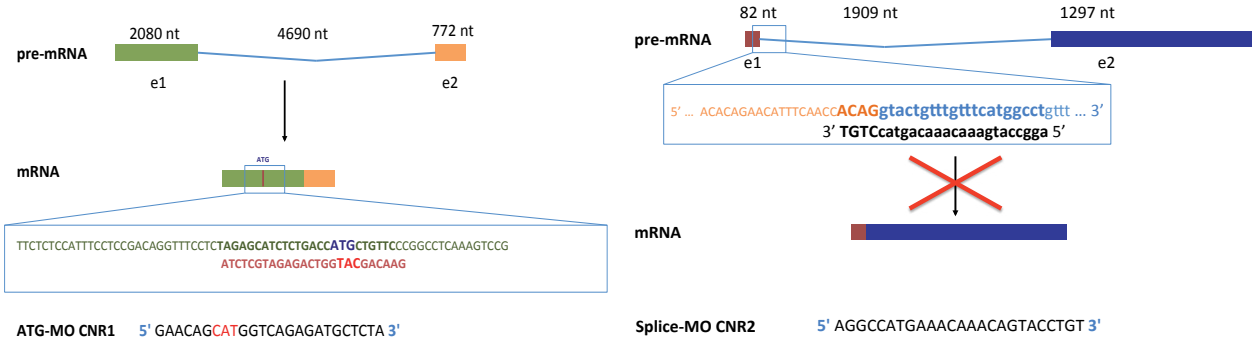


Figure 1: The morpholino antisense oligo to knockdown CNR1 expression in *Danio rerio* was designed against the ATG sequence. The morpholino antisense oligo designed to knockdown CNR2 expression in *Danio rerio* targeted the e1i1 splice junction, blocking the maturation of pre-mRNA.

spectrophotometer (Thermo Scientific, Waltham, MA).

Zebrafish maintenance and injection
All experiments were approved and performed in accordance with the guidelines of the Ethical Committee for Animal Experimentation, K.U. Leuven (project approval number P021/2010). Adult zebrafish (AB strain) and embryos were maintained under standard laboratory conditions. Zebrafish embryo microinjections were made using a FemtoJet injection setup (Eppendorf, Hamburg, Germany). Each injection was made in the 1–4 cell stage of the zebrafish embryo and involved delivery of 2.14 nl of mRNA/MO solution, accom- plished by an injection pressure of less than 4.5 psi, which produced a droplet diameter of 160 mm on a micrometer. Embryos were then stored in E3 solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM Methylene Blue) at 27.5–28.5uC.

Analysis of motor neuron outgrowth
At 30 hours post-fertilization (hpf), morphologically normal zebrafish embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and immunostained using mouse anti-synaptic vesicle 2 (1/200; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IO, USA) and secondary Alexa Fluor 555 anti-mouse antibody (1/500; Molecular Probes, Eugene, OR, USA) in order to visualize motor neurons. Observers blind to injection and treatment conditions measured the axonal length of the first five ventral motor axons after the yolk sac in each embryo using Lucia software (version 4.9) and the average of these five lengths was calculated for each embryo. Motor axons were scored as affected by aberrant branching when two or more axons per embryo branched at, or medial to, the ventral edge of the notochord (all axons on each side of the embryo were checked).

Real time PCR
A real time PCR was used to quantify the amount of *CNR2* mRNA. RNA was extracted from zebrafish embryos (50 embryos per group, with three replicates per condition), using Trizol (Invitrogen, San Diego, CA) and isopropanol purification. cDNA was obtained from the extracted RNA by reverse transcriptase PCR (QuantiTect reverse transcription kit). The amount of Danio rerio CNR2 was quantified by real time PCR using a Taqman assay (Lifetechnologies, ……):

Forward primer:
Reverse primer:

Thermal cycling was performed on a 7300 Sequence Detection System (Applied Biosystems, Carlsbad, CA). All samples were normalized to 18S RNA levels.

Results

To design the morpholinos antisense oligo we did a research of the *Danio rerio CNR1* and *CNR2* genes in the Ensembl genome database (Flicek et al., 2013). (see figure 1)The morpholino antisense oligo against the *CNR1* gene was designed as a 25 base oligo targeting the ATG region. The morpholino antisense oligo against the *CNR2* gene was designed as a 25 base oligo targeting the intronic sequence between e1 and e2 to avoid the correct splicing for the *CNR2*

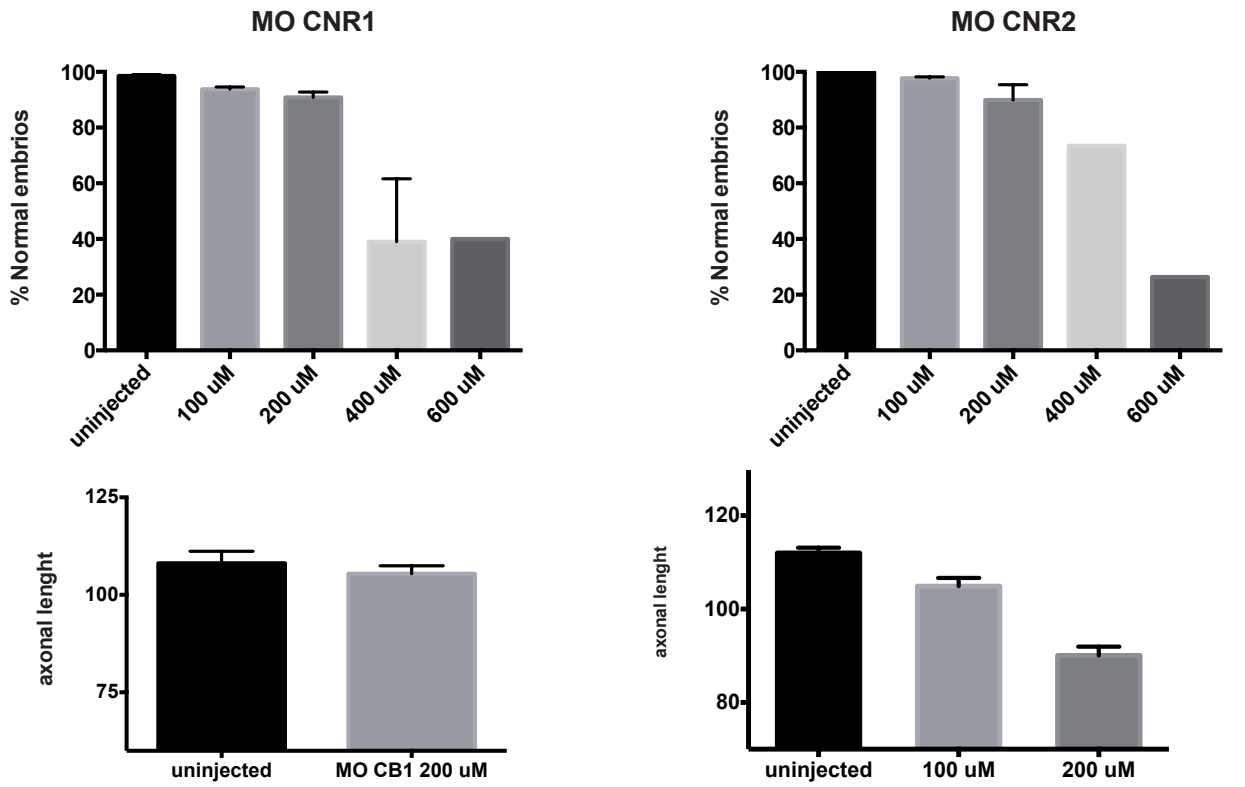
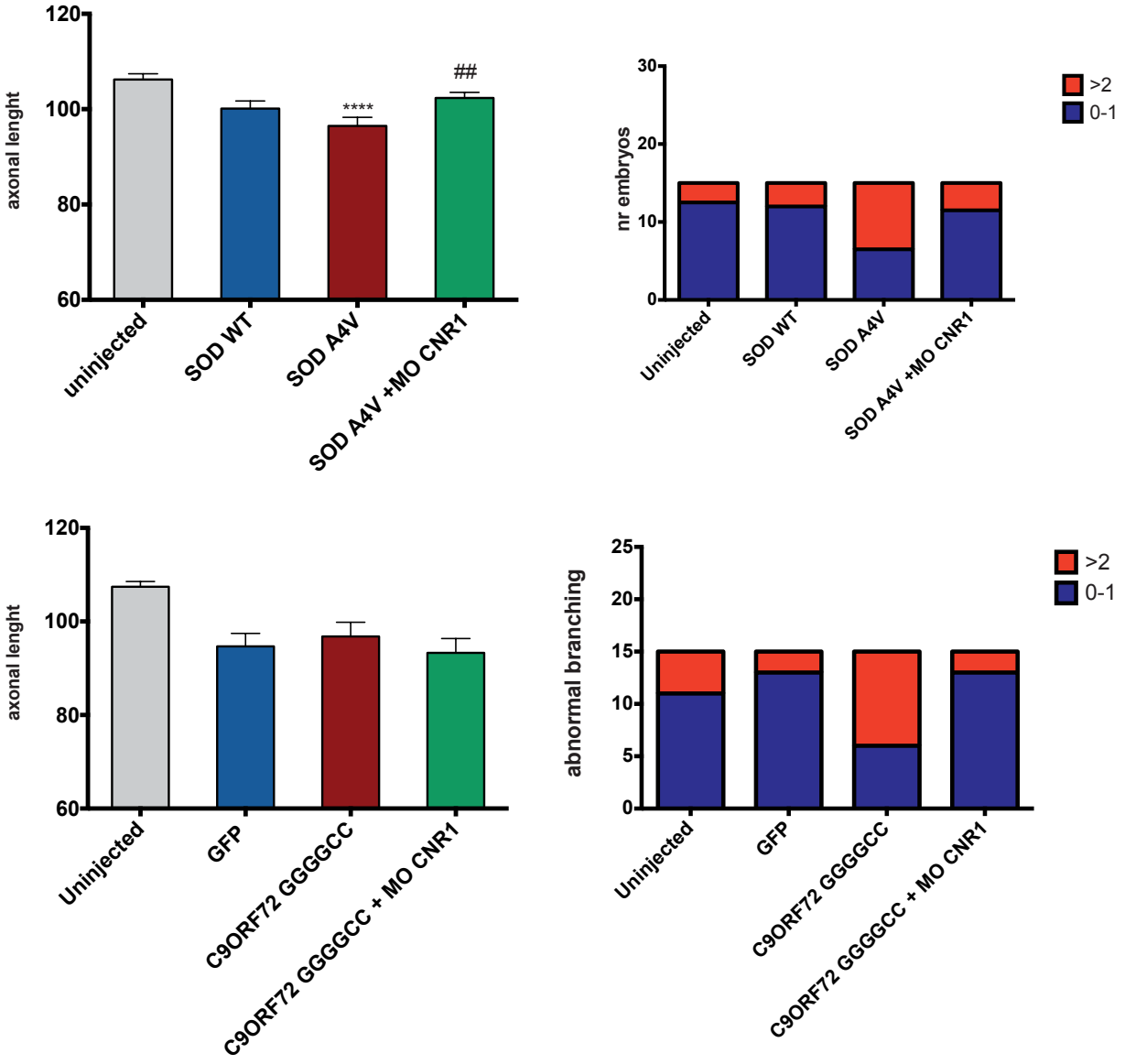


Figure 2: *Left:* Toxic effects of incresing concentrations of the ATG-MO CNR1 in zebrafish embryos. At 200 uM, the axonal lenght of motor neurons measured at 30 hpf was not affected. *Right:* Toxic effects of incresing concentrations of the Splice-MO CNR2 in zebrafish embryos. At 200 uM, the axonal lenght of motor neurons measured at 30 hpf was shortened and this effect was dose dependant.

mRNA. Zebrafish embryos were injected with increasing concentrations of the *CNR1* or *CNR2* morpholinos antisense oligos to check the maximal concentration with no toxic effects in the embryos (see figure 2). The concentration selected for both *CNR1* and *CNR2* was 200 µM. To check if the silencing of CNR1 or CNR2 was affecting the axonal length of the zebrafish embryos we injected 200 µM of the ATG-CNR1 MO or the 200 µM UTR-CNR2 MO in 2 cell-stage embryos. After 30 h we fixed the embryos and stained caudal motor neurons. When we measured the axonal length, we saw that the silencing of CNR1 did not cause a phenotype in motor neurons with values ranging 108,1 ± 3,063 µm in the uninjected embryos vs. 105,4 ± 2,038 µm in the 200 µM ATG-CNR1 MO injected embryos. However, when we silenced *CNR2*, there was a significant decrease in the axonal length that was dose-dependant, with 115,4 ± 1,715 µm for the uninjected embryos, 104,9 ± 1,751 µm for the 100 µM UTR-CNR2 MO injected embryos and 96,11 ± 2,504 µm for the 200 µM UTR-CNR2 MO injected embryos. $p < 0,001$.

As the silencing of *CNR2* had an effect on the motor neuron phenotype, we decided to focus on the silencing of the *CNR1* in the paradigm of axonopathy due to mutated SOD1 expression. To this purpose, we injected zebrafish embryos with 200 ng $SOD1^{WT}$ mRNA, 200 ng $SOD1^{A4V}$ mRNA or 200ng $SOD1^{A4V}$ mRNA and 200 µM ATG-CNR1 MO and measured the axonal length of caudal motor neurons at 30 hpf. The motor neurons showed an axonopathy with shortened axons when injected with the $SOD1^{A4V}$ mRNA compared to the $SOD1^{WT}$

Figure 3: *Left.* Axonal length of motor neurons from Danio rerio embryos injected with SOD A4V, SOD A4V and the morpholino oligo against CNR1 or SOD WT as a control (*top*) or injected with the GGGGCC hexanucleotide repetition (75 repeats), the GGGGCC hexanucleotide repetitio and the morpholino oligo for CNR1 or GFP as a control (*bottom*). Embryos were fixed at 30 hpf and the motor neurons were immunostained with SV2. *Right.* Aberrant branching score in the previous embryos. Embryos were scored as aberrant when 2 or more of its motor neurons presented aditional branching in the proximal length of the axon.



mRNA injected embryos. When co-injected with ATG-CNR1 MO, there was a reversion of the phenotype (see figure 3). We also scored abnormal branching in these embryos. The number of abnormal axon sprouting was higher in the SOD1 A4V mRNA injected embryos than in the SOD1 A4V mRNA co-injected with ATG-CNR1 MO (5.5 vs 3.5).

Discussion

These data show the silencing of the can-

nabinoid receptors in a model of axonopathy due to SOD1^{A4V}. First we have measured the effect of CNR1 and CNR2 silencing in the axonal length of the zebrafish caudal motor neurons. When CNR1 is silenced using an ATG MO, there is no reduction in the axonal length. These results could imply that the CNR1 is not yet expressed in the spinal cord. According to previous data (Lam et al., 2006; Watson et al., 2008), the expression of CB_1R starts lately in the development of the embryo (stage 24hpf) and it is restricted to postmitotic cells in the dorsal telencephalon an the hypothalamus and the pattern expands through development. However in our hands we could detect CB_1R with western blotting detection at 30 hpf. There are no previous data about *CNR2* silencing during development of zebrafish embryos. In our hands, silencing *CNR2* causes an axonopathy in caudal region, with motor neurons showing shortened axons. This data could imply the involvement of the CB_2R in the axonal guiding and would be in accordance with the fact that during development, there is an implication of the CB_2R in neural development and as the neurons get differentiated, there is an expression shift between CB_2R and CB_1R (reviewed in (Galve-Roperh et al., 2013). However our preliminary data shows that in the paradigm of an axonopathy due to SOD1^{A4V} overexpression in zebrafish embryos (Lemmens et al., 2007), the co-injection of SOD1A4V mRNA and ATG-CNR1 MO showed a phenotypic rescue compared with the SOD1^{A4V} mRNA injected embryos. This phenotypic rescue was seen both when we measured the axonal length and the number of embryos with abnormal branching. Although this model is limited because it implies the use of developing embryos, our experimental design shows the improvement of axonopathy due to SOD1^{A4V} expression with the knockdown of *CNR1*. Further experiments need to be done to check if this effect could be due to a CB_1R malfunction due to mutated forms of SOD1, but there is previous data that points to that explanation both in striatum slices of mutated SOD1 mice (Rossi et al., 2010) and with increased survival of SOD-^{G93A}-$CB1^{-/-}$ mice (Bilsland et al., 2006).

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DISCUSSION
Discusión

POTENTIAL THERAPEUTIC ROLE OF THE ECS IN MS

Our results confirm that the activation of cannabinoid receptors, both with the synthetic cannabinoid WIN 55,212-2 and the phytocannabinoids Δ^9-THC-BDS, CBD-BDS and the combination of both in a Sativex-like combination attenuate the neurological decline in mice models of MS. WIN 55,212-2 increases the expression levels of the glutamate transporters GLAST and GLT-1 in the spinal cord of EAE mice, probably enhancing thus the glutamate clearance. RT-PCR of spinal cord lysates show that the treatment with WIN 55,212-2 significantly reduces the expression levels of different cytokynes (i.e. COX2, iNOS and TNFα). WIN 55,212-2 reduces the number of aggregates and the recruitment of macrophages in EAE mice as we see in Nissl stainings and immunohistochemistry of spinal cord sections (i.e. Iba-1 and CD11b). Surprisingly, the effects of WIN-55,212-2 in the acute phase of EAE are reversed by the CB_1R antagonist rimonabant, showing that the cannabinoid receptor that mediates WIN 55,212-2 effect in this acute phase of EAE is CB_1R and not CB_2R.

Δ^9-THC-BDS and CBD-BDS also alter the clinical course of MS in TMEV-IDD mice and EAE mice. In the TMEV-IDD mice there is a reduction of the neurological decline with 5 mg/kg Δ^9-THC-BDS, 5 mg/kg CBD-BDS and the combination of both in 5 mg/kg Sativex-like combination treated animals. The effect of Δ^9-THC-BDS is blocked by the CB_1R antagonist AM251 and partially by the CB_2R antagonist AM630. The effect of CBD is blocked with the PPARγ antagonist T-0070907. Sativex-like combination reduced the infiltration of leukocytes and improved the myelin impairment preventing axonal damage.

In EAE mice 20 mg/kg Δ^9-THC-BDS and 20 mg/kg Sativex-like combination reduce the neurological decline versus vehicle treated mice. However, CBD-BDS treated animals showed no difference compared to vehicle treated animals. This results show that in the EAE model, Sativex-like combination is beneficial to attenuate the clinical course of the disease, with the same effect as Δ^9-THC-BDS treated animals, but with half the amount of psychoactive molecule (10 mg/kg of THC in the Sativex-like combination have the same effect as 20 mg/kg THC). CBD has no overall effect. The blockade of the Δ^9-THC effect by rimonabant reveals the implication of the CB1R.

The CB_1R plays an important role in the regulation of MS. The role of CB_1R in the course of EAE in mice was initially described by (Pryce et al., 2003). WIN 55-22,2 has also successfully been used to delay disease progression in other models of MS (Arévalo-Martín et al., 2012; Downer et al., 2011; Mestre et al., 2009). However there is a different dose-response depending on the animal model. In EAE rats, the 5 mg/kg WIN 55,212-2 was un-effective whereas higher doses had the same effect as the 5 mg/kg WIN 55,212-2 we used in mice (Hasseldam and Fryd Johansen, 2011; Hasseldam and Johansen, 2010). This can be explained due to a different metabolism of cannabinoids or activity of the endocannabinoid system in mice and rats. The effect of Δ^9-THC in EAE is largely known since 1989, when it was published that Δ^9-THC was effective to overcome the symptoms of EAE (Lyman et al., 1989). Different studies have been published since, indicating the amelioration of different parameters of MS in different models, such as tremor and spasticity (Baker et al., 2000), and the reduction of CNS inflammation (Maresz et al., 2007). However, the effectiveness of CBD in animal models of MS is contro-

versial. In our hands, CBD-BDS does not reduce the neurological score of EAE mice, although CBD-BDS is effective in TMEV-IDD, apparently through PPARγ activation and A2A receptors (Mecha et al., 2013). Despite this results, that can be explained due to different pathogenic events that take place in TMEV-IDD and EAE, our results match with those obtained by (Maresz et al., 2007), were Δ^9-THC, from 10 mg/kg but not CBD was effective in treating EAE mice, even if the mice strain (C57BL/6 vs ABH) and the EAE induction (MOG 35-55 vs spinal cord homogenate) differ. In our hands however we could not reproduce the CBD effects in EAE mice obtained in other publications (Kozela et al., 2011).

The use of phytocannabinoids enriched botanical extracts as we use in our study is not devoid of issues. The Δ^9-THC and CBD enriched botanical extract are not pure and thus there is a small percentage (0,8 % - 1,5 %) of other cannabinoids such as cannabigerol, cannabichromene and other phytocannabinoids that are biologically active. These extracts are “dirty drugs” though that can aim different targets of the pathogenic events taking place in MS shuch as excitotoxicity, inflammation and oxidative stress. Besides, the use of pre-clinical models in research has limitations. Although, TMEV-IDD and EAE are the most used models of MS in research, the pathogenic events that take place in those models don’t match exactly with human MS (Croxford et al., 2011). However, MS pathogenic events are not yet fully understood, and every patient has their own “personal” MS. The differential effects of Δ^9-THC-BDS and CBD-BDS in TMEV-IDD and EAE may be a reflection of the differences between both MS models. However, we think that this gives more strength to our study, because the Sativex-like combination is effective in both MS models through different targets. On one side, Δ^9-THC-BDS would be acting through CB_1R and CB_2R and on the other side CBD-BDS would activate PPARγ nuclear receptors.

The lack of effective therapies to reduce the progression of MS urges to look for novel therapeutic targets. Our data adds more weight to the amount of pre-clinical information that points the effectiveness of cannabinoids to alter MS progression. Our data gives insight of the anti-glutamatergic and anti-inflammatory effects of WIN 55,212,2 that are mediated via CB_1R. However, the activation of CB_1R and the undesirable psychoactive effects turns questionable the use of this molecule in clinics. Sativex® however is already licensed as a drug to treat sleep disturbances, neuropathic pain and tremor in MS patients. Its sublingual administration gives low Δ^9-THC plasma levels that derive in none or mild side effects, albeit some of this negative-side effects can be counteracted by CBD.

POTENTIAL THERAPEUTIC ROLE OF THE ECS IN ALS

Our results show some of the implications of the ECS in ALS. First, we have shown the characterization of the ECS in an *in vitro* model that is widely used in ALS, the NSC-34 cells. This motor neuron cell line express CB_1R, and the endocannabinoid synthesis and degradation enzymes NAPE-PLD, DAGL, FAAH and MAGL. In differentiated cells there is a significant increase in FAAH and CB_1R expression levels. This increased levels point to an importance of the endocannabinoid system in controlling the homeostasis in motor neurons. Next we have shown the characterization of the ECS in the SODG93A mice model both in males and females. Transgenic mice overexpress the CB_2R, but there is a differential gender expression in the endocannabinoid synthesis and degradation enzymes between male mice and female mice. Transgenic male mice significantly overexpress the NAPE-PLD enzyme and although not significant, there is a slight decrease in the DAGL levels and an increase in the MAGL levels. In female mice there is no difference in the cannabinoid synthesis and degradation enzymes expression levels. When treated with a Sativex-like combination of Δ^9-THC and CBD at symptom onset, the neurological decline improves in both males and females, especially in the first weeks of treatment. However, in male mice aged 100 days old we could not observe an improvement in hindlimb muscle volume. In end stage animals however, there is a significant increase in the number of large motor neurons in the dorsal horn of Sativex-like treated animals compared to the vehicle treated animals.

We have also obtained for the first time data about the function of CB_1R and CB_2R in the caudal motor neuron of zebrafish. The loss of CB_2R expression with a morpholino antisense oligo gives an axonopathy characterized by shorter axonal length and aberrant branching in zebrafish caudal motor neurons. The CB_1R loss does not affect axonal length of zebrafish caudal motor neurons nor produce an abnormal branching. However, when CB_1R expression is blocked in a model of SOD^{A4V} axonopathy in zebrafish, there is a reversion of the phenotype both in the length and aberrant branching of caudal motor neurons.

The changes in the ECS we have seen in $SOD1^{G93A}$ transgenic mice match previous reports (Bilsland and Greensmith, 2008). The increased expression of the NAPE-PLD enzyme is consistent with the AEA increased levels found in the spinal cord of $SOD1^{G93A}$ mice (Witting et al., 2004). The CB_1R and CB_2R expression in $SOD1^{G93A}$ transgenic mice spinal cord was also concordant with the data published (Shoemaker et al., 2007). However, the treatment with Sativex did not had the same effectiveness as the treatment with other cannabinoids such as cannabinol (Weydt et al., 2005), the CB_2R AM1241 (Shoemaker et al., 2007) or the treatment with Δ^9-THC alone (Raman et al., 2004). The dose of Sativex® we chose was derived from the interesting therapeutic perspective of this last paper, where $SOD1^{G93A}$ injected with 20 mg / kg THC at symptoms onset increased their survival. In our hands, we could not reproduce this increased survival treating with the same content of Δ^9-THC in the Sativex-like combination, and we only had significant improvement of the neurological decline in the firsts weeks of treatment. An explanation could be that CBD in our case is antagonizing the effect of Δ^9-THC, an effect already described (Pertwee, 2008; Turkanis and Karler, 1986). But the loss of function of the CB_1R during the progression of the disease

could be another explanation. Although we and others have not seen changes in CB_1R mRNA expression, there is a decrease in the CB_1R protein level (Shoemaker et al., 2007). This could be just explained with the loss of motor neurons, but others have found an hiper-sensitivity of CB_1R in ALS mice striatum (Rossi et al., 2010). This change in the CB_1R alters the GABA and glutamate release and goes parallel to ALS progression. To our knowledge, this alteration of the CB_1R activity has not be seen in other neurodegenerative diseases. This abnormal sensitivity could explain the paradox increased survival of $CB_1^{-/-}SOD1^{G93A}$ mice described by (Bilsland et al., 2006). Although preliminary and not published, the recovery of the axonal length and the abnormal branching when blocking *CNR1* expression in $SOD1^{G93A}$ injected zebrafish supports this possibility of a missfunction of the CB_1R due to SOD1 mutation. The lack of accurate ALS models apart from the $SOD1^{G93A}$ makes difficult to assure if this missfunction of the CB_1R is characteristic of ALS or just due to an effect between the mutated SOD1 mutation and CB_1R.

ALS is far to be cured. There is an urge for new therapeutic targets that can delay or stop the progression of the disease. Our data supports the idea of an involvement of changes in the ECS due to the disease progression. We have also provided data that points that Sativex®, a licensed drug to treat MS symptoms has mild effects delaying the neurological degeneration in an ALS mouse model, and no effects in increasing the survival. But further experiments should be done, maybe with a different therapeutic approach. The CB_2R agonists has been successful treating ALS symptoms in preclinical research (Shoemaker et al., 2007). An approach using CB_1R antagonists could be successful, as previous data supports the idea of a CB_1R malfunction in ALS (Bilsland et al., 2006; Rossi et al., 2010). There is even a phytocannabinoid, Δ^9-THCV, that has a CB_1R antagonist/CB_2R agonist profile that could be effective delaying the neurological decline of $SOD1^{G93A}$ mice, as it has also been effective treating other symptoms of neurodegeneration in a Parkinson's disease model (García et al., 2011). In any case more research needs to be done but the amount of pre-clinical data urges to take the research with the ECS a step further from pre-clinical models to patients clinical trials.

CONCLUSIONS
Conclusiones

1- WIN 55,212-2 exerts anti-glutamatergic and anti-inflammatory effects through CB1R in the initial stages of EAE in mice.

2- Our data support the therapeutic potential of Sativex® as a pharmacotherapy able to slow MS progression as we observed in TMEV-IDD and EAE.

3- The ECS is altered during ALS progression in $SOD1^{G93A}$ transgenic mice and the use of a Sativex-like combination of Δ^9-THC-BDS and CBD-BDS has mild effect in the initial stages of the disease.

4- The blockade of *CNR1* in the Zebrafish ALS model may reverse the axonopathy caused by $SOD1^{A4V}$ mutations.

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