# UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA
Departamento de Bioquímica y Biología Molecular III

## TESIS DOCTORAL

**Towards a neuroprotective therapy with cannabinoids in Huntington's disease: preclinical and clinical studies**

**Hacia una terapia neuroprotectora con cannabinoides en la enfermedad de Huntington: estudios preclínicos y clínicos**

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR


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**Javier Fernández Ruiz**


**Madrid, 2017**


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MEMORIA PARA OPTAR AL GRADO DE DOCTOR CON MENCIÓN EUROPEA
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**MADRID, 2017**

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CERTIFICAN:

Que la presente Tesis Doctoral titulada "Hacia una terapia neuroprotectora con cannabinoides en la enfermedad de Huntington: estudios preclínicos y clínicos", presentada por Sara Valdeolivas Rojas, Licenciada 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 así conste y a los efectos oportunos, firman el presente certificado en Madrid a 20 de Abril de 2017.

Fdo.: Dra. Onintza Sagredo Ezquioga

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# RESUMEN/SUMMARY

# RESUMEN

## INTRODUCCIÓN

La enfermedad de Huntington (EH) es una enfermedad con herencia autosómica dominante causada por una mutación en el gen IT15, que codifica la proteína huntingtina (HTT). Esta proteína presenta una amplia expresión células de mamíferos y, aunque su función exacta no es totalmente conocida, se sabe que está relacionada con la modulación de procesos celulares tales como transcripción génica, señalización celular, metabolismo energético y neurogénesis. La mutación consiste en una expansión excesiva del tracto de poliglutamina localizado en el extremo *N*-terminal de la proteína, lo cual afecta a su plegamiento y actividad normales, y lleva a la alteración de una serie de procesos celulares y la aparición de eventos citotóxicos (ej. Formación de agregados de la huntingtina mutada (mHTT), desregulación transcripcional, reducción de los niveles de BDNF, excitotoxicidad, alteraciones en la función mitocondrial, alteraciones en la degradación proteica y autofagia, estrés oxidativo, activación glial y respuesta inflamatoria). Todos estos eventos son causados bien por pérdida o por ganandia de función de la mHTT, la cual resulta ser tóxica y provocar la degeneración de subpoblaciones neuronales específicas localizadas en los ganglios basales, especialmente las neuronas medianas espinosas, y también en la corteza cerebral (neuronas piramidales). Además, en fases avanzadas de la enfermedad otras regiones cerebrales se ven también afectadas, tales como la *substantia nigra*, el tálamo y el hipocampo.

Dependiendo de la longitud de la cola de poliglutamina, losprimeros síntomas aparecen generalmente como problemas cognitivos y psiquiátricos, al os que les siguen los trastornos motores característicos, los cuales tienen dos componentes principales: en primer lugar movimientos involuntarios o corea, los cuales comienzan temprano en el transcurso de la enfermedad; y en segundo lugar la incapacidad para realizar movimientos voluntarios, que incluye bradicinesia y falta de coordinación. Los trastornos mentales acaban llevando a demencia en fases avanzadas, y los síntomas se desarrollan inexorablemente hasta el desenlace fatal de la enfermedad, generalmente 15-20 años después de la aparición de los síntomas motores.

Actualmente no existe ningún tratamiento efectivo para la EH, y los pocos tratamientos aprobados están centrados en el control de los síntomas. No existen aún terapias efectivas dirigidas a retrasar o detener el proceso degenerativo. Por este motivo, muchos compuestos centrados en la prevención o reducción del daño cerebral en pacientes de EH están ahora en investigación.

En las últimas décadas los compuestos cannabinoides sido evaluados como potenciales agentes neuroprotectores en muchas enfermedades neurodegenerativas, entre ellas la EH. El denominado sistema cannabinoide endógeno SCE está implicado en una serie de procesos fisiológicos que ayudan al mantenimiento o la recuperación de la homeostasis e integridad celular ante un daño agudo o crónico. Por lo tanto, los diferentes elementos del SCE (receptores cannabinoides, endocannabinoides y sus enzimas de síntesis y degradación) ofrecen nuevas e interesantes dianas terapéuticas para el desarrollo de fármacos neuroprotectores. Los pacientes de EH presentan alteraciones en varios componentes de este sistema a lo largo de la enfermedad, principalmente una reducción en la señalización de receptores $CB_1$ en las neuronas medianas espinosas del estriado (lo cual es un evento temprano en la patología) y un aumento en los niveles de $CB_2$ en células gliales del estriado y la corteza cerebral (lo cual está relacionado con una respuesta inflamatoria). También se han descrito alteraciones en los niveles de endocannabinoides y enzimas de síntesis y degradación, que pueden alterar las acciones de los endocannabinoides sobre sus receptores. Por lo tanto, la manipulación farmacológica de este sistema podría ser terapéuticamente relevante en la EH, debido a la capacidad de los cannabinoides para actuar sobre muchas vías de señalización implicadas en la supervivencia celular, y por tanto limitar los distintos eventos citotóxicos que se dan en la enfermedad. De hecho, cannabinoides con distintos perfiles farmacológicos, tales como agonistas $CB_1$, agonistas $CB_2$ y cannabinoides antioxidantes, han sido ya estudiados en modelos preclínicos

que representan distintos eventos patogénicos que ocurren en los cerebros de los pacientes de EH (ej. Excitotoxicidad, inflamación y estrés oxidativo) y han mostrado resultados muy prometedores.

## OBJETIVOS

La hipótesis principal de esta Tesis Doctoral es que compuestos fitocannabinoides, de manera individual o en combinación, y con un perfil farmacológico de amplio espectro, pueden representar una opción prometedora para abordar los múltiples procesos citotóxicos implicados en la patología de la EH.

Para demostrar esta hipótesis, el objetivo global de esta Tesis es progresar en el desarrollo de una terapia neuroprotectora basada en fitocannabinoides en la EH. Este objetivo global será dividido en 3 objetivos específicos, que serán presentados como 3 capítulos diferentes:

**Capítulo 1: Evaluación preclínica de fitocannabinoides como agentes modificadores de la enfermedad en modelos animales de la EH.**

En este capítulo evaluamos en primer lugar la combinación 1:1, equivalente al Sativex, de extractos botánicos enriquecidos en $\Delta^9$-THC and CBD, en un modelo de degeneración estriatal aguda en rata, generado por la inducción de eventos inflamatorios y activación de la apoptosis provocada por la inyección intraestriatal de malonato. El tratamiento con Sativex redujo el volumen de edema estriatal generado tras la lesión, atenuó la muerte celular y activación glial en el estriado y revertió la expresión de marcadores pro-inflamatorios. También pudimos demostrar que este efecto protector incluía la activación de receptores $CB_1$, pero sobre todo $CB_2$.

A continuación, evaluamos el tratamiento crónico con la misma combinación de fitocannabinoides en el modelo transgénico de ratón R6/2. El tratamiento con Sativex no produjo una mejoría en la coordinación motora pero sí atenuó el grado de “clasping”, lo cual es un marcador de distonia, en estos animales. También atenuó las deficiencias metabólicas observadas en los cerebros de estos animales, e indujo mejoría en algunos marcadores metabólicos relacionados con déficit energético, fallo mitocondrial y deterioro neuronal.

Por último, analizamos el potencial neuroprotector del fitocannabinoide CBG en un modelo agudo en ratón que cursa con activación glial y daño oxidativo, producido por la inyección de 3NP. El tratamiento con CBG mejoró parcialmente varios déficit motores causados por la toxina, impidió casi con totalidad la neurodegeneración estriatal, atenuó la activación microglial y revirtió los cambios en marcadores de inflamación y oxidación en los estriados de estos animales. A continuación, evaluamos los efectos del tratamiento crónico con CBG en ratones R6/2. El tratamiento produjo una pequeña mejoría en la coordinación motora y redujo el número de agregados estriatales de mHTT en este modelo. Asimismo, revirtió el incremento en la expresión génica de $CB_2$ y reducción BDNF y PPARγ, así como de otros marcadores de la patología. Puesto que el CBG presenta muy baja afinidad por receptores cannabinoides, sugerimos que la activación del receptor nuclear PPARγ podría explicar en parte este efecto neuroprotector.

Los resultados de este Capítulo están incluidos en las siguientes publicaciones:

Valdeolivas, S., Satta, V., Pertwee, R.G., Fernandez-Ruiz, J. & Sagredo, O. (2012) Sativex-like combination of phytocannabinoids is neuroprotective in malonate-lesioned rats, an inflammatory model of Huntington’s disease: role of CB1 and CB2 receptors. *ACS Chem Neurosci*, 3, 400-406.

Valdeolivas, S., Sagredo, O., Delgado, M., Pozo, M.A. & Fernandez-Ruiz, J. (2017) Effects of a Sativex-Like Combination of Phytocannabinoids on Disease Progression in R6/2 Mice, an Experimental Model of Huntington’s Disease. *Int J Mol Sci*, 18.

Valdeolivas, S., Navarrete, C., Cantarero, I., Bellido, M.L., Munoz, E. & Sagredo, O. (2015) Neuroprotective properties of cannabigerol in Huntington’s disease: studies in R6/2 mice and 3-nitropropionate-lesioned mice. *Neurotherapeutics*, 12, 185-199.

**Capítulo 2: Evaluación del Sativex en un ensayo clínico piloto con pacientes de EH.**

Los resultados preclínicos obtenidos de modelos celulares y animales sobre la evaluación del potencial neuroprotector de los cannabinoides en EH apoyan la validación de formulaciones de cannabinoides con un amplio espectro farmacológico en estudios clínicos. En este Capítulo, presentamos los resultados de un ensayo piloto en fase II, doble ciego, aleatorizado, cruzado y controlado con placebo en pacientes de EH tratados con Sativex, que se llevó a cabo en el Hospital Ramón y Cajal de Madrid. El objetivo principal del estudio, que era la seguridad y tolerabilidad del Sativex por los pacientes se cumplió, pero no obstante el tratamiento ofreció ninguna mejoría a nivel motor ni cognitivo. Además tampoco se observaron cambios en los biomarcadores examinados con el tratamiento, excepto un incremento en los niveles de expresión génica de $CB_2$ en los linfocitos de los pacientes, durante la fase de tratamiento con Sativex, lo cual se interpretó como una posible respuesta protectora y antiinflamatoria. A la vista de los resultados, proponemos la realización de estudios adicionales, con un mayor número de pacientes y en particular con una duración mayor del tratamiento para confirmar si el Sativex puede producir un beneficio clínico en los pacientes de EH.

Los resultados de este Capítulo están incluidos en la siguiente publicación:

Lopez-Sendón Moreno, J.L., Garcia Caldentey, J., Trigo Cubillo, P., Ruiz Romero, C., Garcia Ribas, G., Alonso Arias, M.A., Garcia de Yebenes, M.J., Tolon, R.M., Galve-Roperh, I., Sagredo, O., Valdeolivas, S., Resel, E., Ortega-Gutierrez, S., Garcia-Bermejo, M.L., Fernandez Ruiz, J., Guzman, M. & Garcia de Yebenes Prous, J. (2016) A double-blind, randomized, cross-over, placebo-controlled, pilot trial with Sativex in Huntington's disease. *J Neurol*, 263, 1390-1400.

**Capítulo 3: Estudio de algunos de los mecanismos implicados en el efecto de compuestos cannabinoides en modelos experimentales de EH.**

En este capítulo nos propusimos estudiar con profundidad algunos de los posibles mecanismos implicados en el efecto protector de los cannabinoides. Nos centramos principalmente en el efecto antioxidante, y la posible activación de NRF2 y la consecuente respuesta antioxidante. Con este objetivo, evaluamos la combinación equivalente al Sativex de extractos botánicos de $\Delta^9$-THC and CBD, en comparación con estos fitocannabinoides por separado, en un modelo celular de EH de neuroblastos de ratón (células $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$). En primer lugar se expuso a estas células a agentes que producían distintos tipos de daño celular que ocurren en el cerebro de pacientes de EH: glutamato (excitotoxicidad), malonato (daño mitocondrial) y peróxido de hidrógeno ($H_2O_2$, daño oxidativo). En todos los casos esta exposición llevó una elevada muerte celular, pero sólo se observó un efecto protector de los fitocannabinoides ante el daño por peróxido de hidrógeno. CBD y, sobre todo, Sativex, protegieron ambas líneas celulares frente ante la exposición a $H_2O_2$, aunque este efecto era mucho más modesto en las células $STHdh^{Q111/Q111}$ que en las $STHdh^{Q7/Q7}$. Junto a este efecto neuroprotector se observó un incremento en la expresión génica de la enzima HO-1, por lo que a continuación estudiamos el posible papel de la vía NRF2/ARE en esta acción de los fitocannabinoides. Nuestros resultados no han podido confirmar la participación de NRF2/ARE en este proceso, por lo que serán necesarios más experimentos para descifrar los posibles mecanismos implicados en esta neuroprotección a las células estriatales ante el daño oxidativo ejercida por el Sativex y CBD.

El segundo objetivo específico en este capítulo consistía en estudiar si los endocannabinoides, en particular el 2-AG, proporcionan neuroprotección en la EH, utilizando el modelo de malonato. A diferencia de lo esperado, observamos que la inhibición de la MAGL, lejos de resultar neuroprotectora, agravaba el daño estriatal, mientras que la inhibición de la DAGL producía el efecto opuesto. Pudimos explicar este inesperado resultado mediante la regulación a la alza de la enzima COX-2 inducida por el malonato, la cual contribuía a la conversión del 2-AG en prostaglandin-gliceril-esters, derivados de este endocannabinoide que son altamente neurotóxicos. Utilizando la línea celular M-213, demostramos que la inducción de COX-2 y consiguiente generación de estos derivados oxigenados del 2-AG contribuía a la muerte neuronal, mientras que la inhibición de COX-2 o el bloqueo de las

dianas de estos derivados reducía la neurotoxicidad.

Los resultados de este Capítulo están incluidos en las siguientes publicaciones:

Valdeolivas, S., Rodriguez-Cueto, C. Sagredo, O. and Fernández-Ruiz, J. Study of the involvement of NRF2-KEAP1/ARE signaling in the antioxidant effect of phytocannabinoids against hydrogen peroxide in a cellular model of Huntington's disease. (Datos preliminares no publicados).

Valdeolivas, S., Pazos, M.R., Bisogno, T., Piscitelli, F., Iannotti, F.A., Allara, M., Sagredo, O., Di Marzo, V. & Fernandez-Ruiz, J. (2013) The inhibition of 2-arachidonoyl-glycerol (2-AG) biosynthesis, rather than enhancing striatal damage, protects striatal neurons from malonate-induced death: a potential role of cyclooxygenase-2-dependent metabolism of 2-AG. *Cell Death Dis*, 4, e862.

## CONCLUSIONES

Los resultados obtenidos en esta Tesis Doctoral nos permiten concluir que:

1. El tratamiento con CBG protege a las neuronas estriatales y mejora el deterioro neurológico en el modelo de daño oxidativo estriatal por 3NP, actuando específicamente en la mejora de la respuesta antioxidante y la reducción de la activación glial. Estos efectos no están mediados por receptores cannabinoides, y podrían deberse a una acción sobre receptores PPAR.

2. El CBG también proporciona neuroprotección en el modelo transgénico R6/2, en este caso, mediante la reducción de la acumulación de agregados de huntingtina, y actuando de manera positiva en la expresión de determinadas neurotrofinas y genes relacionados con desregulación transcripcional en la EH. De nuevo, estos efectos eran independiendtes de receptores cannabinoides.

3. La combinación equivalente al Sativex de extractos botánicos de fitocannabinoides protege a las células estriatales en el modelo de malonato de inflamación aguda y apoptosis, actuando específicamente sobre la reducción de la glía reactiva y mejorando el apoyo neurotrófico. Este efecto implica la activación de receptores $CB_1$ y $CB_2$.

4. La combinación equivalente al Sativex de extractos botánicos de fitocannabinoides también mejora algunas alteraciones motoras y corrige deficiencias metabólicas y marcadores relacionados con déficit energético y daño mitocondrial en el modélo transgénico de EH R6/2.

5. El Sativex es seguro y bien tolerado por pacientes de EH, como muestran los resultados de un ensayo clínico piloto en fase II realizado en una cohorte de pacientes sintomáticos tempranos.

6. No obstante, el Sativex no ha demostrado ningún benedicio clínico en escalas motoras ni cognitivas, así como en biomarcadores de la enfermedad, salvo el aumento en la expresión de receptores $CB_2$, lo cual podría suponer una respuesta protectora. El corto tiempo de tratamiento aplicado en este ensayo sugiere la necesidad de establecer periodos de tratamiento más largos para futuros estudios clínicos.

7. Utilizando una estrategia in vitro, demostramos (células $STHdh^{Q7/Q7}$ y $STHdh^{Q111/Q111}$), demostramos que los efectos neuroprotectores ante el daño oxidativo ejercidos por el CBD en forma de extracto botánico, en solitario o combinado con $\Delta^9$-THC como en el Sativex, podrían implicar una cierta activación de la señalización mediada por NRF2, aunque parece necesaria la contribución de otros mecanismos celulares adicionales.

8. Al contrario de lo esperado, las células $STHdh^{Q111/Q111}$ no son más vulnerables al daño oxidativo, y que la acción de la vía de señalización activada por NRF2 en estas células es limitada.

9. La inhibición de la biosíntesis de 2-AG en ratas lesionadas con malonato, al contrario de lo esperado, previene la degeneración de las neuronas estriatales, mientras que la inhibición de la degradación de 2-AG agrava el daño estriatal.

10. Utilizando este modelo in vivo y una estrategia in vitro (células M-213) demostramos que este resultado podía explicarse por la regulación a la alza de la enzima COX-2, el aumento en los niveles de 2-AG y su transformación mediante esta enzima en prostaglandil-gliceril-esters, los cuales son altamente neurotóxicos.

Todos estos resultados apoyan el potencial neuroprotector de compuestos fitocannabinoides con un amplio espectro farmacológico en modelos experimentales de EH. Estos hallazgos preclínicos sugieren la necesidad de realizar más estudios para dilucidar los mecanismos de neuroprotección de los fitocannabinoides, así como la necesidad de evaluar formulaciones multi-diana de fitocannabinoides, como el Sativex en ensayos clínicos con periodos de tratamiento más prolongados.

# SUMMARY

## INTRODUCTION

Huntington's disease is an inherited autosomal dominant disease caused by a mutation in the IT15 gene that encodes the protein huntingtin (HTT). This protein is widely expressed in mammalian cells and, although its exact function still remains unclear, it appears to be related to the modulation cell processes such as gene transcription, cell signaling, energy metabolism and neurogenesis. The mutation consists in an excessive expansion of the polyglutamine tract of the protein located in its *N*-terminal domain, which alters protein folding and activity and leads to the disturbance of many cellular processes and the appearance of many cytotoxic events (e.g. aggregation of mutant HTT (mHTT), transcriptional dysregulation, reduced BDNF levels, excitotoxicity, altered mitochondrial function, alterations in protein degradation and autophagy, oxidative stress, glial activation and inflammatory responses). All these events are caused by either loss-of-function or gain-of-function in mHTT, which results to be toxic and elicit the degeneration of selective neuronal subpopulations in the basal ganglia, specifically the medium spiny neurons (MSNs), and also in the cerebral cortex (pyramidal cells). In addition, in advanced phases of the disease, other brain regions are also affected, such as *substantia nigra*, thalamus and hippocampus.

Depending on the length of the poliglitamine tract, its earliest symptoms often appear as cognitive and neuropsychiatric issues and are followed by characteristic motor disorders, which have two major components: firstly involuntary movements or chorea, beginning early in course of the pathology; and second the impairment of voluntary movements, which include bradykinesia and lack of coordination. Signs and symptoms develop inexorably, leading mental issues generally to decline into dementia, until the fatal outcome of the disease, generally around 20 years after the appearance of motor impairment.

To date, there is no effective treatment to cure HD, and the very few treatments approved are focused on ameliorating the symptoms. No effective therapies designed to delay or stop the neurodegenerative process are available yet. Therefore, there is an unmet need for neuroprotective strategies aimed at preventing or reducing brain damage in HD patients, and several agents are currently under research.

In the last decades, cannabinoid compounds have emerged as potential neuroprotective agents in many neurodegenerative diseases, including HD. The so-called endocannabinoid system (ECS) is involved in a wide variety of physiological processes that help to maintain or recover cell homeostasis and integrity after acute or chronic damage. Therefore, the different elements of the SCE (cannabinoid receptors, endocannabinoids and their synthesizing and degradative enzymes) offer interesting new therapeutic targets for the development of neuroprotective drugs. HD patients show alterations in various components of this system during the progression of the disease, mainly a dysregulation of $CB_1$ receptors signaling in the MSNs in the striatum (which occurs early in the pathology) and an upregulation of $CB_2$ receptors in glial elements of striatal and cortical areas (which is related to inflammatory responses). Alterations in the levels of endocannabinoids and synthesizing and degradative enzymes have also been reported and may alter the endocannabinoid actions at their receptors. Therefore, the pharmacological manipulation of this system may be therapeutically relevant for HD because of the capability of cannabinoids to act on many signaling pathways involved in cell survival then limiting the different cytotoxic events that occur in the disease. Indeed, cannabinoids with different pharmacological profiles, such as $CB_1$ agonists, $CB_2$ agonists and antioxidant cannabinoids, have already been tested in preclinical models that resemble different pathogenic events that occur in the brains of HD patients (such as excitotoxicity, inflammation and oxidative stress), showing very promising results.

## AIMS

The main hypotheses of this Doctoral Thesis is that phytocannabinoids, alone or in combination, ensuring a broad-spectrum profile, may represent a promising option to arrest the multiple cytotoxic processes involved in HD pathology.

To prove this hypotheses, the **global aim** of this Thesis is to progress in the development of a phytocannabinoid-based neuroprotective therapy in HD. This global objective will be divided into **3 specific aims**, which will be presented as 3 different chapters:

**Chapter 1: Preclinical evaluation of phytocannabinoids as disease modifiers in animal models of HD.**

In this chapter we first evaluated the Sativex-like 1:1 combination of botanical extracts enriched with $\Delta^9$-THC and CBD in a rat model of acute striatal degeneration generated by the induction of inflammatory events and activation of the apoptotic machinery elicited by the intrastriatal injection of malonate. The treatment with Sativex reduced the volume of striatal edema after the lesion, attenuated the striatal cell loss and glial activation and reversed the increase in expression of pro-inflammatory markers. We also demonstrated that $CB_1$, but mainly $CB_2$ receptors were involved in this neuroprotective effect.

Next, we evaluated the chronic treatment with the same Sativex-like combination of phytocannabinoids in the transgenic R6/2 mouse model. Sativex did not produce an improvement in motor coordination but attenuated clasping behavior, which is a marker of dystonia, in these animals. It also attenuated metabolic deficiencies found in the brain of these mice, as well as it improved some prognostic markers related to energy deficit, mitochondrial failure and neuronal deterioration.

Last, we analyzed the neuroprotective potential of the phytocannabinoid CBG in a mouse model of oxidative damage and calpain activation produced by the injection of 3NP. CBG partially improved some motor deficits caused by the toxin, completely prevented striatal cell loss, attenuated microglial activation and reversed the changes in markers of inflammation and oxidative damage in the striata of these mice. Next, we evaluated the effects of chronic treatment with CBG in R6/2 mice. CBG produced a modest improvement in motor coordination and reduced the number of striatal mHTT aggregates in this model. It also partially reversed the increase in $CB_2$ and decrease in BDNF and PPARγ striatal gene expression, as well as other markers reflecting HD pathology. Since CBG has very low affinity for cannabinoid receptors, we propose that the activation of PPARγ may underlie this neuroprotective effect.

The results of this chapter are included in the following publications:

Valdeolivas, S., Satta, V., Pertwee, R.G., Fernandez-Ruiz, J. & Sagredo, O. (2012) Sativex-like combination of phytocannabinoids is neuroprotective in malonate-lesioned rats, an inflammatory model of Huntington's disease: role of CB1 and CB2 receptors. *ACS Chem Neurosci*, 3, 400-406.

Valdeolivas, S., Sagredo, O., Delgado, M., Pozo, M.A. & Fernandez-Ruiz, J. (2017) Effects of a Sativex-Like Combination of Phytocannabinoids on Disease Progression in R6/2 Mice, an Experimental Model of Huntington's Disease. *Int J Mol Sci*, 18.

Valdeolivas, S., Navarrete, C., Cantarero, I., Bellido, M.L., Munoz, E. & Sagredo, O. (2015) Neuroprotective properties of cannabigerol in Huntington's disease: studies in R6/2 mice and 3-nitropropionate-lesioned mice. *Neurotherapeutics*, 12, 185-199.

**Chapter 2: Evaluation of Sativex in a pilot clinical trial with patients of HD.**

The preclinical results obtained from animal and cellular models about the evaluation the potential neuroprotective effect of cannabinoids in HD support the validation of broad-spectrum cannabinoid

formulations in clinical studies. In this Chapter, we present the results of a double-blind, randomized, cross-over, placebo-controlled pilot clinical trial with Sativex in patients with HD, that was performed at "Hospital Ramón y Cajal", Madrid. The primary endpoint of this study, which was the safety and tolerability of Sativex by the subjects was achieved, but it did not show any improvement in motor performance and cognitive and psychiatric measures. Besides, no change was observed in the biomarkers examined, except for an increase in $CB_2$ expression in the patients' lymphocytes during Sativex-treatment phase, which was interpreted as a possible cytoprotective and antiinflamatory response. Additional studies, with more subjects and in particular, longer treatment duration are proposed to further evaluate if Sativex can produce a clinical benefit in HD patients.

The results of this chapter are included in the following publication:

Lopez-Sendon Moreno, J.L., Garcia Caldentey, J., Trigo Cubillo, P., Ruiz Romero, C., Garcia Ribas, G., Alonso Arias, M.A., Garcia de Yebenes, M.J., Tolon, R.M., Galve-Roperh, I., Sagredo, O., Valdeolivas, S., Resel, E., Ortega-Gutierrez, S., Garcia-Bermejo, M.L., Fernandez Ruiz, J., Guzman, M. & Garcia de Yebenes Prous, J. (2016) A double-blind, randomized, cross-over, placebo-controlled, pilot trial with Sativex in Huntington's disease. *J Neurol*, 263, 1390-1400.

**Chapter 3: Study of some of the molecular mechanisms involved in the effect of cannabinoid compounds in experimental models of HD.**

In this chapter we aimed to deeply study some of the possible mechanisms involved in the protective effect of cannabinoids. We focused particularly in the antioxidant effect and the possible involvement of NRF2 activation and promotion of antioxidant response. To this aim, we tested the Sativex-like combination of $\Delta^9$-THC and CBD botanical extracts compared with each phytocannabinoid used individually in a HD cellular model of conditionally immortalized murine striatal neuroblasts ($STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells). Firstly, we exposed these cells to agents that produced different cytotoxic events that occur in HD brains: glutamate (excitotoxicity), malonate (mitochondrial damage) and hydrogen peroxide (oxidative damage). An important extent of cell death was confirmed in all cases, but results showed that protection with phytocannabinoids was only significant against hydrogen peroxide insult. CBD, but mainly Sativex, protected both cell lines against $H_2O_2$, although this effect was much more modest in $STHdh^{Q111/Q111}$ than in $STHdh^{Q7/Q7}$ cells. This effect was accompanied by an increase of HO-1 gene expression, so next we tested the possible involvement of NRF2/ARE pathway in this phytocannabinoid-mediated neuroprotection. Our results did not confirm a role of NRF2/ARE in this process, so further research will be needed to dissect the potential mechanisms involved in the CBD and Sativex –mediated neuroprotection against oxidative damage in striatal cells.

A second specific objective in this chapter consisted in exploring whether endocannabinoids, in particular 2-AG, provide some type of tonic protection in HD, using the malonate model. Unexpectedly, we observed that inhibiting MAGL, rather than being neuroprotective, aggravated striatal damage, whereas the opposite happened with DAGL inhibition. This unexpected result was explained through a malonate-induced COX-2 up-regulation which contributes to convert 2-AG in prostaglandin-glycerol-esters. Such derivatives were highly neurotoxic. Using *in vitro* tools, M-213 cells, we demonstrated the induction of COX-2 and the generation of the 2-AG oxygenated derivatives, as well as that inhibition of COX-2 or the blockade of the targets for these derivatives reduced the neurotoxicity.

The results of this chapter are included in the following publications:

Valdeolivas, S., Rodriguez-Cueto, C. Sagredo, O. and Fernández-Ruiz, J. Study of the involvement of NRF2-KEAP1/ARE signaling in the antioxidant effect of phytocannabinoids against hydrogen peroxide in a cellular model of Huntington's disease. (Datos preliminares no publicados).

Valdeolivas, S., Pazos, M.R., Bisogno, T., Piscitelli, F., Iannotti, F.A., Allara, M., Sagredo, O., Di Marzo, V. & Fernandez-Ruiz, J. (2013) The inhibition of 2-arachidonoyl-glycerol (2-AG) biosynthesis,

rather than enhancing striatal damage, protects striatal neurons from malonate-induced death: a potential role of cyclooxygenase-2-dependent metabolism of 2-AG. *Cell Death Dis*, 4, e862.

## CONCLUSIONS

The results obtained in this Doctoral Thesis allow us to conclude that:

1. CBG protects striatal neurons and improves neurological decline in the 3NP model of acute striatal oxidative damage, by acting specifically on improving antioxidant defenses and reducing glial reactivity, and these effects are exerted by cannabinoid receptor-independent mechanisms, presumably by acting at the PPARs.

2. CBG also exerts neuroprotection in the transgenic R6/2 model, in this case, by reducing the accumulation of huntingtin aggregates and acting positively on specific neurotrophins and specific genes related to HD transcriptional dysregulation. Again the effects are cannabinoid receptor-independent.

3. The Sativex-like combination of phytocannabinoid botanical extracts protects striatal cells in the malonate model of acute inflammatory and apoptotic damage, acting specifically on limiting glial reactivity and improving neurotrophic support. This effect involves the activation of both $CB_1$ and $CB_2$ receptors.

4. The Sativex-like combination of phytocannabinoid botanical extracts also improves some motor deficits and corrects the metabolic deficiencies and prognostic markers related to energy deficit and mitochondrial failure in the R6/2 transgenic model of HD.

5. Sativex is safe and well-tolerated in HD patients as shown in a pilot Phase II clinical trial conducted in a cohort of early symptomatic HD patients.

6. However, Sativex did not exert any clinical benefit in motor and cognitive scales and also in different biomarkers, other than an elevation in the levels of $CB_2$ receptors that could be an endogenous protective response. The short time used for the active treatment in this clinical trial suggests the need to consider longer periods of treatment in future clinical studies.

7. Using in vitro strategies ($STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells), we could demonstrate that the protective effects of CBD against oxidative damage in the form of botanical extract, alone or combined with $\Delta^9$-THC as in Sativex, may involve a certain activation of the NRF2 signaling, although the contribution of additional cellular mechanisms appears to be necessary.

8. Contrarily to the expected result, $STHdh^{Q111/Q111}$ cells are not more vulnerable to the oxidative insult, and the contribution of NRF2 signaling in these cells is rather limited.

9. The inhibition of 2-AG biosynthesis in malonate-lesioned rats, contrarily to the expected result, preserves striatal neurons from death, whereas the inhibition of 2-AG degradation aggravates striatal damage.

10. Using this in vivo model and also an in vitro strategy (M-213 cells), we could demonstrate that such unexpected result was facilitated by the up-regulation of COX-2 enzyme, the increase in 2-AG availability, and its transformation by this enzyme into prostaglandin-glyceryl-esters, which are highly neurotoxic.

Our results support the neuroprotective potential of broad-spectrum phytocannabinoid compounds in different experimental models of HD. These preclinical findings suggest the need of more studies to elucidate the mechanisms of neuroprotection by phytocannabinoids, as well as the need for the evaluation of a multi-target phytocannabinoid formulation, such as Sativex, in additional clinical studies with HD patients but using longer periods of treatment.

# ABBREVIATIONS

# ABBREVIATIONS

| | |
|---|---|
| 2-AG | 2-Arachidonoylglycerol |
| 2-OG | 2-Oleylglycerol |
| 3-NT | 3-Nitrotyrosine |
| 8-OHDG | 8-hydroxydeoxyguanosine |
| AA | Arachidonic acid |
| ACEA | Arachidonoyl-2-chloroethylamide |
| AD | Alzheimer's disease |
| AEA | Anandamide or N-arachidonoylethanolamine |
| AIDS | Acquired immune deficiency syndrome |
| ALS | Amyotrophic lateral sclerosis |
| AMPA | α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ASO | Antisense oligonucleotide |
| ATF2 | Activating transcription factor 2 |
| ATP | Adenosine triphosphate |
| BDNF | Brain derived neurotrophic factor |
| cAMP | Cyclic adenosine monophosphate |
| CB1 | Cannabinoid receptor type 1 |
| CB2 | Cannabinoid receptor type 2 |
| CBC | Cannabichromene |
| CBD | Cannabidiol |
| CBDA | Cannabidiolic acid |
| CBDV | Cannabidivarin |
| CBG | Cannabigerol |
| CBN | Cannabinol |
| CBP | CREB binding protein |
| CBR | Cannabinoid receptor |
| CNS | Central nervous system |
| COX | Ciclooxigenase |
| CREB | cAMP response element binding protein |
| CSF | Cerebrospinal fluid |
| DA | Dopamine |
| DAG | Diacylglycerol |
| DAGL | Diacylglycerol lipase |
| DALN | Desacetyllevonantradol |
| DNA | Desoxyribonucleic acid |
| DSE | Depolarization-induced supression of excitation |
| DSI | Depolarization-induced supression of inhibition |
| ECB | Endocannabinoid |
| ECS | Endocannabinoid system |
| EMA | European Medicines Agency |

| | |
|---|---|
| ERK | Extracellular signal-regulated kinase |
| FAAH | Fatty acid amide hydrolase |
| FDA | Food and Drug Administration |
| FLAT | FAAH-like anandamide transporter |
| GABA | γ-Aminobutyric acid |
| GP | Globus Pallidus |
| GPe | External segment of the globus pallidus |
| GPi | Internal segment of the globus pallidus |
| GPCR | G protein-coupled receptor |
| HD | Huntington's disease |
| HDAC | Histone deacetylase |
| HO1 | Heme oxygenase 1 |
| HTT | Huntingtin |
| IL | Interleukin |
| iNOS | Inducible nitric oxid synthase |
| IP3 | Inositol triphosphate |
| JNK | c-Jun N-terminal protein kinase |
| liso-PI | Lysophosphatidylinositol |
| LOX | Lipooxigenase |
| LTD | Long term synaptic depression |
| MAGL | Monoacylglycerol lipase |
| MAPK | Mitogen-activated protein kinase |
| mHTT | Mutant huntingtin |
| MSN | Medium spiny neuron |
| NADA | N-Arachidonoyldopamine |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |
| NAGly | N-Arachidonoylglycine |
| NAPE | N-Arachidonoyl phosphatidylethanolamine |
| NF-κB | Nuclear factor kappa-licht-chain-enhancer of activated B cells |
| NHR | Nuclear hormone receptor |
| NMDA | N-methyl-D-aspartate |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NPY | Neuropeptide-Y |
| NRF2 | Nuclear factor (erythroid-derived 2)-like 2 |
| OEA | Oleylethanolamide |
| PA | Phosphatidic acid |
| PD | Parkinson's disease |
| PDE10A | Phosphodiesterase 10A |
| PEA | Palmithoylethanolamide |
| PET | Positron emission tomography |
| PGE2 | Prostaglandin E2 |

| | |
|---|---|
| PG-G | Prostaglandin-glycerol-ester |
| PI | Phosphoinositide |
| PI3K | Phosphatidylinositol 3-kinase |
| PIP2 | Phosphatidylinositol biphosphate |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PLA1 | Phospholipase A1 |
| PLC | Phospholipase C |
| PLD | Phospholipase D |
| polyQ | Polyglutamine |
| REST | RE1-Silencing transcription factor |
| PPAR | Peroxisome proliferator-activated receptor |
| RNA | Ribonucleic acid |
| RNAi | Interfering RNA |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RXR | Retinoid X receptor |
| SEA | stearoylethanolamide |
| SNc | *Substantia nigra pars compacta* |
| SNr | *Substantia nigra pars reticulata* |
| SS | Somatostatin |
| SSCI | Substrate-selective COX-2 inhibitor |
| STN | Subthalamic |
| TGF | Transforming grouth factor |
| TNF | Tumor necrosis factor |
| TRP | Transient receptor potential |
| VMAT2 | Vesicular monoamine transporter 2 |
| $\Delta^9$-THC | Δ9-tetradydrocannabinol |
| $\Delta^9$-THCA | Δ9-tetradydrocannabinolic acid |
| $\Delta^9$-THCV | Δ9-tetradydrocannabivarin |

# INTRODUCTION

Huntington's disease (HD) is a devastating neurological disorder that currently has no cure. Along this Introduction, we will review the main aspects of this disease and its current therapies. This will be followed by a description of the alterations observed in the endocannabinoid system in relation with the disease and how different cannabinoid compounds, because of their neuroprotective potential, can modify the course of the disease, what makes them good potential therapies for HD.

# HUNTINGTON'S DISEASE

## Epidemiology and clinical features

Huntington's disease is a hereditary, autosomal dominant disorder caused by a mutation in the exon 1 of the huntingtin (HTT) gene (reviewed in THDCRG, 1993). With a prevalence of 10.6 to 16.7 affected individuals per 100,000 in the Western populations (Evans *et al.*, 2013; Fisher & Hayden, 2014), it is the most common monogenic neurological disorder.

First clinically described by George Huntington in 1872 (Huntington, 2003), the symptoms of this disease usually begin as cognitive and neuropsychiatric issues, such as attention problems, decreased learning, depression and irritability (Peavy *et al.*, 2010; Thompson *et al.*, 2012). As the disease progresses, the typical motor disorders arise (although sometimes they precede cognitive impairment), which have two major components: the first one is the involuntary movements or chorea, beginning early in the course of the pathology; the second is the impairment of voluntary movements, which include incoordination and bradykinesia. This last component is more frequent in the juvenile-onset disease, but also appears in the late phases of the adult illness (Morreale, 2015). Signs and symptoms develop inexorably until the fatal outcome of the disease, which occurs around 15-20 years after motor impairment onset (Ross *et al.*, 2014).

## Etiology and genetics

As introduced above, HD is caused by a mutation in the HTT gene (IT15). This gene is located at chromosome 4p16.3 and encodes the protein huntingtin, whose normal function is not completely understood yet, although it appears to be related to modulation of cell processes like apoptosis, energy metabolism and neurogenesis (Reiner *et al.*, 2003; Cattaneo *et al.*, 2005). This protein has a molecular weight of 348 KDa and contains a polyglutamine (polyQ) tract in its N-terminal domain (THDCRG, 1993). The mutation consists in an excessive number of CAG repeats in the IT15 gene, which results in an expansion of the polyQ tract that affects protein folding and activity. Population not affected by the disease contain 6-35 CAG repeats; between 36 and 39 repeats, the mutation shows reduced penetrance, with some subjects developing HD and others living without showing any clinical sign. When the tract is expanded to 40 or more repeats, the mutation is highly penetrant and inevitably leads to the onset of motor symptoms. The length of the polyQ tract is inversely proportional to the age at onset (Duyao *et al.*, 1993)1993 and, importantly, this is determined by the allele with the longer CAG repeat in a completely dominant manner (Lee *et al.*, 2012). Therefore, juvenile-onset individuals will have a higher number of repeats (usually more than 60) than adult-onset subjects (40-50).

## Mutant huntingtin and mechanisms of neurodegeneration

Huntingtin is ubiquitously expressed in human tissues, with high levels of expression in the central nervous system (CNS). Interestingly, this protein is present in high levels in cortical pyramidal neurons in layers III, V and VI that project to striatal neurons (Hedreen *et al.*, 1991; Strong *et al.*, 1993; Fusco *et al.*, 1999). Forms of the protein are found in the cytoplasm and in the nucleus, and it can change its location between these compartments. Shortly after the cloning of HTT gene in 1993, huntingtin was proved to be essential for the embryonic development of the CNS (Duyao *et al.*, 1995). *In vitro* and *in vivo* studies have shown its pro-survival and anti-apoptotic role (Rigamonti *et al.*, 2000). It has also been demonstrated its involvement in synaptic activity (Smith *et al.*, 2005) as well as in the production and axonal and vesicle transport of the neurotrophin brain-derived neurotrophic factor (BDNF).

Despite what is already known about wild type HTT function, it remains unclear why a single mutation in this protein leads to the selective

neurodegeneration of a specific population of neurons in the striatum: the medium spiny neurons (MSNs). In this context, hypotheses about "loss of function" and "gain of function" have emerged and it appears that both conditions may contribute to the pathogenesis. On the one hand, the loss of normal function of wild type HTT could contribute to the selective vulnerability of these cells and to their degeneration. For example, mutant huntingtin (mHTT) loses the capability to retain RE1-Silencing Transcription factor (REST) factor in the cytoplasm, then allowing its translocation to the nucleus, where it inhibits BDNF gene expression (Zuccato *et al.*, 2003). On the other hand, the single mutation in the HTT gene would mean the acquisition of new toxic functions that also contribute to cell death. For instance, mHTT binds to CREB binding protein (CBP), retaining it in the cytosol and thus counteracting its acetylase activity on specific histones (Jiang *et al.*, 2006). (*Figure 1*. Di Prospero & Tagle, 2000).

A high amount of data indicates that the fragmentation of the mHTT protein is a key early step in the pathogenic mechanism of HD. HTT is translated to produce full-length huntingtin and an amino-terminal HTT exon-1 fragment, as a result of aberrant splicing. Additional protein fragments can be generated after proteolytic cleavage of full-length mHTT. These fragments translocate to the nucleus, where they are retained by self-association, oligomerization and aggregation, leading to the formation of nuclear inclusions that can interfere in the transcriptional machinery. Fragments can oligomerize and aggregate in the cytoplasm as well. The dysregulation of the cellular proteostasis network that occurs during the disease exacerbates this aggregation, and this finally results in an amount of cellular impairments (reviewed in Bates *et al.*, 2015). mHTT aggregates are abundant in the cerebral cortex, mainly in layers V and VI, even before the onset of neurodegeneration. Surprisingly, they are not so abundant in the striatum, the

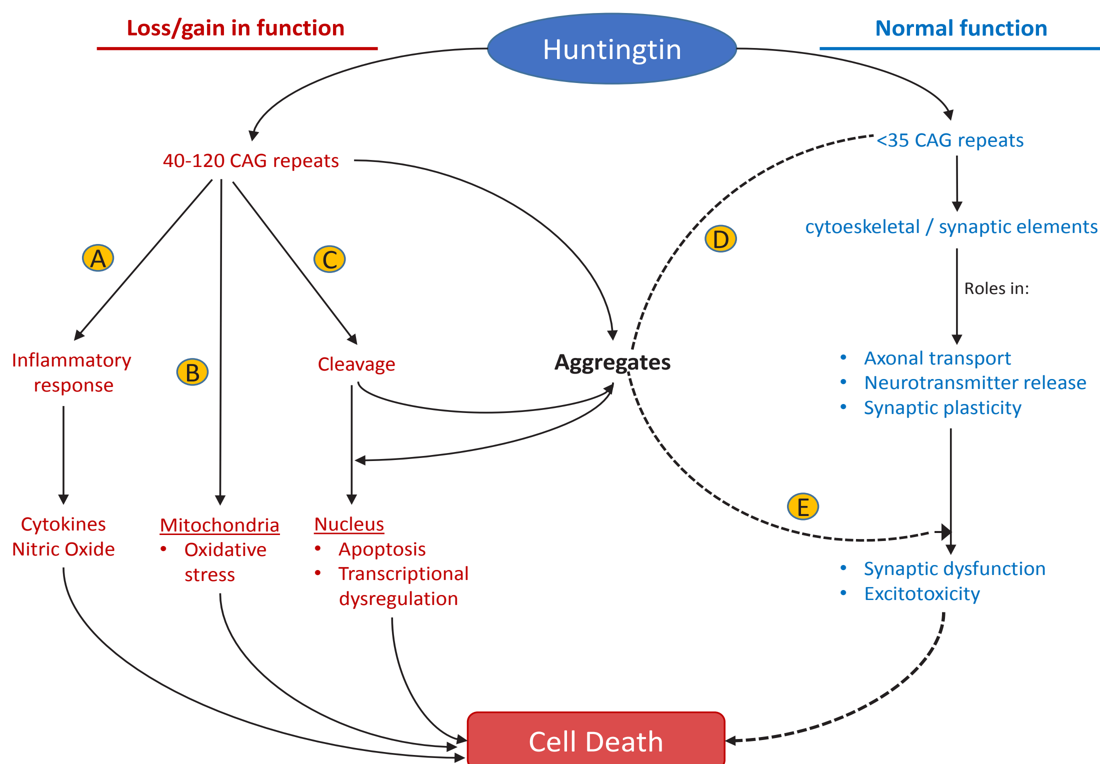


**Figure 1.** mHTT loss and gain of function of hypotheses. Poliglutamine repeat expansion can lead to a toxic gain of function through various mechanisms: A, it may activate an inflammatory response, leading to the release of cytokines and other factors such as nitric oxide, thus exerting a toxic effect; B, it may interfere with energy metabolism triggering oxidative stress; C, it can be abnormally cleaved, creating fragments that aggregate in the nucleus, thus inducing apoptotic processes and transcriptional dysregulation; D, it may form aggregates with itself and other proteins, thus also inducing apoptosis and trascriptional dysregulation; E, it may sequester other proteins, including WT HTT, thereby creating a dominant negative effect while also conferring loss of function (adapted from Di Prospero & Tagle. 2000)

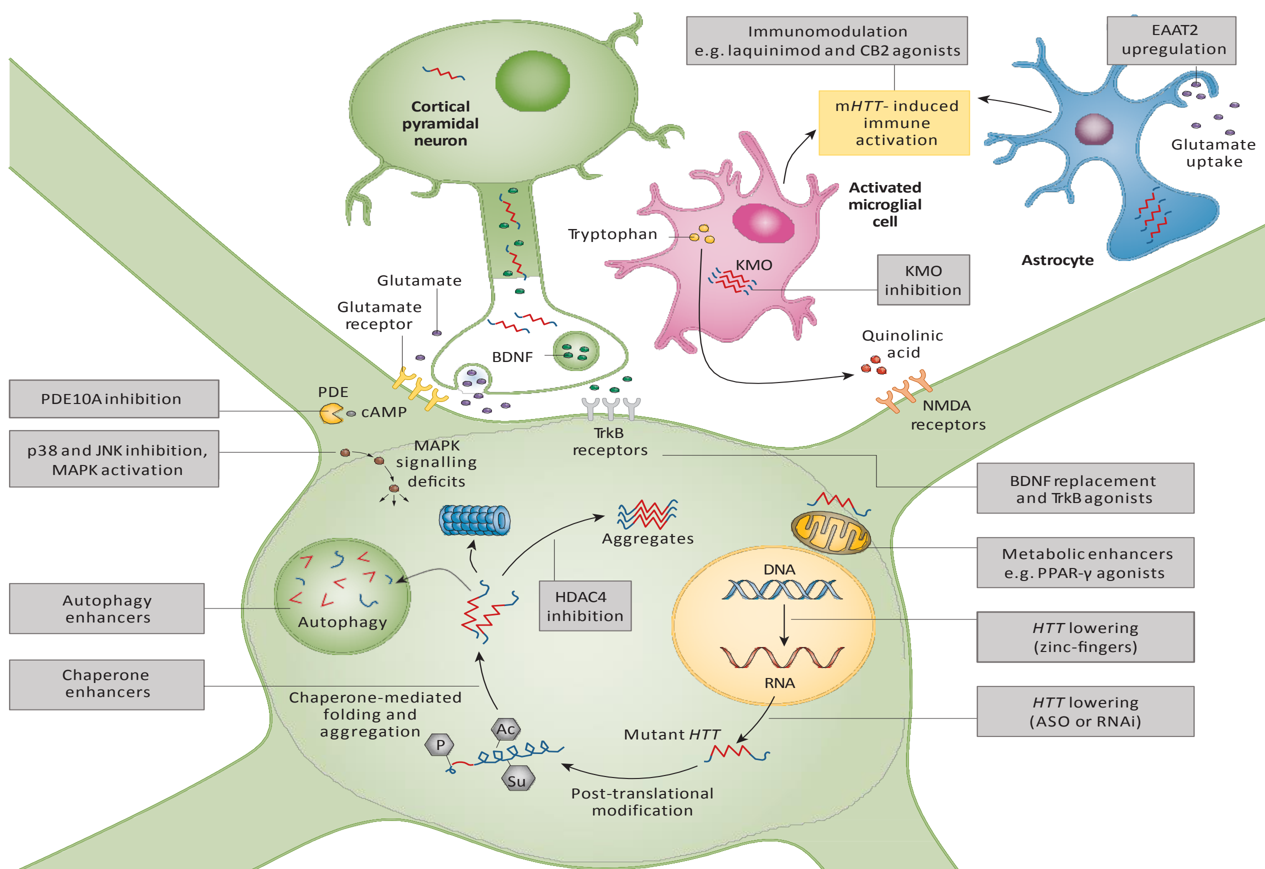


**Figure 2.** Mechanisms of mHTT toxicity and therapeutic targets under investigation. Most recent therapeutic strategies for HD include mHTT lowering, immunomodulation, BDNF, autophagy, chaperone and metabolic enhancers, histone deacetylase, PDE10A and kynurenine 3 monooxygenase (KMO) inhibition and modulation of signalling pathways such as mitogen-activated protein kinases (MAPK), p38 and c-Jun N-terminal kinase (JNK). Adapted from Bates et al., 2016.

most affected structure in the disease, although they can be found in the MSNs. The number of aggregates in the cortex is directly related to the length of the poly-Q tract and therefore inversely related to the age at onset, which confirms its involvement in the etiology of the symptoms. However, there are studies that suggest that large inclusions are not correlated with cell death and might even be neuroprotective (Arrasate *et al.*, 2004). Interestingly, recent reports hypothesize that a cell-to-cell transmission of aggregates between cells can occur in HD, suggesting a prion-like propagation (Frost & Diamond, 2010).

Among the cellular and molecular mechanisms involved in the onset and progression of HD, we can cite the following (reviewed in Zuccato *et al.*, 2010. See *Figure 2*): aberrant proteolysis and aggregation of mHTT; transcriptional dysregulation; reduced BDNF synthesis and transport; excitotoxicity, which is the first pathological mechanism that leads to corticostriatal dysfunction; altered mitochondrial function, mainly affecting the mitochondrial complex II; oxidative stress; alterations in protein degradation and autophagy; and glial activation, together with inflammatory responses. For a description of some of these mechanisms, see *Box 1*.

All these cytotoxic events result finally in the degeneration, specifically and in a greater extent, of the MSNs in the striatum. This neuronal loss is however not exclusive of this structure, as will be discussed in the next sections.

## Neuropathology of HD

The main neuropathological hallmark of the disease, as can be seen in *Figure 3,* is a progressive, bilateral atrophy of the striatum and the cerebral cortex (de la Monte *et al.*, 1988). The striatum belongs to the basal ganglia, whose function is related to the control of movement, therefore the degeneration of this area is directly involved in the motor disturbances observed in HD patients. (for an overview of the basal ganglia organization and pathways, see Box 2).

The majority of neurons in the striatum (approximately 95%) are the medium spiny

**BOX 1. Pathogenic mechanisms in HD**

Among the pathogenic mechanisms that contribute to neurodegeneration in HD, three will be described in this Doctoral Thesis: excitotoxicity, neuroinflammation and oxidative stress.

***Excitotoxicity***

Excitotoxicity is a pathological process initiated by an excessive exposure to glutamate, normally caused by an overactivation of N-methyl-D-aspartate (NMDA) and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. This leads to an uncontrolled influx of $Ca^{2+}$ that activates phospholipases, endonucleases and proteases such as calpain, which are involved in the damage of cell components such as cytoskeleton, DNA and membrane, and definitely provoke cell death (Manev *et al.*, 1989).

Early studies in presymptomatic HD brains showed increased expression of NMDA receptors in the MSNs, which were found to die earlier than interneurons (Graveland *et al.*, 1985). Studies in rodents showed that striatal injection of NMDA receptor agonists results in the selective death of MSNs (Beal *et al.*, 1991; Schwarcz *et al.*, 2010). In HD, mHTT alters neurotransmitter release and the activity of extrasynaptic glutamatergic receptors, especially the NR2B subunit of NMDA (Zeron *et al.*, 2002). mHTT also alters the crosstalk between NMDA and D1/D2 receptors, thus resulting in the selective death of MSNs containing such dopaminergic receptors (Andre *et al.*, 2010). Moreover, mHTT inhibits the expression of mitochondrial transcriptional activators, which alters mitochondrial biogenesis and respiration and reduces its capacity to buffer intracellular $Ca^{2+}$ (Cui *et al.*, 2006). These and other processes contribute to the specific vulnerability of MSNs to excitotoxic damage (Estrada Sanchez *et al.*, 2008).

***Neuroinflammation***

Astrocytes are the most abundant cells in the brain. They contribute to the cellular homeostasis, acting as a trophic support for the neurons, helping in the maintenance of the blood brain barrier and modulating synaptic transmission, but are also involved in the release of certain cytokines and chemokines (Dong & Benveniste, 2001). Microglial cells, on the other hand, are the resident immune cells in the brain (Ransohoff & Perry, 2009). The activation of glial cells as a consequence of an inflammatory event leads to the release of cytotoxic mediators, such as cytokines (e.g., Tumor necrosis factor(TNF)-α, Interleukin(IL)-6, IL-1β), nitric oxide (NO) and reactive oxygen species (ROS)). This process is initially associated with a protective role, eliminating death cells and then allowing damage repair, but a sustained activation of microglia and astrocytes leads finally to an impairment in neuronal homeostasis that drives to cell death.

There is evidence that the expression of mHTT alters the normal function of glial cells, resulting in a pro-inflammatory, overactivated state (Crotti & Glass, 2015). Microglial and astroglial activation has been reported in postmortem brains, and inflammatory mediators are increased in the brain and blood of HD patients (e.g. IL-6, IL-10, TNF-α), and also in the cerebrospinal fluid (CSF) (e.g. complement factors such as C1QC, C2 and C3). Although this immune activation starts years before the onset of symptoms, it is still not clear wether the inflammatory mechanisms are cause or consequence of the neurodegenerative process (Rocha *et al.*, 2016).

***Oxidative stress***

Both acute and chronic brain damage trigger the accumulation of toxic elements, such as ROS and reactive nitrogen species (RNS), which can harm membrane lipids, proteins and the DNA, thus leading to neural death. This process is called oxidative stress, and starts usually as a consequence of an imbalance between the activity of endogenous antioxidant elements (e.g., catalase, heme-oxygenase, superoxide dismutase, glutation) and the oxidative mediators.

Studies in postmortem HD brains show increased levels of components of oxidative processes, such as of 8-hydroxydeoxyguanosine (8-OHDG, a marker of DNA oxidation), 3-nitrotyrosine (3-NT, a protein nitration product) and lipofucsin (marker of lipid peroxidation) among others. Moreover, an elevation of inducible nitric oxide synthase (iNOS) is observed in microglia surrounding dying neurons. This is not only a process restricted to the CNS, since markers of oxidative damage have been observed also in serum, plasma and CSF of patients (Browne & Beal, 2006). They have been used as biomarkers for the different antioxidant therapies that have already been tested in animal models, and some of them in patients (Gil-Mohapel *et al.*, 2014).

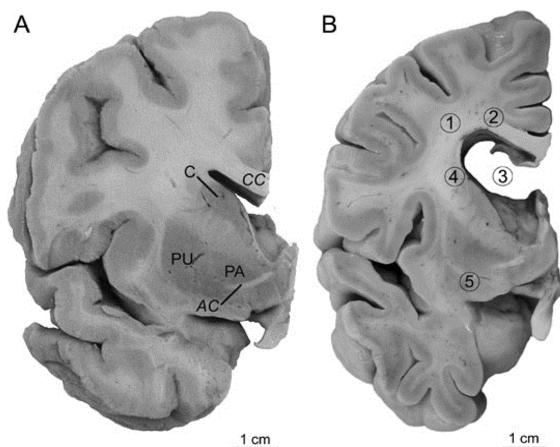


**Figure 3.** Atrophy of the striatum in HD. (A) Frontal section through the right basal forebrain of a representative control individual. (B) Frontal section through the right basal forebrain of a HD patient. Note the loss of deep white matter (1), the narrowed corpus callosum (CC) (2) and widened third ventricle (3). The atrophy of the caudate nucleus (C) (4) and putamen (PU) (5) are also remarkable. AC, anterior commissure; PA, pallidum; PU, putamen. Taken from Rüb et al., 2009.

neurons (MSNs), which are γ-aminobutyric acid (GABA)-ergic projection neurons that reach mainly the globus pallidus (GP) and the substantia nigra pars reticulata (SNr). These are the earliest and most severely affected striatal cell type during HD (Graveland *et al.*, 1985). Other striatal neurons, such as those interneurons being positive for somatostatin (SS), nicotinamide adenine dinucleotide phosphate (NADPH) and neuropeptide-Y (NPY) that modulate the function of the MSNs, are unaffected until late stages of the disease (Ferrante *et al.*, 1985). Large cholinergic neurons are also preserved until late phases (Cicchetti & Parent, 1996). This selective vulnerability of MSNs has been proposed to be due to: differential expression of certain glutamate receptors in these neurons compared to interneurons, which would make them more vulnerable to excitotoxicity (Ferrante *et al.*, 1987; Zeron *et al.*, 2001); a special sensitivity to energy deficit in these cells, which would mean a higher susceptibility to mitochondrial damage (Beal *et al.*, 1993; Gu *et al.*, 1996); and an extreme dependency of BNDF by the MSNs, which make them specially affected by the reduction in BDNF levels caused by mHTT (Zuccato *et al.*, 2003; Gauthier *et al.*, 2004).. Moreover, different MSN subtypes show different vulnerability: MSNs that express encephalin and $D_2$ receptors and project to the external segment of the GP (GPe, "indirect pathway") are the most vulnerable and first to degenerate, which is related to the development of chorea; MSNs hat express dynorphin, substance P and $D_1$ receptors and project to the internal segment of the GP (GPi) and SNr ("direct pathway") degenerate later, with correlates with the appearance of akinesia and rigidity and, in general, a more parkinsonian clinical status (Reiner *et al.*, 1988; Albin *et al.*, 1990).

Although the main structure that experiences neurodegeneration in HD is the striatum, other brain regions are also affected. As indicated before, cortical degeneration has been seen in HD brains, especially in advanced phases in adult-onset patients (40-50 CAG repeats) or earlier (even before the occurrence of chorea) in juvenile-onset patients (>60 CAG repeats). Such cortical degeneration means mainly a significant loss of pyramidal cells in the primary motor cortex (layers V, VI and III), which is involved in corticostriatal pathways (de la Monte *et al.*, 1988). The appearance of cortical degeneration also in early phases suggests that this process is not secondary, but parallel to striatal atrophy (Hedreen *et al.*, 1991). The extent of cortical volume loss correlates with the level of striatal atrophy and the number of polyglutamine repeats, which suggests a relation in the disease processes between these two regions (van Dellen *et al.*, 2005). In late stages of the disease, neuronal loss and atrophy have also been described in other brain structures such as globus pallidus, substantia nigra, subthalamic nucleus, thalamus, hippocampus and cerebellum (Vonsattel *et al.*, 2008; Waldvogel *et al.*, 2015). Most dramatic changes in HD happen in the brain, but degeneration of peripheral tissues has been recently reported as well. Some of these non-brain abnormalities include weight loss, skeletal muscle wasting, insulin sensitivity, testicular degeneration, gastrointestinal disorders and cardiac issues (van der Burg *et al.*, 2009).

**Current therapeutic strategies and clinical trials**

Huntington's disease is caused by a single mutation in one gene. However, there is a bunch of molecular mechanisms involved in the pathogenesis, which makes the search for an effective therapy very difficult. Despite all the efforts and the extensive research performed since the description of the disease,

**BOX 2. Basal ganglia organization and pathways**

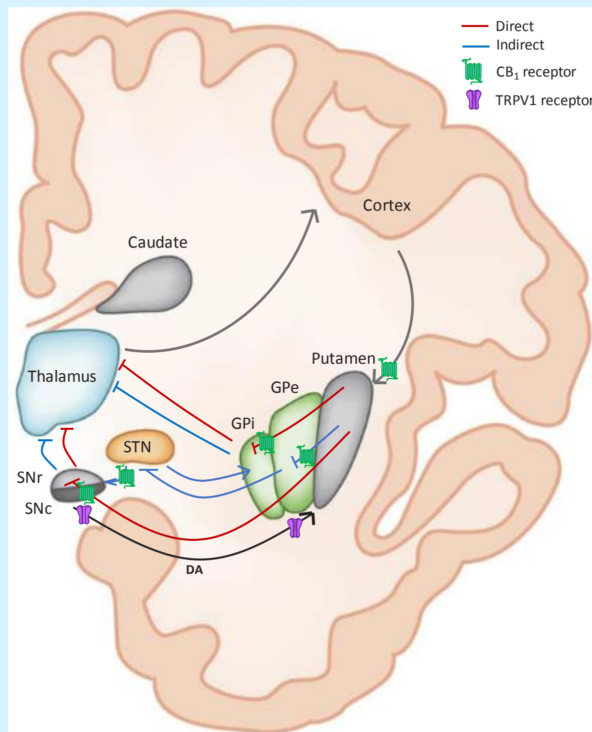


**Figure 4.** Schematic representation of the basal ganglia direct/indirect pathway classical model, and location of CB1 and TRPV1 receptors in the involved structures. Arrows indicate excitatory signalling, blunted arrows indicate inhibitory signalling. DA, dopamine. Adapted from Calabresi *et al*., 2014.

The basal ganglia are a group of nuclei involved in the control of movement and located subcortically at the base of the forebrain. The term involves the striatum (caudate nucleus and putamen), globus pallidus (internal segment -Gpi- and external segment -Gpe), the subthalamic nucleus (STN), and the substantia nigra (pars compacta -SNc- and pars reticulata -SNr).

The basal ganglia are integrated in a forebrain loop that forms a cortical/basal ganglia/thalamus/cortical circuit (*Figure 4*). The main afferent projection to the striatum comes from glutamatergic cortical neurons, but it also receives projections from other areas: glutamatergic inputs from the thalamic nuclei and dopaminergic signals from the SNc which are the regulatory components in the circuit. The canonical model of basal ganglia function states that two pathways, direct and indirect, form by different populations of MSNs that project to distinct output structures. Thus, in the direct pathway, cortical glutamatergic projections activate MSNs, that project to the SNr and GPi. MSNs are GABA-ergic, so they inhibit SNr neurons that are also GABA-ergic and project to the ventrolateral nucleus of the thalamus.

The inhibition of SNr inputs leads to a disinhibition of the thalamic glutamatergic neurons, that project back to cortical areas, then resulting in the activation of the movement. In the indirect pathway, excitatory output from the cortex activates striato-pallidal MSNs, which project indirectly to the SNr through the GPe and STN. The inhibition of the GABA-ergic neurons in the GPe by the activation of GABA-ergic MSNs leads to a disinhibition of the STN, which projects glutamatergic signals to the SNr, activating GABA-ergic neurons that project to the thalamus, thus resulting in an inhibition of the thalamic glutamatergic neurons that reach cortical areas, and this results in the inhibition of movement.

It is interesting to mention that the activation of movement exerted by the direct pathway and the inhibition mediated by the indirect pathway are regulated by nigrostriatal dopaminergic neurons, which enhance movement activation by acting on D1 receptors located at MSNs of the direct pathway, or cause the opposite effect by acting on inhibitory D2 receptors located at MSNs of the indirect pathway.

According to this model, an adequate balance between the direct and indirect pathway would result in a normal control of the movement. In HD, as also happens in other basal ganglia disorders (e.g. Parkinson's disease -PD-), this balance is broken and leads to the appearance of motor symptoms. As explained above, MSNs that express D2 receptors and project to the GPe are the earliest affected in HD. This would mean an alteration of the indirect pathway that would lead to the exacerbation of the movement. Conversely, as the disease progresses, striato-nigral MSNs that express D1 receptors and project to the GPi degenerate, which would affect the direct pathway and ultimately inhibit the thalamic projections to the cortex, resulting in a decreased activation of the movement (Han et al., 2010). These two phases correspond respectively to the early and intermediate grades of the disease, characterized by choreic movements, and to late grades which are more akinetic and resemble parkinsonian symptoms.

Although this canonical view is widely accepted, recent findings have revealed that the circuitry might be much more complex, with the participation of other neurotransmitters, such as serotonin, acetylcholine, neuropeptides, etc. Thus, these two pathways would be structurally and functionally intertwined, and MSNs might either activate or inhibit movement depending on the form of synaptic plasticity expressed at a certain moment (Calabresi et al., 2014).

it still has no cure and very few therapies have passed the clinical evaluation. The majority of drugs approved for HD patients are focused on ameliorating the primary symptomatology (such as psychiatric agents, cognitive enhancers and motor modulators). However, no therapies designed to delay or stop the progression of the neurodegenerative process are available (Pidgeon & Rickards, 2013). The ultimate desired goal is to find a therapy that can be proved to show disease modification (preventing, slowing or even reverting the curse of the pathology), together with the characterization of biomarkers that give early signs of therapeutic success of a drug in pre-symptomatic patients.

Currently, there are several therapeutic agents being pre-clinically and clinically evaluated, mostly acting on the previously mentioned molecular processes affected by the pathology. These strategies include: drugs against excitotoxicity, strategies to increase BDNF, stimulators of huntingtin clearance and autophagy enhancers, drugs against mitochondrial dysfunction and antioxidants, drugs targeting gene transcription (e.g. histone deacetylase (HDAC) inhibitors), modulators of synaptic function (e.g. Phosphodiesterase(PDE)10A inhibitors), immunomodulators and anti-inflammatory agents, mHTT lowering strategies (e.g. Antisense oligonucleotides (ASO), interfering RNA (RNAi), zinc fingers). Stem cell therapies are also under investigation as a neurorepair strategy.

For an overview of some of the last clinical trials performed and currently ongoing, see *Table 1*.

## CANNABINOIDS AND THE ENDOCANNABINOID SYSTEM

The term "cannabinoid" has been used traditionally to name the psychoactive components of the *Cannabis sativa*. Nowadays, cannabinoids can be classified into 3 categories: phytocannabinoids, or natural components of the plant; endocannabinoids, endogenous molecules present in animal tissues and organs,

| Drug | Mechanism of action | Study ID / Identifier | Sponsor | Phase and Status | References |
|---|---|---|---|---|---|
| **IONIS-HTTRx** | Antisense oligonucleotide | ISIS-443139-CS1 / NCT02519036 | Ionis Pharmaceuticals | Phase I/IIa. Recruiting | (Kordasiewicz *et al.*, 2012) |
| **PF-02545920 (MP-10)** | PDE10A inhibitor | NCT02197130 | Pfizer | Phase II. Completed | (Wilson *et al.*, 2015) |
| **Selisistat (SEN0014196)** | Sirt1 inhibitor (inhibition of HDACs) | NCT01521585 | Siena Biotech | Phase II. Completed | (Sussmuth *et al.*, 2015) |
| **Deutetrabenazine (SD-809)** | Vesicular Monoamine Transporter 2 (VMAT2) inhibitor | First-HD / NCT01795859 | Teva Pharmaceutical Industries | Phase III. Completed | (Group *et al.*, 2016) |
| **Pridopidine** | Dopaminergic stabilizer (functional antagonism of $D_2$) | PRIDE-HD / NCT02006472 | Teva Pharmaceutical Industries | Phase II. Completed | (Shannon, 2016) |
| **Laquinimod** | Immunomodulator | LEGATO-HD / NCT02215616 | Teva Pharmaceutical Industries | Phase II. Recruiting | (Dobson *et al.*, 2016) |
| **VX15/2503** | Monoclonal antibody against semaphorin 4D | SIGNAL-HD / NCT02481674 | Vaccinex | Phase II. Ongoing | (Southwell *et al.*, 2015) |
| **Cellavita** | Stem cell therapy | SAVE-DH / NCT02728115 | Azidus Brasil | Phase I. Approved | (Kerkis *et al.*, 2015) |
| **Triheptanoin** | Brain energy metabolism enhancer | TRIHEP3 / NCT02453061 | INSERM | Phase II. Recruiting | (Adanyeguh *et al.*, 2015) |

**Table 1.** Recent clinical trials on HD.

and able to bind cannabinoid receptors; and synthetic cannabinoids, compounds generated in the laboratory that have facilitated the study of the so-called endocannabinoid system (ECS), whose characteristics and mechanisms will be briefly reviewed along this second part of the introduction.

Preparations of the plant *Cannabis sativa* (e.g. marijuana and hashish) have been used during centuries for both recreational and medicinal purposes. The first written description found about medicinal cannabis dates around 2350 B.C and belongs to the Old Kingdom in Memphis, Egypt. There are evidences that prove its use as a drug also in India, the ancient Persia, medieval Arab countries and China, where it was used for numerous indications, such as pain, seizures, asthma and loss of appetite. However, it was not until the 19$^{th}$ century (1839), in Europe, when O'Shaughnessy described its use for the treatment of tetanus in India, and since then it started being used as muscle relaxant and antispasmodic. After all these anecdotal evidences, the medicinal use of cannabis was reduced at the beginning of the 20$^{th}$ century, which hampered significantly the study of its applications (Ligresti *et al.*, 2016).

The key events in the study of cannabinoids occurred in 1963 and 1964, with the isolation and characterization for the first time of the two major constituents of the plant, cannabidiol (CBD) (Mechoulam & Shvo, 1963) and $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) (Gaoni & Mechoulam, 1964) respectively. The evidence of a psychoactive effect of $\Delta^9$-THC boosted the search for endogenous mediators of its action. Due to its lypophilic nature, it was believed to interact nonspecifically with the lipids on the plasma membrane (Lawrence & Gill, 1975). However, in 1988 the first receptor for cannabinoids was discovered, which was formerly called cannabinoid receptor type-1 ($CB_1$) (Devane *et al.*, 1988). The second main receptor of this system was described in 1993, and was called cannabinoid receptor type-2 ($CB_2$) (Munro *et al.*, 1993). The finding of these receptors encouraged the search for their endogenous ligands. Thus, the first endocannabinoids described were the arachidonoylethanolamine (AEA), or anandamide (Devane *et al.*, 1992) and the 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995).

The cannabinoid receptors, their endogenous ligands and the enzymes involved in their synthesis and degradation constitute what we call the ECS, a signaling system involved in many physiological and pathological processes. For this reason, it has become in the last decades an important potential target for the design and development of new therapies.

**Cannabinoid receptors**

As introduced above, two cannabinoid receptors (CBR) have been already cloned and described: $CB_1$ and $CB_2$ receptors (*Figure 5*). They belong to the G protein-coupled receptors (GPCRs), which have 7 transmembrane domains and are coupled to G proteins. They share a global homology of 44%, which increases up to 68% between the transmembrane domains (Montero *et al.*, 2005)2005, and are widely expressed in all vertebrates and also in invertebrates (Elphick, 2012).

$CB_1$ receptor

The $CB_1$ receptor was firstly characterized by radiometric methods firstly in neuroblastoma cell line membranes and furtherly in rat brain membranes (Devane *et al.*, 1988), and then cloned in rat (Matsuda *et al.*, 1990)1990 and later in humans (Gerard *et al.*, 1991)1991. Importantly, its crystal structure has been determined very recently (Hua *et al.*, 2016). The expression of $CB_1$ is particularly abundant in the central nervous system (CNS). Indeed, it is considered the most abundant metabotropic receptor in the brain. The brain areas where its presence is higher are the basal ganglia (SNc, GP and striatum), hippocampus, cerebral cortex and cerebellum, regions that underlie some of the observed effects of cannabinoids, including alterations in memory and cognition, learning and motor behavior. Its expression is more moderate in the amygdala, nucleus accumbens, hypothalamus and brainstem (Herkenham *et al.*, 1991). Besides this wide distribution in different brain areas it can be expressed in different neuronal types, including GABAergic, glutamatergic and serotoninergic. They are located predominantly on presynaptic terminals, but they have been also found postsynaptically, and in glial cells and neuronal progenitors as well (Howlett *et al.*,

2002; Aguado *et al.*, 2005).

From a cellular perspective, $CB_1$ receptors are mainly expressed at the plasma membrane, but recent studies applying advanced imaging techniques have provided evidence of differential activation-dependent distribution of these receptors (Dudok *et al.*, 2015), as well as mitochondrial localization (Benard *et al.*, 2012). Besides its broad distribution in the CNS, $CB_1$ is also expressed in a high number of peripheral tissues, such as heart, spleen, liver, skeletal muscle and gastrointestinal and genitourinary systems (Galiegue *et al.*, 1995; Mackie, 2005).

$CB_2$ receptor

The second cannabinoid receptor was firstly described in spleen (Munro *et al.*, 1993). It is widely expressed particularly in immune tissues such as thymus, tonsils, mast cells and blood cells, including all kind of immune cells (macrophages, polymorphonuclear neutrophils, monocytes, T cells and B cells) (Galiegue *et al.*, 1995; Schatz *et al.*, 1997).

Apart from the immune system, this receptor has also been described in other peripheral tissues, including the gastrointestinal system (Wright *et al.*, 2008), bone cells (Ofek *et al.*, 2006), pulmonary endothelial cells (Zoratti *et al.*, 2003), fibroblasts of cirrhotic liver (Julien *et al.*, 2005) and cardiomyocytes (Shmist *et al.*, 2006).

$CB_2$ receptor has been typically described as the "peripheral" cannabinoid receptor, since at first it was not found in any CNS structure (Lynn & Herkenham, 1994). However, in the last years it has been proved to be expressed in the CNS, mainly associated with astroglial and microglial cells that become activated in pathologies that curse with inflammation (Benito *et al.*, 2003; Nunez *et al.*, 2004; Cabral & Griffin-Thomas, 2009). The presence of $CB_2$ in neuronal cells is a controversial topic that has been discussed in the last few years, maybe due to the lack of specificity of the available antibodies to date (Atwood & Mackie, 2010). Nonetheless, some recent studies have shown their expression in neurons of the basal ganglia (Lanciego *et al.*, 2011), cerebellum (Rodriguez-Cueto *et al.*, 2014), hippocampus (Brusco *et al.*, 2008)2008 and brainstem (Van Sickle *et al.*, 2005), among others.

**Non-cannabinoid receptors activated by cannabinoids**

Some of the effects of cannabinoid compounds cannot be totally explained by the activation of $CB_1$ and $CB_2$ receptors. Therefore, there must be other molecular targets for both endocannabinoids and plant-derived phytocannabinoids. Recent studies have proved that cannabinoid compounds can activate other receptors, such as other GPCRs, ion channels and nuclear hormone receptors (NHRs) (Alexander, 2015).

There are orphan GPCRs that are widely expressed in the CNS and are activated by the action of fatty acid-derived lipids, including certain endocannabinoids among them, despite their limited structural similarity to $CB_1$ and $CB_2$. One of them is GPR18, whose endogenous ligand has been suggested to be N-arachidonoylglycine (NAGly), an active derivative of AEA that does not activate neither $CB_1$ nor $CB_2$. Other ligands of this receptor, with less affinity, could be $\Delta^9$-THC and AEA (McHugh *et al.*, 2012). The cannabinoid-like receptor GPR119 is proposed to be activated by the AEA and 2-AG analogs oleylethanolamide (OEA) and 2-oleoylglycerol (2-OG), respectively (Overton *et al.*, 2006; Hansen *et al.*, 2011). Lastly, GPR55 is the receptor proposed for the endogenous lipid lysophosphatidilinositol, but can also be activated by $\Delta^9$-THC, HU-210, noladin eter, AM-251 and SR141716A (Henstridge *et al.*, 2010).

Direct effects of cannabinoids on ion channels have also been reported. Mainly, on the transient receptor potential channels (TRPs), membrane channels that mediate the transmission of somatosensory stimuli (e.g. pressure, pain and temperature). TRPV1 is activated by AEA, CBD and $\Delta^9$-THC; these last two phytocannabinoids are also agonists of TRPV2, TRPA1 and TRPM8. By the activation of these channels, cannabinoids are suggested to act as pronociceptive agents, in contrast to the antinociceptive effects mediated by $CB_1$ and $CB_2$ (reviewed in De Petrocellis *et al.*, 2011). Furthermore, CBD and $\Delta^9$-THC have also shown to inhibit voltage-gated calcium channels, such as $Ca_v3$ (Ross *et al.*, 2008).

Lastly, cannabinoids can act also on peroxisome

proliferators-activated receptors (PPARs). These are nuclear receptors that, once activated, heterodimerize with the retinoid X receptor (RXR), and act as transcription factors involved in inflammation, cell differentiation and regulation of lipid metabolism. To mention a few, PPARα is activated by AEA, OEA, palmithoylethanolamide (PEA), noladin eter and the synthetic compound WIN55-212,2; PPARγ is activated by WIN55-212,2 and $\Delta^9$-THC. Although most of the PPAR-mediated effects of these compounds have not been elucidated yet, it is suggested that some analgesic, anti-inflammatory and neuroprotective effects of cannabinoids may be partly mediated by the activation of these receptors (Sun & Bennett, 2007; O'Sullivan, 2016).

**Endocannabinoids**

The endogenous compounds that bind cannabinoid receptors are called endocannabinoids (ECBs). These are lipid molecules derived from long chain polyunsaturated fatty acids.

The first endocannabinoid to be described, the so-called anandamide (AEA) (Devane *et al.*, 1992), was purified from porcine brain. It is found in in the CNS but with lower levels of expression than other endocannabinoids (Bisogno *et al.*, 1999), mainly in hippocampus and striatum (where $CB_1$ expression is elevated), but also in brainstem and spinal cord (where $CB_1$ expression is not so high). In the periphery it is found in spleen, heart, testis and uterus (Felder *et al.*, 1996). Pharmacologically, AEA binds to both cannabinoid receptors $CB_1$ and $CB_2$ as a partial agonist, but it can activate GPR55 (Ryberg *et al.*, 2007), TRPV1 (Toth *et al.*, 2009) and PPAR receptors (Sun & Bennett, 2007), as well as inhibit 5-HT3A receptors (Xiong *et al.*, 2008).

The second main endocannabinoid, the so-called 2-arachidonoylglycerol (2-AG), was isolated in 1995 from dog intestine (Mechoulam *et al.*, 1995). It is the most abundant endocannabinoid in the brain, with the highest expression in brain stem, striatum and hippocampus, but also found in the brain cortex and cerebellum (Bisogno *et al.*, 1999). Peripherally, it is expressed in spleen, liver, lung, kidney and pancreas (Mechoulam *et al.*, 1995) (Kondo *et al.*, 1998). It behaves as a full agonist of both $CB_1$ and $CB_2$ receptors, and can also bind to GPR55 (Ryberg *et al.*, 2007) and TRPV1 (Zygmunt *et al.*, 2013).

Other compounds have been identified as potential endogenous ligands of cannabinoid receptors, such as O-arachidonoylethanolamine (or virhodamine), N-arachidonoyldopamine (NADA) and noladin ether (Pacher *et al.*, 2006). Other molecules do not bind cannabinoid receptors but have cannabimimetic activity, and exert an "entourage" effect on main endocannabinoids, enhancing their action (Ho *et al.*, 2008) or avoiding their inactivation, presumably due to the fact that they share the same hydrolytic enzymes with AEA and 2-AG. These are, among others, PEA, OEA, stearoylethanolamide (SEA), 2-linoleylglycerol (2-LG) and 2-OG (Conti *et al.*, 2002; Maccarrone *et al.*, 2002). See in *Figure 5* the structure of various endocannabinoids.

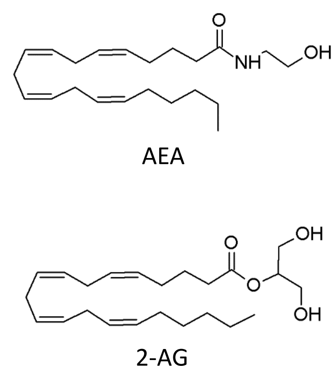


**Figure 5.** Chemical structure of the endocannabinoids AEA and 2-AG.

**Synthesis and release of endocannabinoids**

Unlike classic water-soluble neurotransmitters, which are synthesized and stored in vesicles before being released, endocannabinoids are synthesized "on demand". This means that certain physiological stimuli (usually an increase in the intracellular concentration of $Ca^{2+}$) trigger their production from lipid precursors localized on the inner layer of the plasmatic membrane, and are released immediately after that (Piomelli, 2003). Since the most studied synthesis and degradation pathways of endocannabinoids are the ones for AEA and 2-AG, they will be briefly explained in this and the following section.

The main synthesis route of AEA starts with the activation of a $Ca^{2+}$-inducible

N-acyltransferase that transfers arachidonic acid (AA) to phosphatidylethanolamine to form N-arachidonoyl phosphatidylethanolamine (NAPE). This is then hydrolyzed by a specific phospholipase, $Ca^{2+}$-inducible as well, NAPE-PLD, thus producing phosphatidic acid (PA) and AEA (Di Marzo *et al.*, 1994). Additional mechanisms of AEA production have been recently proposed (Liu *et al.*, 2008). Furthermore, other studies suggest that the synthesis of AEA can also be $Ca^{2+}$-independent (Leung *et al.*, 2006), as well as induced by the activation of certain dopamine, glutamate and acetlcholine receptors (Piomelli, 2003).

The biosynthesis of 2-AG is also $Ca^{2+}$-inducible and is produced from diacylglycerols (DAGs). These molecules can be generated either from the hydrolysis of phosphoinositides (PI) by a phospholipase C (PLC), or PA by the PA phosphohydrolase. DAGs are then hydrolyzed by a DAG-lipase (DAGL), thus producing 2-AG. An alternative pathway involves the formation of the intermediate 2-arachidonoyl-lysophosphatidylinositol (liso-PI) from PI by the phospholipase A1 (PLA1), and the action of the liso-PLC to generate 2-AG (Piomelli, 2003).

**Inactivation of endocannabinoids**

Once released to the synaptic cleft, ECBs exert their action through the binding to cannabinoid or non-cannabinoid receptors (as explained before) and are then rapidly transported back inside the cell to be degraded once ECB signal needs to be terminated. Since they are lipid molecules, they can pass through the membrane by passive diffusion, a process that might be facilitated by a selective transport system. Indeed, the existence of an AEA-specific transporter has been postulated: the fatty acid amide hydrolase (FAAH)-like anandamide transporter (FLAT) would be an inactive cytosolic form of FAAH enzyme that would bind AEA and facilitate its reuptake, although it is not expressed in all tissues, so it cannot be proposed as a global intracellular endocannabinoid carrier (Leung *et al.*, 2013).

Back inside the cell, endocannabinoids are inactivated and degraded by specific enzymes. Anandamide is hydrolyzed by the FAAH enzyme, producing AA and ethanolamine (Cravatt *et al.*, 1996). FAAH is not specific, but it uses other *N*-acylethanolamines as substrates, even 2-AG. It is located mainly postsynaptically and its distribution correlates with the presence of AEA and $CB_1$ receptor (Egertova *et al.*, 2003). 2-AG is mainly degraded (although it also a substrate of FAAH) by the enzyme monoacylglycerol lipase (MAGL), thus producing AA and glycerol (Dinh *et al.*, 2002). It is expressed presynaptically, and in brain regions in which $CB_1$ receptor is highly expressed as well. Other enzymes that can hydrolyze 2-AG, namely ABDH6 and ABDH12, have been identified (Savinainen *et al.*, 2012).

Last, AEA and 2-AG can be inactivated not only by hydrolysis, but also by oxidation by other enzymes involved in lipid metabolism, such as lipooxigenases (LOXs) and ciclooxigenases (COXs). But the reactions catalyzed by these enzymes generate a high amount of new biologically active molecules. The products of COX-2 activity have generated much more interest in the last years. This isoform, inducible by inflammatory stimuli in the brain, can oxygenate both AEA and 2-AG. The oxygenation of AEA produces prostamides such as prostaglandin $E_2$ ($PGE_2$)-ethanolamide, a metabolite that exerts its action by the binding to $PGE_2$ receptors. On the other hand, the oxygenation of 2-AG generates $PGE_2$-glyceryl esters, metabolites that do not act via cannabinoid receptors and that have been suggested recently to play a role in inflammation and excitotoxicity in the brain (Sang *et al.*, 2007; Woodward *et al.*, 2008; Valdeolivas *et al.*, 2013) .

**Pharmacology of the cannabinoid system**

Since the first description of $\Delta^9$-THC, an important part of the cannabinoid research started to focus on the generation of analogous molecules with better pharmacological profile than this natural compound. In addition, this prompted the search for new synthetic molecules that could manipulate the ECS, in order to better understand its physiological activity. The compounds that are currently used for the modulation of the ECS can be classified as cannabinoid receptors agonists, cannabinoid receptors antagonists and endocannabinoid tone modulators, which will be reviewed in this section (see *Figure 6*: chemical structure of synthetic cannabinoids).

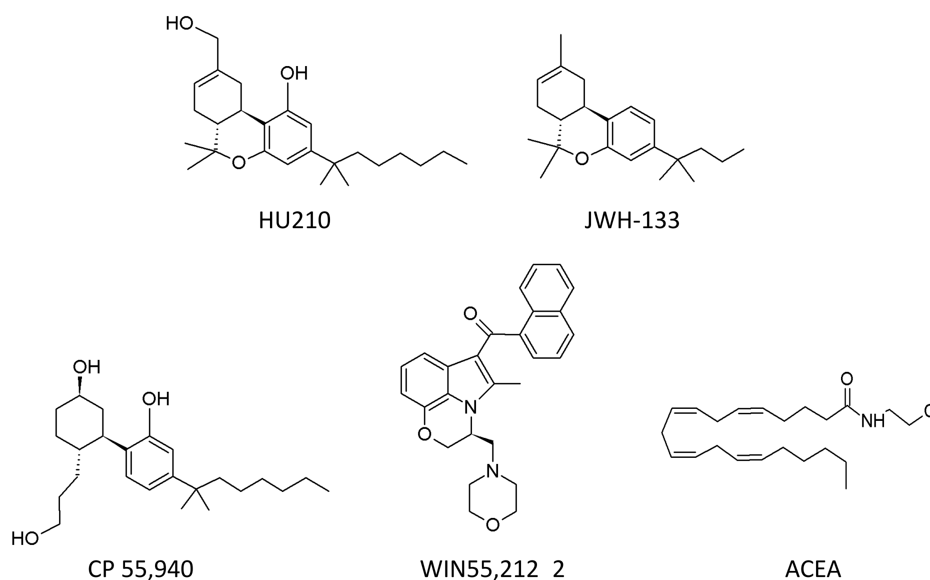


**Figure 6.** Chemical structure of synthetic cannabinoid agonists.

Cannabinoid receptor agonists

Cannabinoid receptors agonists are compounds that bind and activate either $CB_1$, or $CB_2$, or both receptors, with more or less affinity and potency. They can be classified by their chemical structure.

*Classic cannabinoids*

The group of classic cannabinoids involves dibenzopyrene derivatives, which include both natural constituents of cannabis or phytocannabinoids (e.g. $\Delta^9$-THC, $\Delta^8$-THC, cannabinol (CBN)) and synthetic cannabinoids. These include mainly synthetic analogs $\Delta^9$-THC, being HU-210 the most potent and widely characterized, which presents both affinity for $CB_1$ and $CB_2$ (Ottani & Giuliani, 2001). Other $\Delta^9$-THC derivatives include nabilone (Cesamet) and the selective $CB_2$ agonist JWH-133 (Huffman, 2005), among others. For more information on phytocannabinoids, see Box 3.

*Non-classic cannabinoids*

Non-classic cannabinoids are bicyclic and tricyclic $\Delta^9$-THC analogs that lack the pyran ring. The main representative of this group is CP-55,940, which, like $\Delta^9$-THC, is a non-selective agonist of $CB_1$ and $CB_2$, but has around 5 times more potency. Other examples would be CP-22,244, CP-50,556 (levonantradol) and desacetyllevonantradol (DALN) (Howlett *et al.*, 1988).

*Aminoalkylindoles*

This group includes molecules with a chemical structure derived from pravadoline, quite different from the groups previously mentioned. The most widely used is WIN55,212-2, whose affinity is higher for $CB_2$ than for $CB_1$ and has a faster activity than $\Delta^9$-THC (D'Ambra *et al.*, 1992). Other aminoalkylindoles to mention, derived from WIN55,212-2 and $CB_2$-selective are JWH-015 and L-768,242 (Huffman, 2005).

*Eicosanoids*

These cannabimimetic compounds include derivatives of AA, thus their structures are similar to the endocannabinoids AEA and 2-AG. One of these molecules is methaanandamide (AM-356), an AEA analog that is more $CB_1$-selective and, in particular, more resistant to FAAH-mediated metabolism (Abadji *et al.*, 1994). Other compounds in this group are arachidonoyl-2-chloroethylamide (ACEA) (Hillard *et al.*, 1999) and O-1812 (Di Marzo *et al.*, 2001).

Cannabinoid receptor antagonists

The generation of compounds that selectively block $CB_1$ or $CB_2$ receptors, together with the development of knock-out models for these receptors, has been a crucial tool for the study of the endocannabinoid signaling. The first discovered CB1-selective antagonist is SR141716A or "rimonabant" (Rinaldi-Carmona *et al.*, 1994). Other molecules derived from SR141716A are AM-251 (Gatley *et al.*, 1996) and

**BOX 3. Phytocannabinoids**

More than 100 cannabinoid compounds have been identified in *Cannabis sativa*, in different amounts. These plant-derived natural compounds are called phytocannabinoids. The most abundant in cannabis are $\Delta^9$-THC and CBD, and of the tens of compounds left in variable amounts, only a few have generated pharmaceutical interest so far: cannabichromene (CBC), CBG, cannabidivarin (CBDV), $\Delta^9$-tetrahydrocannabivarin ($\Delta^9$-THCV), $\Delta^9$-tetrahydrocannabinolic acid ($\Delta^9$-THCA) and cannabidiolic acid (CBDA). For the purpose of this Doctoral Thesis, we will focus in this Box on $\Delta^9$-THC, CBD and CBG (See *Figure 7*).

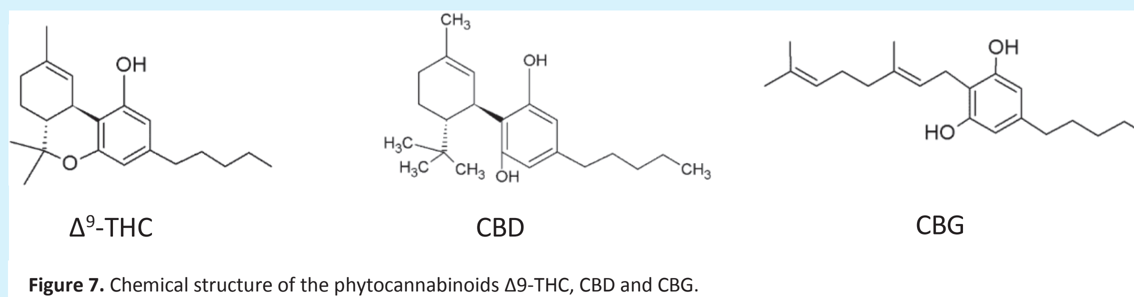


**Figure 7.** Chemical structure of the phytocannabinoids Δ9-THC, CBD and CBG.

The main constituent of the plant, and accountable for its psychoactive effects, is $\Delta^9$-THC. This phytocannabinoid is a high-affinity, partial agonist of both $CB_1$ and $CB_2$, so most of it central effects are due to this cannabinoid receptor activation, but it has shown activity on other receptors as well. For example, it is suggested to activate GPR55 and GPR18. It acts as an allosteric modulator of $5\text{-}HT_{3A}$ serotonin receptors, and glycine receptors. Regarding vanilloid receptors, it can activate TRPV2, TRPA1 and inhibit TRPM8. $\Delta^9$-THC can also act as an inhibitor of ion channels, such as Cav3, $K_v1.2$ and voltage-gated sodium channels. This variable pharmacological activity allows $\Delta^9$-THC to alter many different central and peripheral processes, but due to the activation of cannabinoid receptors, it has shown positive effects on analgesia, food intake, nociception, emesis, inflammation, anxiety, sleep and modulation of neuronal excitability among others, besides the neuroprotective effects on neurodegeneration that will be addressed on further sections. Nevertheless, the activation of $CB_1$ receptor is also the main reason for the appearance of psychotropic effects, the development of tolerance and dependence, as well as the risk of psychiatric and cardiovascular complications, which have limited its application in therapy (Costa, 2007).

CBD is the most abundant non-psychoactive phytocannabinoid, which has aroused the interest for its therapeutic potential in the last years. It is usually called an "atypical cannabinoid", since it does not elicit the classic CB1-mediated cannabinoid "tetrad" (catalepsy, analgesia, hypolocomotion, hypothermia), unlike $\Delta^9$-THC. It has been reported classically to have very low affinity for cannabinoid receptors, although recent studies suggest its activity as an allosteric inhibitor of $CB_1$ and week inverse agonist of $CB_2$. In addition, it can increase the endocannabinoid levels by inhibiting FAAH enzyme. CBD also acts as a partial agonist of GPR18 and antagonist of GPR55. It can activate TRPV1, TRPV2 and TRPA1, as well as antagonize TRPM8. Like $\Delta^9$-THC, it shows positive allosteric modulation of glycine receptors. CBD can activate PPARγ and $5\text{-}HT_{1A}$ (in this case presumably acting as an allosteric modulator), as well as inhibit the metabolism of AA. Moreover, due to its chemical structure, it has an interesting antioxidant profile. Because this moderate activity exhibited on a wide number of molecular targets CBD is currently investigated for its potential therapeutic properties on anxiety, psychosis, depression, epilepsy, emesis, inflammatory disorders, arthritis, neurodegeneration and cancer, among others (see Pisanti *et al.*, 2017 for review).

CBG was isolated and synthesized in 1964 (Gaoni & Mechoulam, 1964) and has been much less studied than the other previous phytocannabinoids so far. Like CBD, it does not induce psychoactive effects, since it has negligible activity at the $CB_1$ receptor and appears also inactive at $CB_2$ (Granja *et al.*, 2012). It has been reported to act as a TRPM8 antagonist and TRPV1 and TRPA1 agonist (Borrelli *et al.*, 2014). CBG can also activate α2 adrenergic receptors and inhibit $5\text{-}HT_{1A}$ (Cascio *et al.*, 2010).

For a deeper insight on the pharmacology and therapeutic potential of phytocannabinoids, see a review in Ligresti *et al.*, 2016.

SR147778 or "surinabant" (Rinaldi-Carmona *et al.*, 2004). Rimonabant is the most widely used $CB_1$ antagonist for basic research and was one of the first cannabinoid-related compounds to be commercialized (named Acomplia) for the treatment of obesity and metabolic syndrome (Topol *et al.*, 2010), but had to be withdrawn in 2008 due to serious secondary effects that included depressive/suicidal behavior. Nowadays, it is accepted that this might be provoked by the capacity of these compounds to behave as inverse agonists, thus not only attenuating the effects of cannabinoid receptors, but also inducing the contrary effects (Pertwee, 2010). Peripherally-restricted antagonists (not crossing the blood-brain barrier) or neutral antagonists are being developed to avoid these issues (Kirilly *et al.*, 2012).

Among $CB_2$-selective antagonists, the most used are SR144528, a rimonabant analog (Rinaldi-Carmona *et al.*, 1998), and the aminoalkylindole AM-630 (Howlett *et al.*, 2002).

<u>Endocannabinoid tone modifiers</u>

Besides the direct activation/inhibition of cannabinoid receptors, other mechanisms have been described to enhance or decrease endocannabinoid signaling. For example, a new pharmacological approachment to the study of the endocannabinoid system that is currently increasing is the search for potential allosteric modulators. That is, compounds that do not bind to the orthosteric binding site of the receptor but to allosteric sites, searching thus for a different action than the subsequent to the classical activation, or for the reduction of secondary effects derived from orthosteric binding. Some of these compounds are ORG27569 and the endogenous molecule lipoxin A4, which are positive allosteric modulators, and PSNCBAM-1, which is a negative allosteric modulator (reviewed in Morales *et al.*, 2016). These are selective for $CB_1$, but there is research in progress trying to generate $CB_2$ allosteric modulators as well.

Other way to alter the levels of endocannabinoids is by the inhibition of the enzymes involved in their synthesis or, in particular, their degradation. Regarding the metabolism of AEA, some of the most used FAAH inhibitors are URB-597, OL-135, OL-92 and PF-750, which enhance the actions of ECBs, particularly AEA, at their receptors. Regarding 2-AG, DAGL inhibitors have been synthesized, such as O-3841 and OMDM188. To block 2-AG degradation, MAGL-inhibitors such as OMDM169 and JZL184 are used (reviewed in Di Marzo, 2009).

Last, another way to enhance the action of endocannabinoids would be the inhibition of their reuptake to the cell. Although the existence of the anandamide transporter, as explained before, is still controversial, a few inhibitors of endocannabinoid-reuptake have been synthesized, such as UCM707, UCM119, AM404, OMDM1 and OMDM2 (reviewed in Glaser *et al.*, 2005).

**Cannabinoid receptor-coupled signaling**

As introduced in previous sections, cannabinoid receptors are membrane proteins with 7 transmembrane domains, coupled to G-proteins in the inner face of the cellular membrane (see Turu & Hunyady, 2010 for review).

The activation of $CB_1$ receptor leads to the recruitment and activation of three different types of G-proteins: $G\alpha_{i/o}$, $G\alpha_s$ and $G\alpha_q$. The activation of $G\alpha_{i/o}$ induces the inhibition of the adenylyl cyclase (AC), reducing the production of cyclic adenosine monophosphate (cAMP) and thus the activity of cAMP-dependent protein kinase (PKA), which participates in the control of many biological processes, representing the major intracellular signaling pathway associated with the $CB_1$ receptor (*Figure 8*). The activation of this subunit has been also reported to modulate the function of $Ca^{2+}$ channels, as well as different $K^+$ channels (reviewed in Howlett *et al.*, 2002). In certain cells, the activation of $CB_1$ can cause the increase of cAMP levels through $G\alpha_s$. This occurs after the treatment with pertussis toxin, which maintains $G\alpha_{i/o}$ inactive (Abadji *et al.*, 1999). The coupling to $G\alpha_q$ induces the activation of the membrane protein phospholipase C (PLC), which hydrolyzes the phosphatidylinositol biphosphate (PIP2), producing inositol triphosphate (IP3) and DAG, both products involved in the release of $Ca^{2+}$ from the endoplasmic reticulum and the activation of protein kinase C (PKC) (Lauckner *et al.*, 2005).

On the other hand, the released βγ subunits

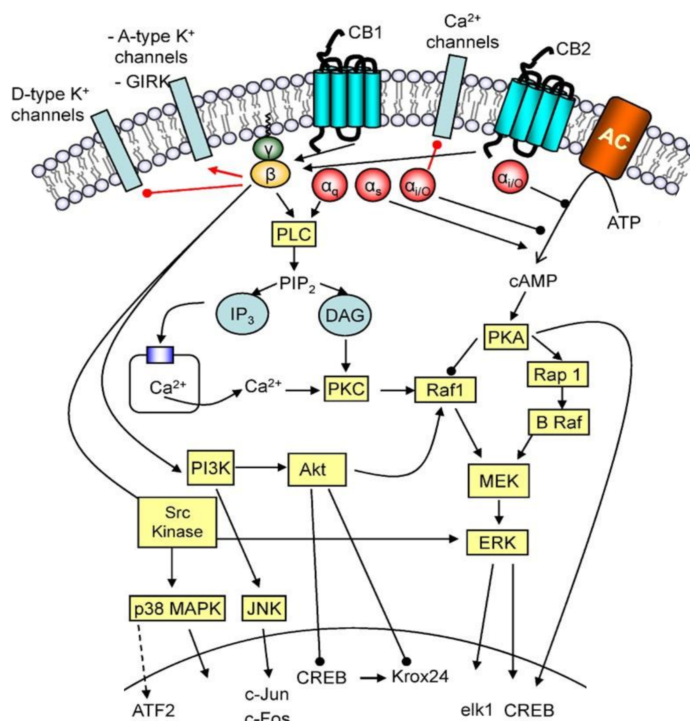


**Figure 8.** CB1 and CB2 receptors-mediated signalling. ATF2, ctivating transcription factor 2; ATP, adenosine triphospate; CREB, cAMP response element binding protein; PKA, protein kinase A. Arrows indicate activation, blunted arrows indicate inhibition. Adapted from Andre & Gonthier, 2010.

can induce several pathways. They can activate the mitogen-activated protein kinases (MAPK) p38, c-Jun N-terminal protein kinase (JNK) (Liu *et al.*, 2000; Rueda *et al.*, 2000), extracellular signal-regulated kinase (ERK) (Galve-Roperh *et al.*, 2002) and phosphatidylinositol 3-kinase (PI3K) - Akt pathways (Molina-Holgado *et al.*, 2002), involved in cellular differentiation and death. Cannabinoid ligands can also modulate other pathways involved in cell proliferation and survival, such as ceramide biosynthesis (involved in apoptosis and cell-cycle arrest) (Guzman *et al.*, 2001) and the modulation of nitric oxide synthase (NOS) enzymes (Stefano *et al.*, 2000; Esposito *et al.*, 2006).

Regarding $CB_2$ receptor, it has only been reported to couple $G\alpha_{i/o}$, and thus lead to the activation of MAPK and PI3K pathways. Moreover, $CB_2$ activation can lead to the increase of intracellular $Ca^{2+}$, not through the activation of ion channels, but by the activation of the PLC pathway (Zoratti *et al.*, 2003).

Last, the research on the formation and activity of cannabinoid receptor dimers has increased in the last few years. Homomers of $CB_1$ and $CB_1$-$CB_2$ and $CB_2$-GPR55 heteromers have been reported (Wager-Miller *et al.*, 2002; Callen *et al.*, 2012; Balenga *et al.*, 2014), as well as with other receptors, such as $D_2$, A2a, 5-$HT_{2A}$ and OX-1 (Carriba *et al.*, 2007; Pinna *et al.*, 2014; Vinals *et al.*, 2015; Imperatore *et al.*, 2016). The formation of these receptors can affect the canonical signaling of cannabinoid receptors alone and it can also influence the pharmacological management of the different receptors being part of a specific heteromer. Moreover, their presence and activity has been reported to be affected in certain pathologies. This has enhanced their interest as potential therapeutic targets, and thus prompted further investigation on this topic (Franco *et al.*, 2013).

### Physiological role of the ECS

The wide expression of the ECS throughout all kind of tissues and organs indicates its crucial value as a modulator of multiple physiological processes in the body. Although its components are present mostly in the nervous system, endocannabinoids and cannabinoid receptors have been found also in peripheral organs, such as the immune system, liver, kidney, muscle and bones, among others. For an overview of the distribution of the ECS in the periphery and potential cannabinoid therapies for related pathologies, see *Table 2*.

<table>
<tr><th>Peripheral organ</th><th colspan="2">Related pathologies</th><th>Potential therapies</th></tr>
<tr><td>Cardiovascular system</td><td colspan="2">Cardiomyopathies, heart failure, atherosclerosis, stroke</td><td>$CB_1$ antagonists (peripheral)<br>$CB_2$ agonists</td></tr>
<tr><td rowspan="2">Gastrointestinal tract</td><td colspan="2">Irritable bowel syndrome, inflammatory bowel disease, gastric ulcer</td><td>$CB_1$ agonists (peripheral)<br>$CB_2$ agonists<br>FAAH inhibitors<br>MAGL inhibitors</td></tr>
<tr><td colspan="2">Obesity, metabolic syndrome</td><td>$CB_1$ antagonists (peripheral)</td></tr>
<tr><td>Liver</td><td colspan="2">Fatty liver disease</td><td>$CB_1$ antagonists (peripheral)<br>$CB_2$ agonists</td></tr>
<tr><td>Muscle</td><td colspan="2">Muscular dystrophy</td><td>$CB_1$ antagonists</td></tr>
<tr><td>Bone</td><td colspan="2">Osteoporosis</td><td>$CB_2$ agonists</td></tr>
<tr><td>Skin</td><td colspan="2">Atopic dermatitis, prurigo, uremic itch</td><td>PEA</td></tr>
<tr><td>Kidney</td><td colspan="2">Diabetic and other neprhopathies and tubulopathies</td><td>$CB_1$ antagonists (peripheral)<br>$CB_2$ agonists</td></tr>
<tr><td>Reproductive system</td><td colspan="2">Infertility</td><td>$\Delta^9$-THC<br>PEA</td></tr>
<tr><td rowspan="3">Immune system</td><td rowspan="3">Immune-mediated diseases</td><td>Multiple sclerosis</td><td>$\Delta^9$-THC, $CB_2$ agonists</td></tr>
<tr><td>Arthritis</td><td>CBD, $CB_2$ agonists</td></tr>
<tr><td>Allergic asthma</td><td>$\Delta^9$-THC, CBN</td></tr>
</table>

**Table 2.** Localization and therapeutic potential of the ECS in the periphery (Adapted from Croxford & Yamamura, 2005; Maccarrone *et al*., 2015).

The ECS as a neuromodulator system in the CNS

The most important function of the endocannabinoids in the CNS is their action as modulators of synaptic transmission by acting retrograde signaling system. This action is mostly mediated by the activation of $CB_1$ receptor, which is the most abundant GPCR in the brain, although recent evidence also situates $CB_2$ receptor in a similar function in specific synapses and/or CNS areas (Stempel *et al.*, 2016). As explained previously, $CB_1$ is mainly located at presynaptic terminals, while the enzymes for the synthesis of endocannabinoids are found postsynaptically. The depolarization of the postsynaptic membrane, as a consequence of an increase of $Ca^{2+}$ entry to the cell stimulates the synthesis of endocannabinoids, which are released "backwards" to the synaptic cleft and activate presynaptically-located $CB_1$ receptors. If the neurotransmitter whose release is inhibited is glutamate this effect is called depolarization-induced suppression of excitation (DSE); if it is GABA, it is called depolarization-induced suppression of inhibition (DSI). In both cases, the role of the ECS is clearly protective, since it is addressed to maintain synapse homeostasis, attenuating both excessive neuronal excitation (glutamate) or inhibition (GABA). Retrograde cannabinoid signaling has also been reported to be involved in long term synaptic depression (LTD). This form of synaptic plasticity is followed by a low frequency, long patterned stimulus, which leads to a $CB_1$-dependent, long-lasting decrease of neurotransmitter release, mainly as a consequence of a decrease in postsynaptic receptor density (see Castillo *et al.*, 2012 for a deep review).

The synaptic modulation by the ECS can be exerted also by non-retrograde signaling. For example, postsynaptic AEA produced as a consequence of the synaptic activation of metabotropic glutamate receptors can instead act on postsynaptic TRPV channels, which have shown to trigger LTD in the dentate gyrus through a $Ca^{2+}$ -dependent endocytosis of AMPA receptors (Chavez *et al.*, 2010). Also, an intracellular $CB_2$ activation at pyramidal cells of the medial prefrontal cortex produces an autocrine inhibition (den Boon *et al.*, 2012).

It has been widely proved in the last years that not only neurons, but also glial cells can play a

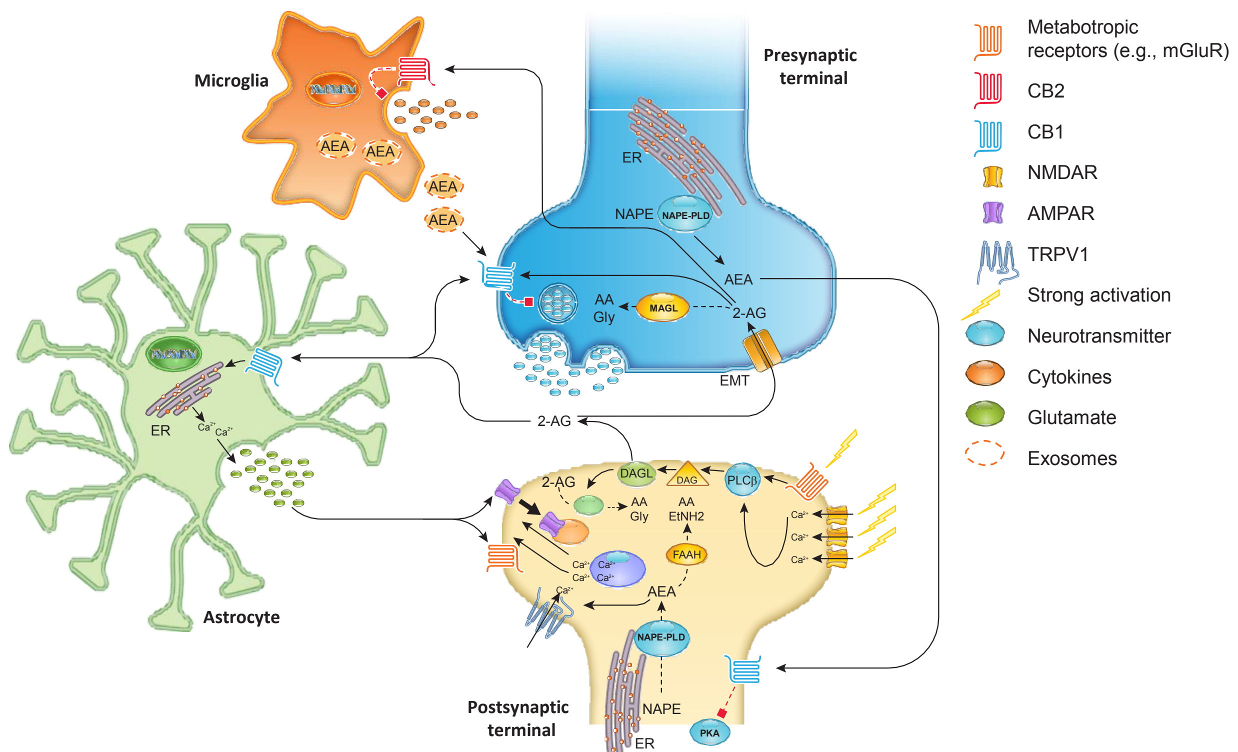


**Figure 9.** Neuromodulatory activity of the endocannabinoid system at the tripartite synapse. Black arrows indicate stimulation, and red blunted arrows indicate inhibition. Dotted black arrows indicate enzymatic transformation. Taken from Ligresti *et al*., 2016.

direct role in synaptic plasticity. Astrocytes have been reported recently not only to act as support agents, but be direct interplayers in the synaptic communication between neurons, what has been called the "tripartite synapse" (Covelo & Araque, 2016). Endocannabinoids can also act on this astrocyte-neuron communication. The activation of astroglial $CB_1$ receptors leads to an influx of $Ca^{2+}$ through activation of PLC, and this ends in the release of glutamate by astrocytes that acts on neurons (Navarrete *et al.*, 2014). On the other hand, among the widely known roles of microglial cells, they can also prune developing synapses and thus regulate synaptic function and plasticity (Hong *et al.*, 2016). Interestingly, recent reports show that microglial-AEA, released in vesicles, is able to act presynaptically on GABAergic neurons (Gabrielli *et al.*, 2015). For an overview of the neuromodulatory activity of the endocannabinoid system, see *Figure 9*.

Since the ECS is located in many structures along the CNS, and modulates both inhibitory and excitatory transmission, it is involved in all kind of neurobiological processes that include cognitive function, anxiety, motor control and nociception, among others. Likewise, dysregulations of the ECS have been documented in the pathologies related to all of these processes, which turns it into a potential therapeutic target for these conditions. An overview of the presence of the ECS and its potential applications is presented in *Table 3*.

## Cannabinoids and neuroprotection

One key function of the ECS in the nervous system is the regulation of the homeostasis, integrity and survival of neurons and glial cells. This is especially important, since all neurodegenerative disorders and acute brain damage curse with cell loss and the capability of replacement of neural cells is very limited.

Indeed, the ECS has been reported to be altered in brain pathologies, and the pharmacological manipulation of this system has provided potential therapeutic approaches in different models of acute brain damage and neurodegenerative diseases. These include ischemia (Hillard, 2008), brain trauma (Shohami *et al.*, 2011), Alzheimer's disease (Aso & Ferrer, 2014), Huntington's disease (Sagredo *et al.*, 2012), Parkinson's disease (More & Choi, 2015), multiple sclerosis (Pryce & Baker, 2012) and amyotrophic lateral sclerosis (Pryce & Baker,

| Process | Functions assigned to the ECS | Location of ECS elements | Pathologies for the application of cannabinoids | References |
|---|---|---|---|---|
| **Motor control** | Stimulation of movement at low doses; inhibition at high doses | Basal ganglia, cerebellum | HD, multiple sclerosis (MS)*, PD, Tourette syndrome, dyskinesia | (Fernandez-Ruiz *et al.*, 2011; Muller-Vahl, 2013) |
| **Cognitive processes** | Motivation, emotion, reward system | Brain cortex, limbic system | Addiction, psychiatric disorders | (Laviolette & Grace, 2006) |
| **Learning and Memory** | Memory extinction, effects on LTP and LTD | Hippocampus | Memory impairment disorders (Alzheimer's disease (AD), post-traumatic stress syndrome) | (Maroof *et al.*, 2013; Neumeister *et al.*, 2015) |
| **Stress and anxiety** | Anxiolytic effect | Central amygdala, paraventricular hypothalamic nucleus | Anxiety, psychiatric disorders | (Rubino *et al.*, 2015; Lee *et al.*, 2016) |
| **Sleep** | Regulation of sleep and sleep-wake cycles | Anterior and lateral hypothalamus | Sleep disorders (insomnia, somnolence) | (Prospero-Garcia *et al.*, 2016) |
| **Pain** | Spinal and supra-spinal analgesia | Brain and spinal areas involved in nociception, sensory nerve terminals | Neuropathic pain | (Romero-Sandoval *et al.*, 2015; Maldonado *et al.*, 2016) |
| **Neuronal development** | Normal neural differentiation and proliferation during development | Embryonic stem cells, neuronal progenitors | Epilepsy, possible interest in neuro-repair | (Dow-Edwards & Silva, 2017; Rosenberg *et al.*, 2017) |
| **Body temperature** | Regulation of body temperature | Hypothalamic nuclei | Unknown | (Rawls & Benamar, 2011) |
| **Appetite and food intake** | Regulation of appetite and energy balance | Hypothalamic nuclei and limbic areas | Increase of appetite in AIDS and cancer patients; decrease of appetite in obesity patients | (Gatta-Cherifi & Cota, 2015; Ostadhadi *et al.*, 2015) |
| **Emesis** | Vomit and nausea control | Area postrema | Reduction of nausea and vomit in cancer patients | (Rock & Parker, 2016) |
| **Neuroendocrine regulation** | Adenohypophyseal hormones release | Mediobasal hypothalamus | Unknown | (Pagotto *et al.*, 2006) |

**Table 3.** Overview of the physiological processes of the CNS in which the ECS is involved, and potential applications of endocannabinoid modulators in related pathologies. *Although MS is not a motor disorder in nature, those symptoms that are treated with cannabinoids (e.g. spasticity) may be interpreted as motor symptoms.

2015). When considering the manipulation of the ECS as a neuroprotective strategy it is important to take into account the following: endogenous protective responses (such as enhanced endocannabinoid generation or up-regulation of $CB_2$ receptors) should be enhanced by pharmacological treatments, as they represent an adaptive response aimed at restoring cell and tissue homeostasis; however, dysregulated responses (such as elevated FAAH and MAGL or reduced $CB_1$ receptor signaling) should be corrected by pharmacological treatments, as they represent a maladaptive mechanism eventually contributing to disease symptoms and progression.

Among the multiple cellular mechanisms involved in the neuroprotective role of cannabinoids, that can be cannabinoid receptors –dependent or not, three will be reviewed for their relevance for the purpose of this Doctoral

Thesis: attenuation of excitotoxicity, oxidative stress and neuroinflammation, three neurotoxic events introduced previously that are strongly related to each other, as well as present among most neurodegenerative disorders (*Figure 10*).

*Cannabinoids and excitotoxicity*

Cannabinoids can act as antiexcitotoxic agents through different mechanisms. As explained previously, the release of cannabinoids activates $CB_1$ receptors present in presynaptic glutamatergic terminals, thus inhibiting glutamate release and reducing excitotoxicity. $CB_2$ may be also involved in this action, as HU-210 has proved to act as antiexcitotoxic in a manner dependent of both cannabinoid receptors (Docagne *et al.*, 2007). Moreover, certain cannabinoids, such as the synthetic HU-211 and anandamide can directly antagonize NMDA receptors (Feigenbaum *et al.*, 1989; Hampson *et al.*, 1998). In addition, cannabinoid agonists can also directly act on postsynaptic terminals by closing voltage-gated channels to prevent $Ca^{2+}$ entry and the activation of $Ca^{2+}$ dependent damaging pathways.

*Cannabinoids and neuroinflammation*

Cannabinoids can have a double effect on neuroinflammation, reducing the production of pro-inflammatory cytokines and stimulating the release of anti-inflamatory and pro-survival mediators, such as transforming growth factor(TGF)-β and IL-10 by glial cells (reactive microglia and activated astrocytes). Interestingly, $CB_2$ is typically overexpressed in these cells in neuroinflammatory events, and thus the anti-inflammatory effects mediated by cannabinoids are proposed to be mediated mostly by the action on this receptor. However, some studies indicate that $CB_1$ agonists are also effective in this sense. Other cannabinoids can act by a cannabinoid receptor-independent manner, for example by activating PPAR nuclear receptors, in particular PPAR-γ, and inhibiting Nuclear factor kappa-licht-chain-enhancer of activated B cells (NFκB) (Fernandez-Ruiz *et al.*, 2010).

*Cannabinoids and oxidative stress*

Certain cannabinoids, because of its chemical

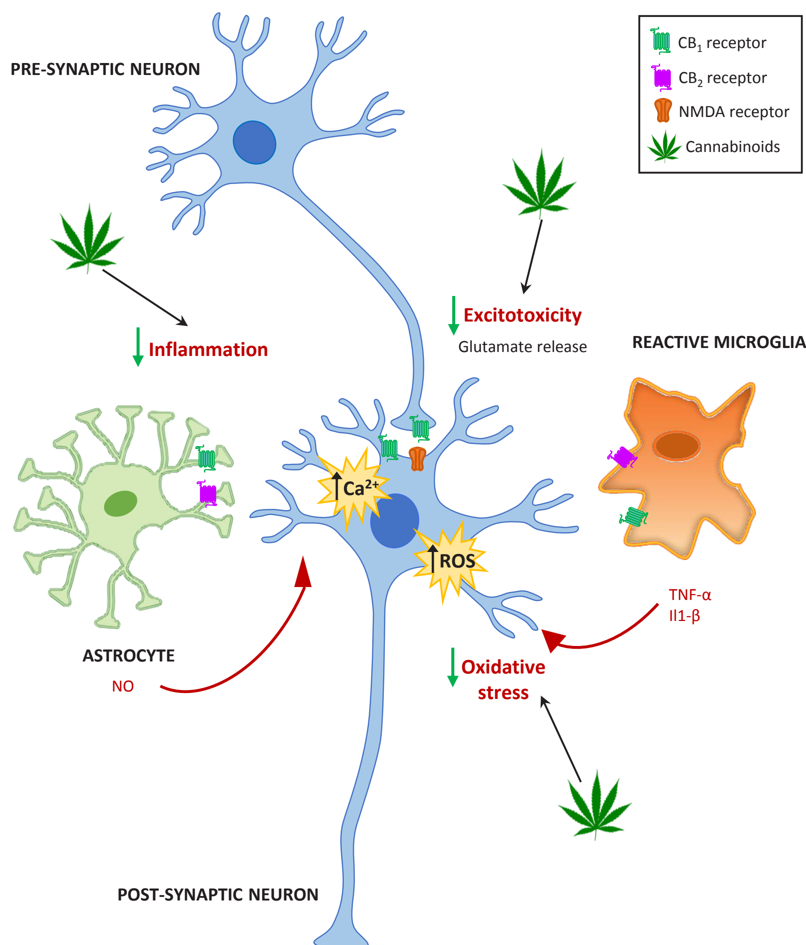


**Figure 10.** Schematic view of the main mechanisms of neuroprotection by cannabinoids that may be translated to HD.

**BOX 4. Clinical development of cannabinoid drugs**

Years of preclinical research have lead finally to the approval of some cannabinoid drugs, based on synthetic compounds or on phytocannabinoids, for different indications.

**Cesamet**: Constituted by the synthetic $\Delta^9$-THC analog nabilone. It was originally approved in Canada in 1981 and by the FDA (Food and Drug Administration) in 1985, for the treatment of nausea and vomiting in patients undergoing cancer treatment (Ware *et al.*, 2008). It is currently being evaluated for chronic pain as well (Tsang & Giudice, 2016).

**Marinol**: Constituted by synthetic $\Delta^9$-THC, not obtained from the plant and named dronabinol. Originally approved by the FDA) for nausea (1985) and for the stimulation of appetite (1992). It has been also approved in Canada for cancer-related nausea and vomiting (1988) (May & Glode, 2016) and for AIDS-related anorexia (2000) (Badowski & Perez, 2016).

**Acomplia**: Constituted by the $CB_1$ inverse agonist rimonabant, or SR141716A. As explained in previous sections, it was initially approved by the European Medicine Association (EMA) in 2006 for the treatment of obesity and metabolic syndrome (Topol *et al.*, 2010). It was finally withdrawn from market in 2008 due to negative psychiatric side effects reported during follow-up studies.

**Sativex**: Mouth spray composed by a 1:1 combination of $\Delta^9$-THC and CBD botanical extracts, but contains other phytocannabinoids in minimal amounts. It was approved to treat MS-related spasticity in 11 countries in Europe, besides Canada (where it is also approved for neuropathic and oncologic pain) and Israel (Patti *et al.*, 2016). In the US, the FDA granted in 2014 "Fast Track" designation to Sativex for the treatment of pain in cancer patients (Johnson *et al.*, 2013). It is currently being evaluated for chronic non-cancer or neuropathic pain (Topol *et al.*, 2010; Russo *et al.*, 2016).

**Epidiolex**: The last available cannabinoid medicine is an oral solution of pure CBD. It received orphan designation by the FDA in 2013 and the EMA in 2014 for the treatment of infantile epilepsies (Lippiello *et al.*, 2016) such as Dravet syndrome and Lennox-Gastaut syndrome (Hussain *et al.*, 2015; Devinsky *et al.*, 2016).

structure that includes a phenolic ring (such as CBD, $\Delta^9$-THC, CBG, dexanabinol) can act as ROS scavengers and thus attenuate oxidative damage. It has been clasically accepted that cannabinoid action on oxidative damage was only due to this receptor-independent activity, but recent studies have suggested that their antioxidant action might also be mediated by the action on PPAR receptors and transcription factors, such as Nuclear factor (erythroid-derived 2)-like 2 (NRF2) and NFκB. Moreover, AEA, which is not a ROS scavenger by structure, has shown to reduce oxidative stress, maybe by the activation of $CB_1$. Therefore, the involvement of cannabinoid receptors on the antioxidant properties of cannabinoids cannot be ruled out (Fernandez-Ruiz *et al.*, 2015).

## CANNABINOIDS AND HUNTINGTON'S DISEASE

One of the neurological disorders that has been more intensively investigated for the development of a cannabinoid therapy, mainly addressed to delay disease progression, is HD. Indeed, some clinical trials have been conducted with different cannabinoid formulations, one of them included as a part of this Doctoral Thesis.

### Endocannabinoid signaling in the basal ganglia

As explained before, $CB_1$ receptors are highly expressed throughout the basal ganglia. Indeed, they are located in the presynaptic terminals of striatonigral and striatopallidal neurons, the two major neural subpopulations that degenerate in HD, which belong to the so-called direct and indirect pathways respectively, and are both GABAergic. Intrinsic striatal neurons do not contain $CB_1$, with the exception of some parvalvumin-positive GABAergic interneurons and a few cholinergic neurons. $CB_1$ receptors are also located in the terminals of glutamatergic cortico-striatal projection neurons and also in those that project from the subtalamic nucleus to the substantia nigra. However, nigro-striatal dopaminergic neurons do not express $CB_1$, but TRPV1 instead. This receptor is also involved, as explained in previous sections, in

endocannabinoid signaling, and is present in the striatum and globus pallidus as well.

The presence of $CB_1$ receptor, mainly presynaptically, in all these areas enables its ligands to directly or indirectly modulate the activity of the neurotransmitters involved in the function of the basal ganglia. Thus, cannabinoids can, by retrograde signaling, inhibit GABA or glutamate release depending on the neuronal-type, in a $CB_1$-dependent manner, while cannabinoids that are TRPV1 agonists may directly reduce dopamine release. Al these effects result in the inhibition of motor activity (see Fernandez-Ruiz, 2009 for review) (*Figure 4*).

As regards to $CB_2$ receptors, they have not been found in significant levels in motor areas, except for certain neuronal populations in the cerebellum (Rodriguez-Cueto *et al.*, 2014) and substantia nigra (Garcia *et al.*, 2015), as well as in perivascular microglia (Nunez *et al.*, 2004). However, their expression in the basal ganglia is exacerbated in pathological conditions, which suggests an important role of these receptors in the modulation of motor disorders.

The levels of endocannabinoids such as AEA and 2-AG are also higher in the basal ganglia, especially in the substantia nigra and globus pallidus (Giuffrida *et al.*, 2000).

Alterations of the ECS in Huntington's disease

Autoradiographic and immunohistochemical studies in *post-mortem* tissues reveal a downregulation in the expression of $CB_1$ receptors in the MSNs, both in human samples of HD patients (Glass *et al.*, 2000), transgenic mice (McCaw *et al.*, 2004) and in neurotoxin-generated models (Lastres-Becker *et al.*, 2002a). This downregulation occurs early in the pathology, before the appearance of clinical symptoms, which indicates that this event is one of the most relevant neurochemical alterations of these neurons, and potentially a key pathogenic factor in this pathology (Blazquez *et al.*, 2011). Interestingly, this decrease of $CB_1$ levels has been reported recently to occur also in NPY/nNos-expressing striatal interneurons (Horne *et al.*, 2013), while it does not occur in the glutamatergic corticostriatal projection neurons (Chiodi *et al.*, 2012; Chiarlone *et al.*, 2014).

Conversely, the expression of $CB_2$ receptors has been found to be strongly increased in human striatal and cortical samples, some transgenic models (Palazuelos *et al.*, 2009; Bouchard *et al.*, 2012) and in inflammatory acute models of the disease (Sagredo *et al.*, 2009), and such elevation has been situated in vascular endothelium of postmortem samples (Dowie et al., 2014) and in glial elements in animal models (activated astrocytes and reactive microglia).

On the other hand, alterations in the levels of endocannabinoids and the enzymes involved in their metabolism have also been reported. Although, these alterations vary in one way or another depending on the brain structure in the case of human tissues, and between different models of the disease (see Morera-Herreras *et al.*, 2016 for review). It is important to remark that some controversial data have been recorded with the endocannabinoid inactivating enzymes, in particular FAAH, and this may have an important influence in pharmacological treatments targeting the endocannabinoid system, as suggested by Dowie *et al.*, 2010. For example, FAAH levels have been found to change in opposite directions in HD, decreasing in human lymphocytes (Battista *et al.*, 2007) and increasing its activity in lymphocytes of genetic murine models like R6/2 mice (Bari *et al.*, 2013). Regarding the caudate-putamen, FAHH levels have been reported to be increased both in *postmortem* human tissue and in the striatum of R6/1 and R6/2 mice (Blazquez *et al.*, 2011), which is accompanied by reduced levels of endocannabinoids (Bisogno *et al.*, 2008).

All these described alterations in the components of the endocannabinoid system in the brains of HD patients and in animal models suggest first that losses of $CB_1$ receptor signaling may be a pathogenic event, thus facilitating the development of excitotoxicity or other neurotoxic events, so that it should be pharmacologically corrected by the treatment with selective $CB_1$ agonists, also useful to reduce chorea. They also suggest that the elevated levels of $CB_2$, rather than being a pathogenic event, appears more an endogenous protective response that should be potentiated by pharmacological treatment. Therefore, the collected evidences suggest that both $CB_1$ and $CB_2$ receptors could be interesting pharmacological targets in HD. Thus, considering

these premises, the manipulation of classic cannabinoid receptors could mean a potential strategy to attenuate cell death and alleviate motor symptoms in this disease. However, this possibility also demands to investigate the status of the endocannabinoid hydrolyzing enzymes, whose inhibition may be protective or harmful depending on the cellular context

Therapeutic potential of cannabinoids in Huntington's disease

Different approaches have been followed so far in the preclinical evaluation of cannabinoids as disease modifiers in HD. Firstly, the hypokinetic activity of $CB_1$ agonists or compounds able to enhance the endocannabinoid tone (e.g. inhibition of ECB inactivation) suggested that they could be efficient anti-hyperkinetic, and thus symptom-relieving agents. In this sense, positive results have been obtained in acute models of the disease (Lastres-Becker *et al*., 2002b). However, the $\Delta^9$-THC analog nabilone was clinically evaluated for its action on motor symptoms, and contradictory results were reported (Muller-Vahl *et al.*, 1999; Curtis & Rickards, 2006), although the last clinical trial performed with this compound showed improvements in various motor and cognitive parameters (Curtis *et al.*, 2009). Interestingly, the inhibition of ECB inactivation was effective against choreic movement in preclinical models, but their effects were not $CB_1$-receptor dependent, but involved the action of TRPV1 receptors (Lastres-Becker *et al.*, 2003), so these receptors could be another potential target for the management of motor symptoms in HD.

Another therapeutical approach would be to prevent or delay the progressive cell death that occurs in the brains of HD patients. The neuroprotective and neuromodulator effect of cannabinoid compounds makes them interesting as potential disease modifiers. Along the last years, several preclinical studies have shown the protective capability of cannabinoids against brain damage in different models of HD. For example, a non-selective agonist, the phytocannabinoid $\Delta^9$-THC, showed positive results in a mouse model of excitotoxicity and in R6/2 transgenic mice (Palazuelos *et al.*, 2009; Blazquez *et al*., 2011) and both $CB_1$ and $CB_2$ receptors were proposed to be involved in this effect. $CB_2$ agonists have also provided neuroprotection in inflammatory models (Sagredo *et al.*, 2009). Moreover, non-cannabinoid receptor dependent mechanisms have been reported to be also contributing to this neuroprotection. The phytocannabinoids $\Delta^9$-THC and CBD alone, but particularly in a 1:1 combination, proved to be effective in a rat model of oxidative damage, in a manner that was not dependent on neither $CB_1$ nor $CB_2$ activation, but presumably because of the potent intrinsic antioxidant properties of these phytocannabinoids (Sagredo *et al.*, 2011). Indeed, CBD had been evaluated in a clinical trial many years before, unfortunately without benefits on symptoms or disease progression (Consroe *et al.*, 1991).

In conclusion, several cannabinoid compounds, with different pharmacological profiles, have been evaluated and proved efficiency as neuroprotective agents in different models of HD. Despite the promising results at the preclinical level, further evidences are needed to move to the clinical field. Given the multi-target profile of cannabinoids as well as the evidence collected with individual cannabinoids in preclinical models, it appears that the combination of some of these compounds may result in a broad-spectrum treatment that can approach various of the many neuropathological events that occur in the brains of the patients, making them interesting novel therapeutic strategies for HD.

# AIMS

Huntington's disease is a devastating neurodegenerative disorder that currently has no cure and for which only a few therapies against specific motor and psychiatric symptoms are available yet, so there is an urgent need for new medicines than can delay the progression of the neurodegenerative process. Since the discovery of alterations of different elements of the ECS in HD patients, there has been a huge increase in the research studies performed to evaluate the potential of cannabinoid compounds as symptom relieving and disease modifying therapies in this disease. Cannabinoids have proven to prevent various deleterious events that take place in the brains of HD patients and that are involved in the neurodegenerative process, such as excitotoxicity, oxidative stress and inflammation. Our hypotheses is that phytocannabinoids, alone or in combination, but always with a broad-spectrum profile, may represent a promising option to approach the multiple cytotoxic processes involved in HD neuropathology. In order to obtain robust evidences that support these compounds, a deep preclinical evaluation must be developed, from different cellular and animal models to finally reach the evaluation in patients.

In this context, the **global aim** of this Thesis is to validate the neuroprotective potential of phytocannabinoids in Huntington's disease.

This global objective will be divided into 3 **specific aims**, which will be represented in this Thesis as 3 different chapters:

**1. Preclinical evaluation of phytocannabinoids as disease modifiers in animal models of HD.** The different cytotoxic events that lead to striatal neurodegeneration can be reproduced by different experimental models. We used acute models of neuroinflammation and oxidative stress, as well as a transgenic murine model of HD. The selected phytocannabinoids were the 1:1 Sativex-like combination of $\Delta^9$-THC-BDS and CBD-BDS, as well as CBG administered in pure form.

**2. Evaluation of Sativex in a pilot clinical trial with patients of HD.** The results obtained from preclinical studies supported the evaluation of this 1:1 combination of $\Delta^9$-THC and CBD botanical extracts present in Sativex. This was conducted with subjects recruited at "Hospital Universitario Ramón y Cajal", Madrid.

**3. Study of some of the molecular mechanisms involved in the effect of cannabinoid compounds in experimental models of HD.** To this aim, we used first a model of knock-in HD cell lines that were exposed to different cytotoxic agents and treated with the Sativex-like combination of $\Delta^9$-THC-BDS and CBD-BDS, as well as each phytocannabinoid alone. In a second study, we investigated the status of the endocannabinoid signaling in experimental models of HD, paying emphasis in 2-AG and its metabolism, and we explored how changes in the availability of this endocannabinoid may alter its effects from the expected protective action towards an increase in striatal damage.

# RESULTS

# CHAPTER 1

## Preclinical evaluation of phytocannabinoids as disease modifiers in animal models of HD.

In this Chapter we evaluated the neuroprotective potential of phytocannabinoids in different animal models of HD.

First, we evaluated the effect of the 1:1 combination of botanical extracts enriched with $\Delta^9$-THC and CBD, which are the main constituents of Sativex, in rats lesioned with the complex II inhibitor malonate. This model is characterized by an acute striatal degeneration by mechanisms that involve apoptosis and glial activation. The treatment with Sativex was able to reduce the volume of striatal edema caused by the lesion. It also partially attenuated the striatal cell loss and the microglial and astroglial activation caused by the lesion, and completely prevented the increase in expression of the pro-inflammatory marker iNOS. Our results also supported the involvement of $CB_1$, but mainly $CB_2$ receptors in these effects.

In the next paper, we evaluated the same Sativex-like combination of $\Delta^9$-THC and CBD botanical extracts by a daily treatment from $4^{th}$ to $12^{th}$ week of age in the transgenic R6/2 mouse model. This model is characterized by an early and fast development of motor symptoms as well as a marked striatal atrophy. To analyze the effect of Sativex in these mice, we evaluated the extent of motor disturbances, the brain metabolic activity by positron emission tomography (PET) imaging and the levels of brain metabolic markers by $H^+$-MRS. Sativex did not produce any recovery in rotarod performance, but clearly attenuated clasping behavior, thus improving dystonia in these animals. It also attenuated the reduction in brain metabolic activity and recovered the changes in some metabolic markers of energy failure and neuronal deterioration observed in these animals.

In the last paper, we analyzed the neuroprotective potential of pure CBG in mice lesioned with the complex II inhibitor 3NP. In this model the acute striatal cell death is associated with calpain activation, mitochondrial dysfunction and oxidative stress. CBG partially improved some motor deficits caused by the toxin, completely reverted striatal cell loss and attenuated the microglial activation. It also reverSed the changes in markers of inflammation and oxidative stress in the striata of these mice. Next, we evaluated the effects of CBG in the transgenic mouse model R6/2. CBG produced a modest improvement in motor performance measured by the rotarod test. It also partially reversed the increase in $CB_2$ and decrease in BDNF and PPARγ striatal gene expression, as well as other markers of HD pathology. Last, it reduced the number of mHTT aggregates in the striata of these mice. This work was performed in collaboration with the group led by Eduardo Muñoz at "Universidad de Córdoba".

**Papers included in this Chapter:**

Valdeolivas, S., Satta, V., Pertwee, R.G., Fernandez-Ruiz, J. & Sagredo, O. (2012) **Sativex-like combination of phytocannabinoids is neuroprotective in malonate-lesioned rats, an inflammatory model of Huntington's disease: role of CB1 and CB2 receptors.** *ACS Chem Neurosci*, **3**, 400-406.

Valdeolivas, S., Sagredo, O., Delgado, M., Pozo, M.A. & Fernandez-Ruiz, J. (2017) **Effects of a Sativex-Like Combination of Phytocannabinoids on Disease Progression in R6/2 Mice, an Experimental Model of Huntington's Disease.** *Int J Mol Sci*, **18**.

Valdeolivas, S., Navarrete, C., Cantarero, I., Bellido, M.L., Munoz, E. & Sagredo, O. (2015) **Neuroprotective properties of cannabigerol in Huntington's disease: studies in R6/2 mice and 3-nitropropionate-lesioned mice.** *Neurotherapeutics*, **12**, 185-199.

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# Sativex-like Combination of Phytocannabinoids is Neuroprotective in Malonate-Lesioned Rats, an Inflammatory Model of Huntington's Disease: Role of $CB_1$ and $CB_2$ Receptors

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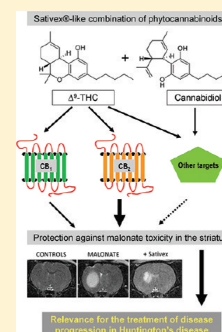
**ABSTRACT:** We have investigated whether a 1:1 combination of botanical extracts enriched in either $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) or cannabidiol (CBD), which are the main constituents of the cannabis-based medicine Sativex, is neuroprotective in Huntington's disease (HD), using an experimental model of this disease generated by unilateral lesions of the striatum with the mitochondrial complex II inhibitor malonate. This toxin damages striatal neurons by mechanisms that primarily involve apoptosis and microglial activation. We monitored the extent of this damage and the possible preservation of the striatal parenchyma by treatment with a Sativex-like combination of phytocannabinoids using different histological and biochemical markers. Results were as follows: (i) malonate increased the volume of edema measured by in vivo NMR imaging and the Sativex-like combination of phytocannabinoids partially reduced this increase; (ii) malonate reduced the number of Nissl-stained cells, while enhancing the number of degenerating cells stained with FluoroJade-B, and the Sativex-like combination of phytocannabinoids reversed both effects; (iii) malonate caused a strong glial activation (i.e., reactive microglia labeled with Iba-1, and astrogliosis labeled with GFAP) and the Sativex-like combination of phytocannabinoids attenuated both responses; and (iv) malonate increased the expression of inducible nitric oxide synthase and the neurotrophin IGF-1, and both responses were attenuated after the treatment with the Sativex-like combination of phytocannabinoids. We also wanted to establish whether targets within the endocannabinoid system (i.e., $CB_1$ and $CB_2$ receptors) are involved in the beneficial effects induced in this model by the Sativex-like combination of phytocannabinoids. This we did using selective antagonists for both receptor types (i.e., SR141716 and AM630) combined with the Sativex-like phytocannabinoid combination. Our results indicated that the effects of this combination are blocked by these antagonists and hence that they do result from an activation of both $CB_1$ and $CB_2$ receptors. In summary, this study provides preclinical evidence in support of a beneficial effect of the cannabis-based medicine Sativex as a neuroprotective agent capable of delaying signs of disease progression in a proinflammatory model of HD, which adds to previous data obtained in models priming oxidative mechanisms of striatal injury. However, the interest here is that, in contrast with these previous data, we have now obtained evidence that both $CB_1$ and $CB_2$ receptors appear to be involved in the effects produced by a Sativex-like phytocannabinoid combination, thus stressing the broad-spectrum properties of Sativex that may combine activity at the $CB_1$ and/or $CB_2$ receptors with cannabinoid receptor-independent actions.




**KEYWORDS:** *Phytocannabinoids, cannabidiol, $\Delta^9$-tetrahydrocannabinol, $CB_1$ and $CB_2$ receptors, Huntington's disease, malonate, basal ganglia, neurodegeneration, neuroprotection*

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by motor abnormalities, cognitive dysfunction, and psychiatric symptoms.[1] The primary cause of the disease is a mutation in the huntingtin gene consisting of a CAG triplet repeat expansion translated into an abnormal polyglutamine tract in the amino-terminal portion of this protein that becomes toxic for striatal and cortical neuronal subpopulations.[2] At present, there is no specific pharmacotherapy to alleviate motor and cognitive symptoms and/or to arrest/delay disease progression in HD. Thus, even though a few compounds have produced encouraging effects in preclinical studies (i.e., minocycline, coenzyme Q10, unsaturated fatty acids, inhibitors of histone deacetylases) none of the findings obtained in these studies have yet led on to the development of an effective medicine.[3] Importantly, therefore,

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following on from an extensive preclinical evaluation using different experimental models of HD, clinical tests are now being performed with cannabinoids. The preclinical studies with cannabinoids demonstrated preservation of striatal neurons by several agonists against different cytotoxic stimuli that operate in HD pathogenesis,[4,5] effects that were exerted through multiple mechanisms of action, some of which involve the activation of $CB_1$ and/or $CB_2$ receptors and others of which do not. For example, cannabinoids with antioxidant profile, that is, $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and cannabidiol (CBD), protected striatal neurons against toxicity caused by the mitochondrial complex II inhibitor 3-nitropropionic acid (3NP) that primes oxidative injury.[6,7] However, the activity of $\Delta^9$-THC at the $CB_1$ and $CB_2$ receptors enables this phytocannabinoid to induce neuroprotection in other experimental models, for example, the transgenic mouse model of HD, R6/2, in which the effects of $\Delta^9$-THC are likely produced through the activation of $CB_1$ receptors[8] and possibly through the activation of $CB_2$ receptors too, as selective agonists of this receptor type preserved striatal neurons in this genetic model[9] and also in malonate-lesioned rats,[10] a model priming pro-inflammatory events. Selective agonists of $CB_1$[8,11] and $CB_2$[9] receptors also preserved striatal neurons in in vitro or in vivo excitotoxic models. By contrast, other studies showed no effects in R6/1 mice using $\Delta^9$-THC, the synthetic agonist HU-210 and the inhibitor of the endocannabinoid metabolism URB597.[12] All these data suggest that the evaluation of cannabinoids as disease-modifying agents in patients should be necessarily conducted with a broad-spectrum cannabinoid or with combinations of various cannabinoids with different and complementary pharmacological profiles. Sativex, a cannabis-based medicine recently licensed for the treatment of spasticity and pain in multiple sclerosis patients,[13,14] has an appropriate profile for HD as it can activate $CB_1$ and $CB_2$ receptors due to the presence of $\Delta^9$-THC, but it can also exert cannabinoid receptor-independent antioxidant properties due to $\Delta^9$-THC and, in particular, to CBD.[15] We recently initiated some experiments with the combination of $\Delta^9$-THC and CBD botanical extracts present in Sativex in those animal models of HD, in which individual cannabinoid agonists have proved to be effective,[6−11] with the objective of determining whether this mixture also works in these models. We have just published the first data obtained in rats subjected to 3NP intoxication,[16] a model priming, as mentioned above, calpain activation and oxidative injury as major cytotoxic mechanisms, and in which pure $\Delta^9$-THC[6] and CBD[7] administered separately, have already been found to display neuroprotective properties. The Sativex-like combination of phytocannabinoids also preserved striatal neurons from death caused by 3NP intoxication, and this effect was, as expected, independent of $CB_1$ and $CB_2$ receptors.[16] These findings prompted us to extend our research to rats lesioned with malonate, an acute model of HD in which striatal damage is produced primarily by apoptosis and glial activation/inflammatory events and in which selective $CB_2$ receptor agonists have been shown to be effective.[10] To this end, we lesioned rats with an intrastriatal injection of malonate and used these animals for two different sets of experiments. First, we examined the neuroprotective effects of a 1:1 combination of botanical extracts of $\Delta^9$-THC and CBD, a combination that approximates to the 1.0:0.93 mixture of these extracts that is present in Sativex. The level of neuroprotection was evaluated by measuring the following parameters: (i) the volume of edema measured by in vivo NMR imaging; (ii) the number of Nissl- and FluoroJade B-stained cells which correlate with the number of surviving and degenerating cells, respectively, in the striatal parenchyma; (iii) the presence of reactive microgliosis, by using Iba-1 immunohistochemistry, and astrogliosis labeled with GFAP immunostaining; and (iv) the expression of various biochemical markers that have been found previously to be altered in this and other HD models, i.e. inducible nitric oxide synthase (iNOS), the neurotrophin IGF-1 and the $CB_1$ receptor.[8,10,16,17] In the second set of experiments, we explored whether the neuroprotective effects observed in this model with the combination of $\Delta^9$-THC and CBD present in Sativex involved the activation of $CB_1$ receptors, $CB_2$ receptors, or both, using selective antagonists for these two receptors (SR141716 and AM630, respectively). For this second set of experiments, we monitored the number of Nissl-stained cells, since this constitutes a very selective and sensitive marker of malonate damage in the striatal parenchyma.

## RESULTS AND DISCUSSION

Only a few clinical studies have been performed to determine whether cannabinoid compounds are efficacious in HD, and the results they have yielded are rather controversial.[18−21] Possibly, the reason for such controversy is that these clinical studies concentrated more on HD symptoms rather than on disease progression. Recent animal studies, however, have demonstrated that combinations of different cannabinoids or the use of a broad-spectrum cannabinoid may delay disease progression by preserving striatal neurons from death in different animal models of HD, thus stressing again the need for new clinical studies directed now at testing whether the neuroprotective effects that certain cannabinoids induce in experimental models of HD[8−11,16] are also produced in HD patients. As mentioned in the introduction, we recently initiated some experiments with a 1:1 combination of the $\Delta^9$-THC and CBD botanical extracts that are present in Sativex in those animal models of HD, in which individual cannabinoid agonists have been found to be protective,[6−11] with the objective to determine whether this mixture also works in these models. We used first rats subjected to 3NP intoxication, a model priming calpain activation and oxidative injury as major cytotoxic mechanisms, and in which pure $\Delta^9$-THC[6] and pure CBD[7] administered by themselves, had displayed neuroprotective properties. The Sativex-like combination of phytocannabinoids also protected striatal neurons from death caused by 3NP intoxication and its effects were, as expected, independent of $CB_1$ and $CB_2$ receptors.[16] In this Article,[16] we already presented preliminary evidence that the Sativex-like combination of phytocannabinoids might also work in additional models of HD, as it was capable to attenuate malonate-induced iNOS up-regulation in rats lesioned with this neurotoxin, but this was the only parameter analyzed in these rats.[16] The goal of the present study was to corroborate the potential of Sativex-like combination of phytocannabinoids in this model of HD, in which striatal damage is primarily produced by apoptosis and glial activation/inflammatory events,[10] by measuring different histopathological and biochemical parameters that have been more directly related to striatal degeneration in this HD model.

**Effects of Phytocannabinoids on Malonate-Induced Striatal Damage.** The first objective was to establish that we could produce the characteristic striatal damage associated with the local administration of malonate.[10] As expected, the administration of malonate damaged the striatal parenchyma as indicated by the increased edema volume recorded in in vivo

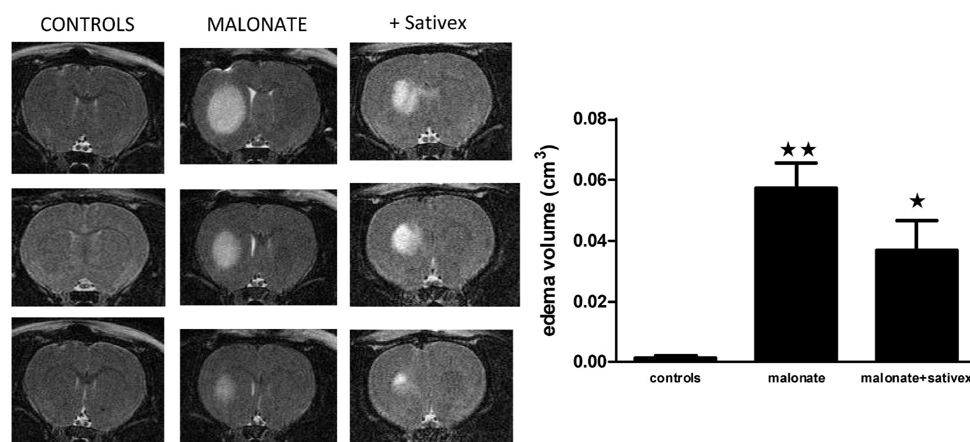
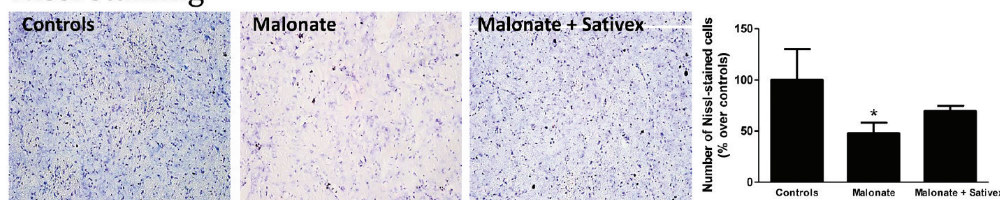



**Figure 1.** Volume of edema measured by in vivo NMR imaging procedures in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), or vehicle (Tween 80-saline). Details in the text. The 1:1 mixture $\Delta^9$-THC- and CBD-enriched botanical extracts is referred to as Sativex in this figure. Values are expressed as means ± SEM for 5−6 animals per group. Data were subjected to one-way analysis of variance followed by the Student−Newman−Keuls test (*$p < 0.05$, **$p < 0.005$ compared with controls). Representative in vivo NMR images for each experimental group are included in the left panel.

Nissl staining




Fluoro-Jade staining

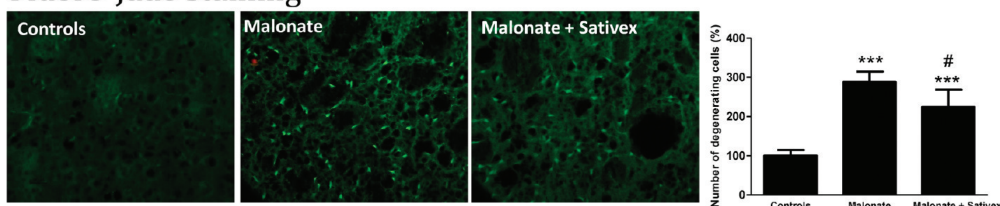
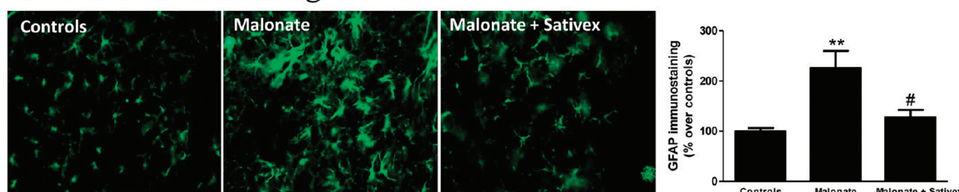



**Figure 2.** Nissl and FluoroJade B staining measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), or vehicle (Tween 80-saline). Details in the text. The 1:1 mixture $\Delta^9$-THC- and CBD-enriched botanical extracts is referred to as Sativex in this figure. Values correspond to percent over the contralateral nonlesioned side and are expressed as means ± SEM for 5−6 animals per group. Data were subjected to one-way analysis of variance followed by the Student−Newman−Keuls test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ compared with controls; $^{\#}p < 0.05$ compared with malonate + vehicle). Representative Nissl- and FluoroJade B-stained microphotographs for each experimental group are included in the left panel. Magnification = 20×.

NMR imaging ($F(2,19) = 8.86$, $p < 0.005$; see Figure 1), the reduction in the number of surviving cells determined by Nissl staining ($F(2,10) = 6.36$, $p < 0.05$; see Figure 2), the increase in the level of degenerating cells determined by FluoroJade B staining ($F(2,10) = 32.87$, $p < 0.0005$; see Figure 2), and the extent of reactive microgliosis labeled with Iba-1 immunohistochemistry ($F(2,12) = 4.652$, $p < 0.05$; see Figure 3) and astrogliosis labeled with GFAP immunohistochemistry ($F(2,11) = 9.25$, $p < 0.01$; see Figure 3). Malonate toxicity also affects the expression of different markers related to inflammation (i.e., iNOS: $F(2,11) = 5.78$, $p < 0.05$) and neurotrophins (i.e., IGF-1: $F(2,15) = 4.41$, $p < 0.05$) (see Figure 4). These changes, although characteristic of a specific experimental model of HD, have also been found in HD patients using CSF, blood cell, and post-mortem brain samples,[22,23] thus supporting the idea that glial activation associated with inflammatory events is part in the pathogenic process that occurs in HD patients. Lastly, malonate toxicity is also associated with a reduction in the expression of $CB_1$ receptors ($F(2,25) = 4.863$, $p < 0.05$; see Figure 4) an effect largely related to HD pathogenesis in different animal models.[6,8,16]

The administration of a 1:1 combination of $\Delta^9$-THC- and CBD-enriched botanical extracts that are present in Sativex attenuated, to a different extent, all these malonate-induced changes. For example, it slightly reduced the volume of edema measured with in vivo NMR imaging procedures 24 h after

GFAP immunostaining




Iba-1 immunostaining

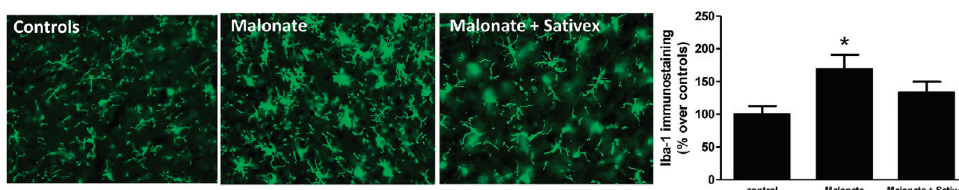



**Figure 3.** Iba-1 and GFAP immunostainings measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), or vehicle (Tween 80-saline). Details in the text. The 1:1 mixture $\Delta^9$-THC- and CBD-enriched botanical extracts is referred to as Sativex in this figure. Values correspond to percent over the contralateral nonlesioned side and are expressed as means ± SEM for 3−4 animals per group. Data were subjected to one-way analysis of variance followed by the Student−Newman−Keuls test ($*p < 0.05$, $**p < 0.01$ compared with controls; $^{\#}p < 0.05$ compared with malonate + vehicle). Representative Iba-1 and GFAP immunostained microphotographs for each experimental group are included in the left panel. Magnification = 20×.

malonate application and 24 h before animal death (Figure 1). These in vivo results correlated well with the data obtained after the histopathological analysis of animal brains. For example, the treatment with a Sativex-like combination of phytocannabinoids increased the number of Nissl-stained cells that had been reduced by malonate (see Figure 2), while reducing the high number of degenerating cells stained with FluoroJade-B (see Figure 2). In a similar way, the Sativex-like combination of phytocannabinoids attenuated the activation of glial elements caused by malonate (i.e., reactive microglia labeled with Iba-1, and astrogliosis labeled with GFAP; see Figure 3), which was particularly abundant and generalized along the whole striatal parenchyma in this experimental model. Lastly, the Sativex-like combination of phytocannabinoids attenuated the malonate-induced up-regulatory responses in iNOS (already described in ref 16) and IGF-1 gene expression in the striatal parenchyma (see Figure 4), whereas it partially recovered the malonate-induced reduction in $CB_1$ receptors (see Figure 4). These findings support the notion that the combination of both phytocannabinoid-botanical extracts may be effective as a neuroprotective therapy in malonate-lesioned rats as it was previously found when individual cannabinoid agonists (i.e., HU-308) were used in the same HD model.[10] In this study,[10] however, we found that CBD administered alone was not active against malonate-induced striatal damage, but, according to the present data, its combination with $\Delta^9$-THC resulted in highly positive effects presumably by enhancing the activity of this last phytocannabinoid at the $CB_1$ and $CB_2$ receptors (see below). The rationale for such combination has been largely discussed in previous reviews.[24]

**Involvement of $CB_1$ and/or $CB_2$ Receptors in the Beneficial Effects of Phytocannabinoids on Malonate-Induced Striatal Damage.** In our previous study that used an experimental model of HD that primes oxidative injury and calpain activation as major causes of striatal damage, we detected protective effects of the Sativex-like combination of phytocannabinoids, that seemed to result from the antioxidant and cannabinoid receptor-independent properties of both phytocannabinoids rather than from any interaction with $CB_1$ and/or $CB_2$ receptors. We wanted to see if this was also the case in the experimental model of HD used in the present study. To this end, we conducted additional experiments with selective antagonists for $CB_1$ and $CB_2$ receptor types, that is, SR141716 and AM630, respectively, combined with the Sativex-like mixture of phytocannabinoids. The same or similar antagonists had been already tested after being administered alone in the same animal model of HD, showing no effect in the case of $CB_2$ receptor blockade[10] or even aggravating striatal damage in the case of SR141716.[25] In our present study, the blockade of $CB_1$ receptors with SR141716 or the blockade of $CB_2$ receptors with AM630 reversed the phytocannabinoid-induced decrease in the ability of malonate to attenuate Nissl-staining of cells ($F(5,23) = 6.11$, $p < 0.005$; see Figure 5). Interestingly, this reversal was no greater when both antagonists were administered together (see Figure 5). However, it is important to note that, although SR141716 and AM630, both separately and together, reversed the effects of the Sativex-like combination of $\Delta^9$-THC- and CBD-enriched botanical extracts, the lowest number of Nissl-stained cells was observed after the treatment with AM630, administered alone or in combination with SR141716. This finding, together with our previously published data showing that $CB_2$ receptors underwent an up-regulation in glial elements located in the striatal parenchyma after malonate,[10] whereas $CB_1$ receptors underwent a down-regulation in the same animal model in the present study (see Figure 4) and also in other models of HD,[8,16] suggests that even though $\Delta^9$-THC can activate both $CB_1$ and $CB_2$ receptors, the $CB_2$ receptor plays a greater role than the $CB_1$ receptor in the protection from malonate-induced striatal damage that the $\Delta^9$-THC- and CBD-enriched botanical extracts produce.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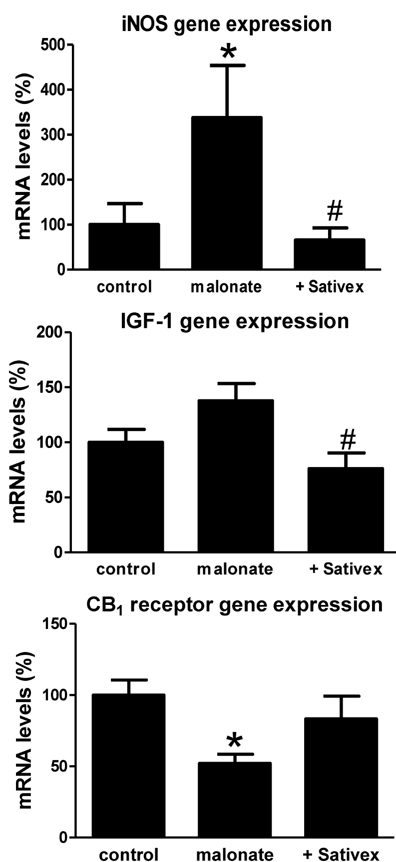


**Figure 4.** Gene expression for iNOS, IGF-1, and $CB_1$ receptors measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), or vehicle (Tween 80-saline). Details in the text. The 1:1 mixture $\Delta^9$-THC- and CBD-enriched botanical extracts is referred to as Sativex in this figure. Values correspond to percent over the contralateral nonlesioned side and are expressed as means ± SEM for 5−6 animals per group. Data were subjected to one-way analysis of variance followed by the Student−Newman−Keuls test ($^*p < 0.05$ compared with controls; $^\#p < 0.05$ compared with malonate + vehicle).

## CONCLUSION

Collectively, this study provides preclinical evidence in support of a beneficial effect of the cannabis-based medicine Sativex as a neuroprotective agent capable of delaying disease progression in a proinflammatory model of HD, which adds to previous data obtained in models priming oxidative mechanisms of striatal injury. However, the interest here is that, in contrast to these previous data, we have now obtained evidence that both $CB_1$ and $CB_2$ receptors are likely to be involved in Sativex effects, thus stressing the broad-spectrum properties of this medicine that may combine activity at the $CB_1$ and/or $CB_2$ receptors with cannabinoid receptor-independent actions. Moreover, our previous data showing that, in this proinflammatory model of HD, $CB_2$ receptors were up-regulated[10] whereas $CB_1$ receptors were down-regulated (also demonstrated here), as well as our observation that the protective effect of the Sativex-like combination of $\Delta^9$-THC- and CBD-enriched botanical extracts could be blocked by the selective $CB_2$ receptor antagonist AM630, suggest that $CB_2$ receptors play a particularly important role in this protective effect, a hypothesis that is also in concordance with the previous literature (see ref 26 for review). Lastly, given that HD is a disorder that is currently poorly managed in the clinic, the present data obtained with malonate-lesioned rats, together with previously published data obtained using an oxidative model of HD,[16] provide a strong justification for mounting clinical trials with cannabinoids that produce positive effects in these preclinical models of HD.

## MATERIALS AND METHODS

**Animals and Surgery.** Adult (12 week old; 350−400 g) male Sprague−Dawley rats (Harlan Ibérica, Barcelona, Spain) were used for the experiments. 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 86/609/EEC) and approved by the "Comité de Experimentación Animal" of our university. Rats were subjected to unilateral lesions of the striatum with the complex II inhibitor malonate, following a procedure described previously.[10] Rats were injected stereotaxically (coordinates: +0.8 mm anterior, +2.9 mm lateral from the bregma, −4.5 mm ventral from the dura mater) into the left striatum with 2 M malonate (dissolved in PBS 0.1 M, pH = 7.4) in a volume of 1 $\mu$L. The contralateral striatum of each animal remained unaffected, when data generated in this experimental model are expressed as percent of the lesioned side over the corresponding nonlesioned side.

**Treatments and Sampling.** We followed the same procedure used in previous studies with cannabinoids in malonate-lesioned rats.[10,25] Thus, 30 min before and 2 h after the intrastriatal injection of malonate, animals were treated with combinations of botanical extracts enriched with either $\Delta^9$-THC, kindly provided by GW Pharmaceuticals Ltd., Cambridgeshire, U.K. ($\Delta^9$-THC botanical extract contains 67.1% $\Delta^9$-THC, 0.3% CBD, 0.9% cannabigerol, 0.9% cannabichromene, and 1.9% other phytocannabinoids) or CBD, also provided by GW Pharmaceuticals Ltd., Cambridgeshire, U.K. (CBD botanical extract contains 64.8% CBD, 2.3% $\Delta^9$-THC, 1.1% cannabigerol, 3.0% cannabichromene, and 1.5% other phytocannabinoids). The total dose of cannabinoid administered was always 4.63 mg/kg (equivalent to 3 mg/kg of pure CBD + $\Delta^9$-THC), a dose within the range of effective doses of both compounds when they were administered in pure form in this and other experimental models of HD.[8−11] This was also the effective dose in our previous study with Sativex-like combination of phytocannabinoids in another HD model.[16] Cannabinoids were prepared in Tween 80-saline solution (1:16) and they were administered i.p. Malonate-lesioned rats administered with vehicle, as well as sham-operated animals, were also included in this experiment. In a further experiment, rats injected with malonate, following the same procedure described above, and also injected with the 1:1 combination of $\Delta^9$-THC- and CBD-enriched botanical extracts used in the previous experiment, were coadministered (10 min before each injection of Sativex-like combination) with the $CB_1$ receptor antagonist SR141716 (1 mg/kg), kindly provided by Sanofi-Aventis (Montpellier, France) or the $CB_2$ receptor blocker AM630 (1 mg/kg), purchased from Tocris (Biogen Científica, Madrid, Spain), both prepared in Tween 80-saline (1:16). In both experiments, animals were killed 48 h after the administration of malonate and their brains were rapidly removed, the two striata dissected and frozen separately in 2-methylbutane cooled in dry ice, and stored at −80 °C for subsequent qRT-PCR analysis. Animals to be used for histological analysis were transcardially perfused with saline followed by fresh 4% paraformaldehyde [in 0.1 M phosphate buffered-saline (PBS)], and their brains were collected and postfixed overnight at 4 °C, and then immersed in antifreeze solution and stored at −20 °C for Nissl and FluoroJade B staining and immunohistochemical analysis. In all experiments, at least 5−6 animals were used per experimental group.

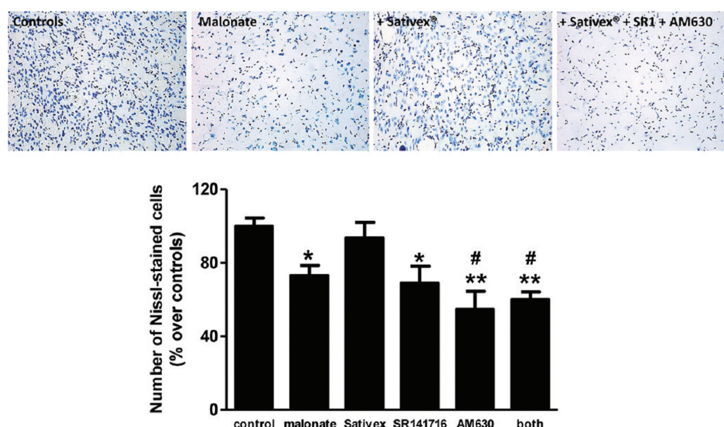


**Figure 5.** Nissl staining measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), selective antagonists for the $CB_1$ (SR141716) and/or the $CB_2$ (AM630) receptor, or vehicle (Tween 80-saline). Details in the text. The 1:1 mixture $\Delta^9$-THC- and CBD-enriched botanical extracts is referred to as Sativex in this Figure. Values correspond to percent over the control group and are expressed as means ± SEM for 5−6 animals per group. Data were subjected to one-way analysis of variance followed by the Student−Newman−Keuls test ($*p < 0.05$, $**p < 0.01$ compared with controls; $^{\#}p < 0.05$ compared with malonate + Sativex).

**In Vivo NMR Imaging Procedures.** In the first experiment consisting of injections of Sativex-like combinations of phytocannabinoids to malonate-lesioned rats, animals from the three experimental groups were used, at 24 h after malonate lesion and 24 h before animal death, for in vivo NMR imaging studies in which the volume of striatal edema was calculated. Experiments were performed at the Nuclear Magnetic Resonance Centre of Complutense University (Madrid, Spain) using a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany) operating at 4.7 T, equipped with a 12 cm actively shielded gradient system. Rats were anesthetized with oxygen:isoflurane and subsequently placed in prone position inside a cradle. The animal head was immobilized and placed underneath a 4 cm surface coil. A respiration sensor was used to control the animals. First global shimmer was assessed, and then three gradient-echo scout images in axial, sagittal, and coronal directions were acquired (TR/TE = 100/3.2 ms, matrix = 128 × 128). A 3D fast spin−echo experiment with axial slice orientation was subsequently performed using the following acquisition parameters: TR = 3000 ms, effective TE = 86.5 ms, NA = 2, FOV = 2.56 × 2.56 × 1.28 $cm^3$, matrix size = 256 × 128 × 32. The reconstructed matrix size was 256 × 256 × 32. The total time of the acquisition experiment was 27 min.

**Histological Analyses.** Coronal brain sections (25 μm thick) were obtained with a vibratome and collected as floating slices at the level of the caudate-putamen. They were used for Nissl (see details in ref 27) and FluoroJade B (see details in ref 10) staining, which permitted determination of the effects of particular treatments on cell number, and for immunohistochemical analysis of Iba-1, a marker of microglial cells, and GFAP, a marker of astrocytes. For immunohistochemistry, sections were incubated overnight at 30 °C with (i) monoclonal antirat Iba-1 antibody (Wako, Neuss, Germany) used at 1:300, or (ii) monoclonal antirat GFAP (Sigma-Aldrich, Madrid, Spain) used at 1:400. After incubation with the corresponding primary antibody, sections were washed in 0.1 M PBS and incubated for 2 h at 37 °C with a mouse highly cross-adsorbed AlexaFluor 488 secondary antibody (Invitrogen, Carlsbad, CA) for GFAP immunostaining, and with a rabbit biotinylated secondary antibody (Sigma-Aldrich, Madrid, Spain) followed by incubation for 2 additional hours with streptavidin 488 (Molecular Probes, Paisley, U.K.) for Iba-1 immunostaining. Both secondary antibodies were used at 1:200. 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 confocal microscope and a Nikon DXM 1200F camera were used for slide observation and photography, and all image processing, including cell counting, was done using ImageJ, the software developed and freely distributed by the U.S. National Institutes of Health (Bethesda, MD). For this purpose, multiple sections, selected from levels located approximately 200 μm from the middle of the lesion, were obtained from each brain and used to generate a mean value per subject.

**Real Time qRT-PCR Analysis.** Total RNA was isolated from striata using RNATidy reagent (AppliChem. Inc., Cheshire, CT). 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, whereas its integrity was confirmed in agarose geles. After genomic DNA was removed (to eliminate DNA contamination), single-stranded cDNA 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 RT-PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) to quantify mRNA levels for IGF-1 (ref Rn99999087_m1), iNOS (ref Rn00561646_m1), or $CB_1$ receptor (ref Rn00562880_m1), using β-actin expression (ref Rn00667869_m1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), and the threshold cycle was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA).

**Statistics.** Data were assessed by one-way ANOVA, followed by the Student−Newman−Keuls test.

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### Author Contributions

#Both authors have contributed equally to this work.

### Author Contributions

▽Both authors shared the senior authorship of this study.

### Author Contributions

O.S., R.G.P., and J.F.-R. designed the experiments included in this Article, which were conducted by S.V. and V.S. and supervised by O.S. and J.F.-R. J.F.-R. wrote the different versions of the manuscript, which were revised by the other

authors. All authors have approved the final version for submission.

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**Notes**

The authors declare the following competing financial interest(s):Authors have formal links with GW Pharmaceuticals that funds some of their research.

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## ■ ABBREVIATIONS

3-NP, 3-nitropropionate; CBD, cannabidiol; GFAP, glial fibrillary acidic protein; HD, Huntington's disease; IGF-1, insulin like growth factor-1; iNOS, inducible nitric oxide synthase; PBS, phosphate buffer saline; $\Delta^9$-THC, $\Delta^9$-tetrahydrocannabinol

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# Effects of a Sativex-Like Combination of Phytocannabinoids on Disease Progression in R6/2 Mice, an Experimental Model of Huntington's Disease

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**Abstract:** Several cannabinoids afforded neuroprotection in experimental models of Huntington's disease (HD). We investigated whether a 1:1 combination of botanical extracts enriched in either $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) or cannabidiol (CBD), which are the main constituents of the cannabis-based medicine Sativex®, is beneficial in R6/2 mice (a transgenic model of HD), as it was previously shown to have positive effects in neurotoxin-based models of HD. We recorded the progression of neurological deficits and the extent of striatal deterioration, using behavioral, in vivo imaging, and biochemical methods in R6/2 mice and their corresponding wild-type mice. The mice were daily treated, starting at 4 weeks after birth, with a Sativex-like combination of phytocannabinoids (equivalent to 3 mg/kg weight of pure CBD + $\Delta^9$-THC) or vehicle. R6/2 mice exhibited the characteristic deterioration in rotarod performance that initiated at 6 weeks and progressed up to 10 weeks, and elevated clasping behavior reflecting dystonia. Treatment with the Sativex-like combination of phytocannabinoids did not recover rotarod performance, but markedly attenuated clasping behavior. The in vivo positron emission tomography (PET) analysis of R6/2 animals at 10 weeks revealed a reduced metabolic activity in the basal ganglia, which was partially attenuated by treatment with the Sativex-like combination of phytocannabinoids. Proton nuclear magnetic resonance spectroscopy ($H^+$-MRS) analysis of the ex vivo striatum of R6/2 mice at 12 weeks revealed changes in various prognostic markers reflecting events typically found in HD patients and animal models, such as energy failure, mitochondrial dysfunction, and excitotoxicity. Some of these changes (taurine/creatine, taurine/*N*-acetylaspartate, and *N*-acetylaspartate/choline ratios) were completely reversed by treatment with the Sativex-like combination of phytocannabinoids. A Sativex-like combination of phytocannabinoids administered to R6/2 mice at the onset of motor symptoms produced certain benefits on the progression of striatal deterioration in these mice, which supports the interest of this cannabinoid-based medicine for the treatment of disease progression in HD patients.

**Keywords:** phytocannabinoids; cannabidiol; $\Delta^9$-tetrahydrocannabinol; Huntington's disease; R6/2 mice; basal ganglia; neuroprotection

## 1. Introduction

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by motor alterations (chorea followed by akinesia), but also cognitive dysfunction and psychiatric symptoms [1]. HD is primarily caused by a mutation in the huntingtin gene consisting of a CAG triplet repeat expansion translated into an abnormal polyglutamine tract in the N-terminus of this protein, which becomes toxic for striatal and cortical neuronal subpopulations [2]. The available pharmacotherapy to alleviate HD symptoms is poor (e.g., tetrabenazine for chorea), whereas there are no available treatments able to arrest/delay disease progression in HD. In recent years, several compounds (e.g., minocycline, coenzyme Q10, unsaturated fatty acids, inhibitors of histone deacetylases) have been investigated and produced encouraging effects in preclinical studies, but none of the findings obtained in these studies have yet led to the development of an effective medicine [3].

Continuing on from an extensive preclinical evaluation using different experimental models of HD, clinical tests are now being performed with cannabinoids [4]. Such preclinical evaluation demonstrated preservation of striatal neurons by several cannabinoid agonists against different cytotoxic stimuli that operate in HD pathogenesis [5,6]. These beneficial effects were exerted through multiple mechanisms of action, some of which involve the activation of $CB_1$ and/or $CB_2$ receptors and others of which do not. For example, cannabinoids with an antioxidant profile (i.e., $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), cannabidiol (CBD), or cannabigerol (CBG)) protected striatal neurons against oxidative injury caused by the mitochondrial complex II inhibitor 3-nitropropionic acid (3NP), and this occurred through effects that were $CB_1$/$CB_2$ receptor-independent [7–9]. Given its activity at the $CB_1$ and $CB_2$ receptors, $\Delta^9$-THC was also investigated with positive results in other experimental models, for example, R6/2 mice, a transgenic mouse model of HD [10]. The effects of $\Delta^9$-THC in these mice are likely produced through the activation of $CB_1$ receptors [10], but they could also involve the activation of $CB_2$ receptors, as selective agonists of this receptor type preserved striatal neurons in this genetic model [11] and also in malonate-lesioned rats [12], a model priming apoptotic death and glia-driven inflammation. Selective agonists of $CB_1$ [10,13] and $CB_2$ [11] receptors also preserved striatal neurons in in vitro or in vivo excitotoxic models, whereas other authors did not find any beneficial effect in R6/1 mice using $\Delta^9$-THC, the synthetic agonist HU-210, and the inhibitor of the endocannabinoid metabolism URB597 [14].

The data collected from these studies support the interest of evaluating cannabinoids as disease modifiers in HD in patients, but they also suggest that this should be done with a broad-spectrum cannabinoid. As an alternative, a combination of cannabinoids having complementary pharmacological effects would also be adequate. Such a profile can be found in Sativex, a cannabis-based medicine which has been licensed for alleviating specific symptoms (spasticity, pain) in patients affected by multiple sclerosis [15,16]. $\Delta^9$-THC present in Sativex may activate $CB_1$/$CB_2$ receptors and exert receptor-independent antioxidant effects, which would be markedly enhanced by CBD also present in Sativex [17]. A few years ago, we initiated experiments with the Sativex-like combination of these two phytocannabinoids in different animal models of HD. We used those models in which individual cannabinoids had been active [7,8,10–13]. We worked first with rats subjected to 3NP intoxication [18], in which calpain activation and oxidative injury are important cytotoxic mechanisms. In this model, the administration of pure $\Delta^9$-THC [7] or pure CBD [8] displayed neuroprotective properties. We found that, using the Sativex-like combination of these two phytocannabinoids, striatal neurons were also preserved against the 3NP intoxication and, again, we found that such a neuroprotective effect was $CB_1$/$CB_2$ receptor independent [18]. Next, we conducted similar studies in a second model of HD, rats unilaterally-lesioned with malonate,

in which activation of the apoptotic machinery and glial activation/inflammatory events were responsible for the striatal damage. We had previously described that selectively activating the $CB_2$ receptor resulted in a reduction of the striatal damage [12], whereas this was aggravated after $CB_1$ receptor blockade [19]. The Sativex-like combination of phytocannabinoids also preserved striatal neurons from death caused by malonate, and this effect was dependent on both $CB_1$ and $CB_2$ receptors [20].

These positive effects prompted us to extend our research to transgenic mouse models of HD, specifically the R6/2 mice, which are frequently used for the evaluation of potential neuroprotective compounds that warrant investigation at the clinical level. To this end, we subjected R6/2 mice to daily treatment with Sativex-like combination of phytocannabinoids (equivalent to 3 mg/kg weight of pure CBD + $\Delta^9$-THC) or with vehicle. Treatments commenced at 4 weeks and were prolonged up to 12 weeks after birth, the age at which animals were euthanized. The progression of neurological deficits (e.g., rotarod performance) was recorded during the treatment period, whereas additional motor markers (e.g., clasping behavior) were recorded just before animals were euthanized together with the in vivo imaging analysis of local metabolic activity using positron emission tomography (PET). Once euthanized, brains were collected and the striatum dissected and used for ex vivo proton magnetic resonance spectroscopy ($H^+$-MRS) analysis, which provides a series of detectable biomarkers reflecting: (i) oxidative damage (e.g., reduced glutation/creatine ratio (GSH/Cre)); (ii) energy failure (e.g., elevated lactate/*N*-acetyl-aspartate ratio (Lac/NAA), reduced NAA/choline ratio (NAA/Cho)); (iii) excitotoxicity (e.g., increased glutamate/NAA ratio (Glu/NAA)); and (iv) other events typically found in HD (e.g., taurine/Cre ratio (Tau/Cre), Tau/NAA ratio, all of them being relatively prognostic for brain integrity/damage) [21–26].

## 2. Results

### *2.1. Behavioral Analysis of R6/2 Mice Treated with the Sativex-Like Combination of Phytocannabinoids*

Despite the possibility of differences by gender in disease progression and treatment responses in R6/2 mice, our experiment was conducted exclusively in males to avoid a potential influence of ovarian steroid fluctuations when using females. Compared to wild-type animals, R6/2 mice exhibited a characteristic loss of weight gain that was initiated at 9 weeks of age and deteriorated at 10–11 weeks and up to 12 weeks of age when animals were euthanized (age: $F(8,200) = 279.1$, $p < 0.0001$; genotype/treatment: $F(2,200) = 4.497$, $p < 0.05$; interaction: $F(16,200) = 10.64$, $p < 0.0001$; Figure 1A). This loss of weight has been widely reported in R6/2 mice [9,10] and also in other transgenic models of HD [27,28]. In parallel, there was a deterioration in rotarod performance that was already evident at the age of 6 weeks after birth, then occurring before the loss of weight, and reaching its maximum at the last age analyzed (10 weeks after birth) (age: $F(5,139) = 21.57$, $p < 0.0001$; genotype/treatment: $F(2,139) = 187.3$, $p < 0.0001$; interaction: $F(10,139) = 15.30$, $p < 0.0001$; Figure 1B). The treatment with the Sativex-like combination of phytocannabinoids did not produce any recovery in both rotarod performance and weight gain (Figure 1A,B). By contrast, the Sativex-like combination of phytocannabinoids was highly effective in attenuating clasping behavior detected in R6/2 mice at the age of 10 weeks ($F(2,17) = 13.88$, $p < 0.0005$; <95% confidence intervals for wild-type mice, and R6/2 animals treated with vehicle or Sativex-like combination of phytocannabinoids were 0, 1.12. and 0.04, respectively; >95% confidence intervals were 0, 2.88, and 1.53, respectively; Figure 1C), a response that reflects dystonia and is absent in wild-type animals. The statistical relevance of these differences was confirmed using different post-hoc assays (Student-Newman-Keuls, Tukey, and Bonferroni), all reaching similar statistics.

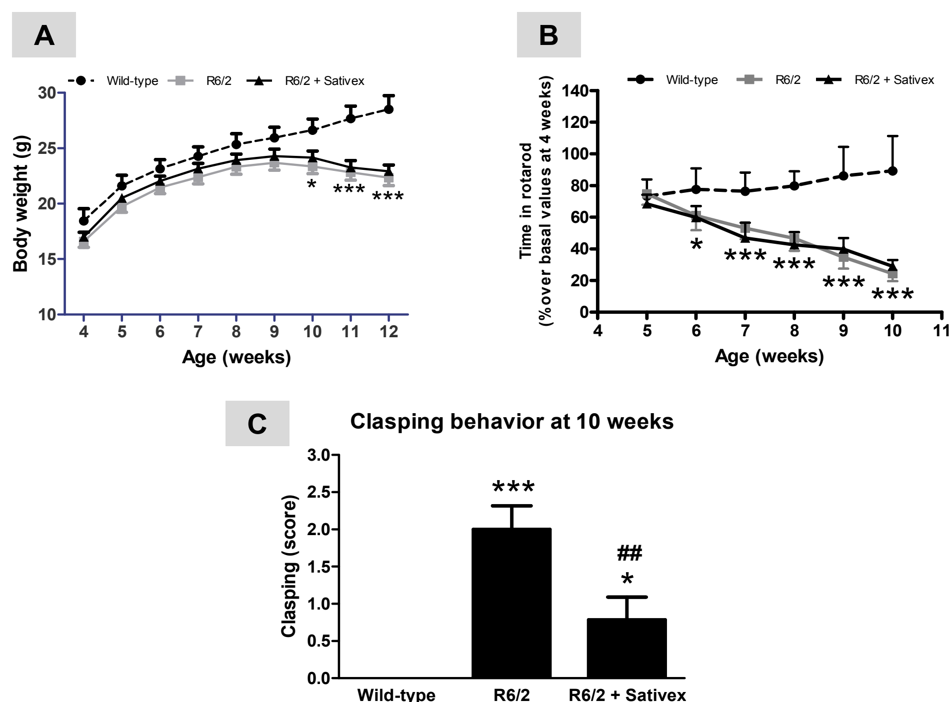


**Figure 1.** Weight gain (**A**), rotarod performance (**B**), and clasping behavior (**C**) in R6/2 mice treated from the age of 4 weeks after birth with $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC)- and cannabidiol (CBD)-enriched botanical extracts combined in a Sativex®-like ratio 1:1 (4.5 mg/kg equivalent to 3 mg/kg of pure CBD + $\Delta^9$-THC), or vehicle (Tween 80-saline), and the corresponding wild-type animals. Details in the text. Values are expressed as means ± SEM for six to eight animals per group. Data were subjected to one- (clasping) or two-way (rotarod and weight) analysis of variance followed by the Student-Newman-Keuls test (* $p < 0.05$, *** $p < 0.005$ compared with wild-type mice; ## $p < 0.01$ compared to R6/2 mice treated with vehicle).

### 2.2. *In Vivo Imaging Analysis of Regional Brain Metabolic Activity in R6/2 Mice Treated with the Sativex-Like Combination of Phytocannabinoids*

The PET analysis with [$^{18}$F]-fluoro-deoxy-glucose ([$^{18}$F]FDG) of R6/2 mice proved a reduced metabolic activity in the whole brain at 10 weeks ($F(2,14) = 4.609$, $p < 0.05$; Figure 2A). Such reduction was also evident in those brain areas more affected in R6/2 mice such as the caudate-putamen ($F(2,14) = 4.593$, $p < 0.05$; Figure 2B) and the globus pallidus ($F(2,14) = 4.794$, $p < 0.05$; Figure 2C), but it was also present in the cerebral cortex, cerebellum, amygdala, hypothalamus, and other forebrain regions (data not shown). In all cases, the reduction in regional metabolic activity in the R6/2 mice was attenuated by treatment with the Sativex-like combination of phytocannabinoids (Figure 2A–C). In the case of the whole brain, the difference between R6/2 mice treated with vehicle and treated with the Sativex-like combination of phytocannabinoids reached statistical significance (<95% confidence intervals for wild-type mice, and R6/2 animals treated with vehicle or Sativex-like combination of phytocannabinoids were 12.99, 10.01, and 10.79, respectively; >95% confidence intervals were 18.57, 12.80, and 19.12, respectively; Figure 2A), but in the case of the caudate-putamen and globus pallidus, the effect was evident only in the loss of statistical significance when compared to wild-type animals (caudate-putamen: <95% confidence intervals for wild-type mice, and R6/2 animals treated with vehicle or Sativex-like combination of phytocannabinoids were 13.95, 10.19, and 10.52, respectively; >95% confidence intervals were 20.56, 13.56, and 20.27, respectively; Figure 2B; globus pallidus: <95% confidence intervals for wild-type mice, and R6/2 animals treated with vehicle or Sativex-like combination of phytocannabinoids were 13.98, 10.48, and 10.86, respectively; >95% confidence intervals were 20.53, 13.51, and 20.14, respectively; Figure 2C). Effects similar to the caudate-putamen and the

globus pallidus by the treatment with the Sativex-like combination of phytocannabinoids were also evident in the cerebellum (data not shown), other Central Nervous System (CNS) structure that has been related to the control of motor activity. In other brain regions (e.g., septum nuclei, amygdala) the differences between R6/2 mice treated with vehicle or with the Sativex-like combination of phytocannabinoids were statistically significant (data not shown), as for the whole brain, but these regions are not related to the control of movement but to emotional and cognitive processes which, although also affected in HD patients, were not reproduced in R6/2 mice. Representative PET images for the three groups investigated are included in Figure 2D.

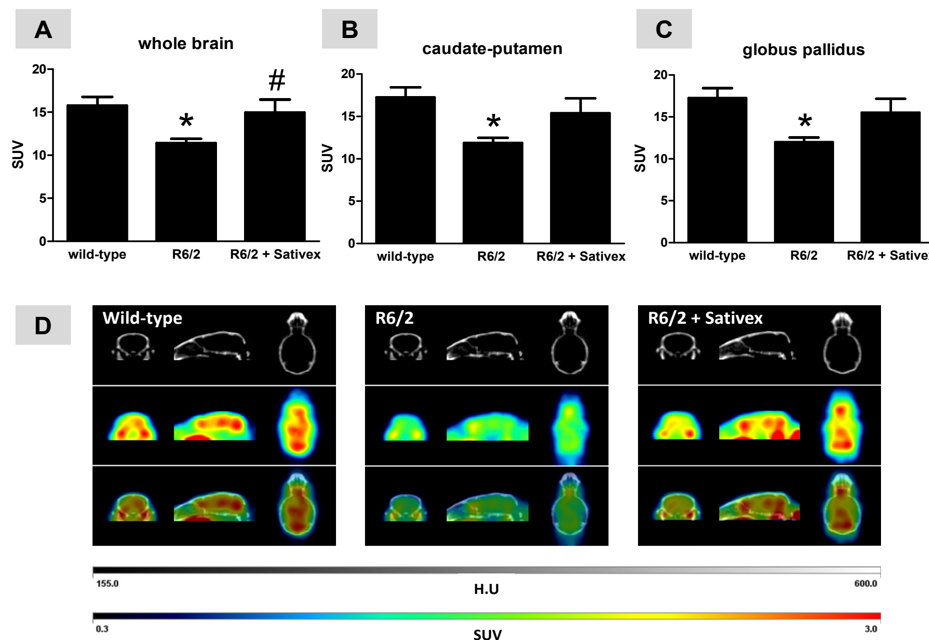


**Figure 2.** In vivo glucose metabolic rates measured by positron emission tomography (PET) analysis in the whole brain (**A**), caudate-putamen (**B**), and globus pallidus (**C**), including representative PET images in (**D**) of R6/2 mice (at 10 weeks after birth) treated from the age of 4 weeks after birth with $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex®-like ratio 1:1 (4.5 mg/kg equivalent to 3 mg/kg of pure CBD + $\Delta^9$-THC), or vehicle (Tween 80-saline), and the corresponding wild-type animals. Details in the text. Values correspond to standard uptake value (SUV) and are expressed as means ± SEM of five subjects per group and age. Data were subjected to one-way analysis of variance followed by the Student-Newman-Keuls test (* $p < 0.05$ compared with wild-type mice; # $p < 0.05$ compared to R6/2 mice treated with vehicle).

### *2.3. H⁺-MRS Analysis of Several Markers of Brain Integrity in R6/2 Mice Treated with the Sativex-Like Combination of Phytocannabinoids*

The $H^+$-MRS analysis of the post-mortem striatum of R6/2 animals at 10 weeks after birth demonstrated important changes in specific markers of HD pathology. For example, we found an elevation in Lac/NAA ratio ($F(2,14) = 10.99$, $p < 0.005$; Figure 3A), which is an indirect marker of a possible energy deficit and mitochondrial dysfunction due to elevated Lac generation, a result also found by other authors [24,25], and/or reduced NAA levels possibly reflecting neuronal dysfunction/damage [24]. Lowered NAA levels also may reflect mitochondrial dysfunction, as this metabolite is synthesized in mitochondria [24]. This elevation tended to be reduced, although modestly (without reaching statistical significance) by the treatment with the Sativex-like combination of phytocannabinoids (Figure 3A). However, this treatment completely recovered the reduction in NAA/Cho ratio found in R6/2 mice ($F(2,12) = 11.62$, $p < 0.005$; (<95% confidence intervals for wild-type mice, and R6/2 animals treated with vehicle or Sativex-like combination

of phytocannabinoids were 0.84, 0.55, and 0.88, respectively; >95% confidence intervals were 1.16, 0.72, and 1.26, respectively; Figure 3B), which is also a potential predictive marker of energy failure [24,25]. We also found an elevation in Glu/NAA ratio (F(2,14) = 24.81, $p < 0.0001$), which potentially reflects excitotoxicity [21–23,26], but this response was not altered by the treatment with the Sativex-like combination of phytocannabinoids (Figure 3C). The $H^+$-MRS analysis also revealed elevated Tau/Cre (F(2,14) = 4.572, $p < 0.05$; Figure 3D) and Tau/NAA (F(2,14) = 30.08, $p < 0.0001$; Figure 3E) ratios in R6/2 mice. Elevated Tau levels—presumably reflecting an endogenous protective response, as well as changes in cell volume regulation, and being frequently associated with lower Cre concentrations potentially reflecting reduced energy availability—have been found in HD patients using metabolomic analysis [29] and in other mouse models of HD [26]. These changes were attenuated by the treatment with the Sativex-like combination of phytocannabinoids, in particular for Tau/NAA ratio (<95% confidence intervals for wild-type mice, and R6/2 animals treated with vehicle or Sativex-like combination of phytocannabinoids were 0.76, 2.00, and 0.94, respectively; >95% confidence intervals were 1.24, 2.34, and 1.81, respectively; Figure 3E) and, to a lesser extent (only losing statistical significance with respect to wild-type mice), for Tau/Cre ratio (<95% confidence intervals for wild-type mice, and R6/2 animals treated with vehicle or Sativex-like combination of phytocannabinoids were 0.70, 1.28, and 0.93, respectively; >95% confidence intervals were 1.30, 1.37, and 1.35, respectively; Figure 3D). No changes were noted in GSH/Cre ratio (F(2,14) = 1.583, ns), potentially reflecting oxidative damage [30], in R6/2 mice compared to wild-type animals, which agrees with the poor oxidative stress response described in these mice [9]; treatment with the Sativex-like combination of phytocannabinoids did not modify this ratio (Figure 3F).

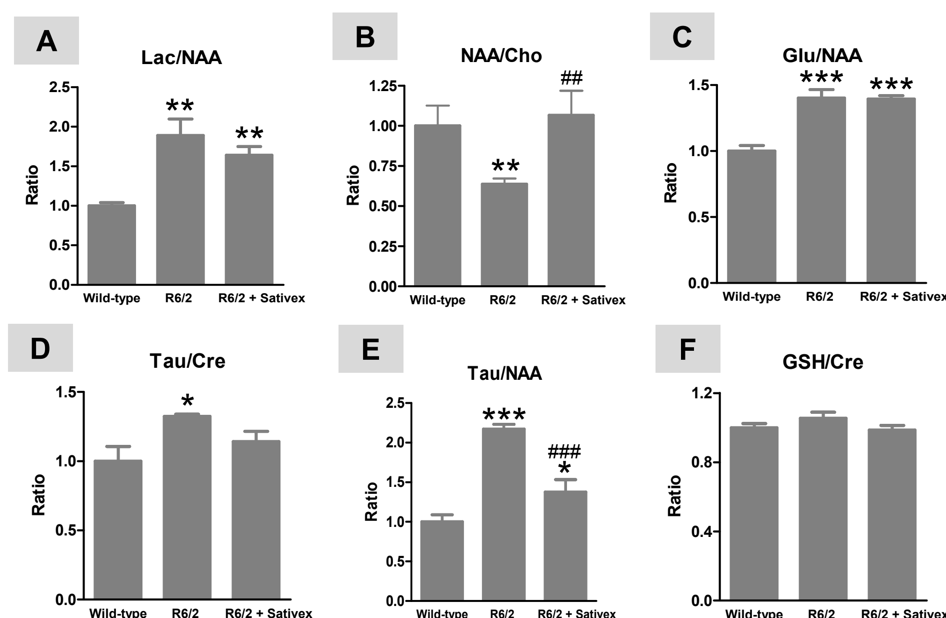


**Figure 3.** Lactate/*N*-acetyl-aspartate (Lac/NAA) (**A**); NAA/ choline (Cho) (**B**); glutamate (Glu)/NAA (**C**); Tau/Cre (**D**); Tau/NAA (taurine/creatine) (**E**); and (reduced glutation) GSH/Cre (**F**) ratios measured by proton nuclear magnetic resonance spectroscopy ($H^+$-MRS) analysis in the striatum of R6/2 mice (at 12 weeks after birth) treated from the age of 4 weeks after birth with $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex®-like ratio 1:1 (4.5 mg/kg equivalent to 3 mg/kg of pure CBD + $\Delta^9$-THC), or vehicle (Tween 80-saline), and the corresponding wild-type animals. Details in the text. Values are expressed as means ± SEM of five animals per group. Data were subjected to one-way analysis of variance followed by the Student-Newman-Keuls test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared with wild-type mice; ## $p < 0.01$, ### $p < 0.005$ compared to R6/2 mice treated with vehicle).

## 3. Discussion

Only a few clinical studies have been performed to determine whether cannabinoid compounds have beneficial effects in HD [31–34]. These clinical studies concentrated more on HD symptoms (e.g., choreic movements, behavioral disturbances) rather than on disease progression. Recent animal studies, however, have demonstrated that using a broad-spectrum cannabinoid or, alternatively, combinations of cannabinoids having complementary profiles, it is possible to delay the progression of the disease and to preserve the integrity of striatal neurons, and this has been found in different animal models of HD [8–13]. Such observations demand new clinical studies directed at testing in patients these disease-modifying properties demonstrated by certain cannabinoids. To provide more support to this idea, we recently demonstrated neuroprotective effects, using the Sativex-like combination of $\Delta^9$-THC and CBD botanical extracts, in rats subjected to 3NP intoxication [18] and also in malonate-lesioned rats [20], two classic experimental in vivo models of HD in which striatal damage is caused by different primary mechanisms (e.g., calpain activation/oxidative injury, apoptosis/gial activation/inflammation, respectively).

In the present study, we used R6/2 mice, a genetic model which is frequently used in the preclinical evaluation of novel therapies for HD because of the rapid disease progression of these animals, and in which different cannabinoids administered individually, including $\Delta^9$-THC but not CBD, were shown to be active [10,11]. Our results suggest certain benefits with the Sativex-like combination of phytocannabinoids in R6/2 mice in line with those observed in neurotoxin-based models [18,20]. In our hands, the mixture of both phytocannabinoids attenuated clasping behavior, which represents a classic motor symptom found in these mice, although it was inactive against the progressive worsening in rotarod performance. This lack of Sativex benefits in mouse rotarod performance was relatively surprising, as we have previously found that this parameter, being markedly reduced in R6/2 mice, was attenuated by the treatment with pure $\Delta^9$-THC [10]. In our opinion, it is not a problem of insufficient dose, as we have preliminary and unpublished data indicating that higher doses (5 and 10 mg/kg) for the Sativex-like combination of phytocannabinoids led to the same lack of improvement. We do not consider that this improvement may be elicited by increasing the number of subjects in both experimental groups, as they were relatively homogeneous with their progressing lines for the rotarod performance showing a high overlapping. In order to find an explanation it is better to look at other studies that have also described no improvement in rotarod performance but beneficial effects in other behavioral parameters and recovery at the neuropathological level. This was the case of our previous study with the phytocannabinoid CBG, which proved a poor and statistically non-significant recovery in the worsened rotarod response typical of R6/2 mice, but it produced a much more marked reduction in the presence of mutant huntingtin aggregates as well as an increase in different prosurvival factors (e.g., neurotrophins) [9]. Similar findings derive from a study in R6/1 mice, a similar genetic HD model, although with a less aggressive phenotype due to a shorter CAG repeat length in the transgene, in which authors were unable to find rotarod improvements with $\Delta^9$-THC, despite some benefits found in other behavioral parameters such as motor responses in exploratory tests [14].

The benefits we found in clasping behavior with the Sativex-like combination of phytocannabinoids had a biochemical correlate in the improvement of metabolic activity recorded in the basal ganglia using in vivo imaging PET analysis, as well as in different $H^+$-MRS indices, potentially reflecting energy failure and neuronal deterioration. It is true that we did not observe any improvement in excitotoxicity and oxidative stress markers measured with $H^+$-MRS with the Sativex-like combination of phytocannabinoids, despite the potential of this combination against both processes. This can be possibly explained by the fact that excitotoxic damage and, in particular, oxidative stress are not particularly relevant cytotoxic processes in R6/2 mice, as found here and as has been described before in other studies [9,35]. On the other hand, it would have been interesting to have data of animal survival after the treatment with the Sativex-like combination of phytocannabinoids, but this was not possible once the animals were euthanized for collecting tissues for $H^+$-MRS analysis.

Collectively, the improvement in some behavioral and neurochemical parameters that have been found here in R6/2 mice after the Sativex-like combination of phytocannabinoids, together with the data obtained in neurotoxin-based models [18,20], fueled the interest in the clinical evaluation of Sativex as a disease-modifying therapy in HD patients. In fact, such clinical evaluation was initiated before our present experiment in R6/2 mice was concluded, and it was derived from the collaboration of a group of Spanish neurologists, GW Pharmaceuticals (the British pharma company that developed Sativex), and various groups of basic researchers including us. The clinical study was developed in a small population of HD patients (all of them early symptomatic), designed as a crossover trial with two different treatment patterns: (i) placebo (12 weeks), washout period (4–6 weeks), and Sativex (12 weeks); and (ii) Sativex (12 weeks), washout period (4–6 weeks) and placebo (12 weeks). The dosing of Sativex®/placebo was 12 sprays/day. The primary endpoint was safety of Sativex in HD patients, whereas the secondary endpoint was to obtain any evidence of slower disease progression in the patients during the active treatment phases. We observed that Sativex, in concordance with previous data obtained in control subjects, patients of other pathologies, and laboratory animals (reviewed in [36]), was safe in HD patients. However, we were unable to detect any evidence of slower disease progression [4]. It is possible that the efficacy of Sativex constituents needs periods of treatments longer than the relatively short 12 weeks used in this clinical trial. A similar situation happened with creatine, which was also a very active neuroprotective compound in preclinical studies in HD [37,38], but failed when passed to clinical validation [39]. Authors concluded that the duration of the study was the critical factor as one year, a treatment period longer than in our study with Sativex [4], was considered not sufficiently long to have clinically detectable impact in HD patients [39]. With the aim of progressing in this direction, we are presently designing a novel initiative that will attempt to recruit a higher population of HD patients, to involve different hospitals in Europe, and to work with more than one Sativex dosing regimen and longer treatment periods.

## 4. Materials and Methods

### *4.1. Animals*

R6/2 and wild-type mice, produced from initial breeders obtained from Jax (Jackson Laboratories, Bar Harbor, ME, USA), were housed in rooms with controlled photoperiod (08:00–20:00 light) and temperature (22 ± 1 °C) and with free access to standard food and water. Offspring were genotyped for the transgene containing the mutated huntingtin following a procedure described previously [10]. Male R6/2 (160 ± 5 CAG repeats) and wild-type animals were used at the age of 4 weeks to record disease progression and basal ganglia deterioration in pharmacological studies using a Sativex-like combination of phytocannabinoids. All experiments were conducted according to local and European rules (directive 86/609/EEC) and approved by the "Comité de Experimentación Animal" of our university (CEA-UCM 56/2012; 8 March 2012).

### *4.2. Treatments and Sampling*

Animals included in our pharmacological studies were treated with the 1:1 combination of botanical extracts enriched with either $\Delta^9$-THC, kindly provided by GW Research Ltd., Cambridge, UK ($\Delta^9$-THC botanical extract contained 69.6% $\Delta^9$-THC, 0.3% CBD, 0.9% CBG, 0.9% cannabichromene, and 1.9% other phytocannabinoids) or CBD, also provided by GW Research Ltd., Cambridge, UK (CBD botanical extract contained 64.3% CBD, 2.3% $\Delta^9$-THC, 1.1% CBG, 3.0% cannabichromene, and 1.5% other phytocannabinoids). The total dose of cannabinoid administered was always 4.5 mg/kg (equivalent to 3 mg/kg of pure CBD + $\Delta^9$-THC), a dose within the range of effective doses of both compounds when they were administered in pure form in this and other experimental models of HD [10–13], and also close to the clinical uses of Sativex [4,36]. This was also close to the doses used in our previous studies with Sativex-like combination of phytocannabinoids in other HD models, although these studies used rats instead mice [18,20]. Cannabinoids were prepared in Tween 80-saline

solution (1:16) and they were administered intraperitoneally (i.p.). R6/2 mice administered with vehicle, as well as counterpart wild-type animals, were also included in this experiment. To include an additional group of wild-type mice treated with Sativex would have been desirable. However, due to the need to consider the new 3R recommendations on the use of experimental animals and given that previous studies have reported no relevant differences between wild-type animals treated with vehicle or with different cannabinoids [9–11], such a group was not included in our present study. The treatment was initiated when animals were 4 weeks old (presymptomatic) and was repeated every day up to the age of 12 weeks, the age at which the animals were euthanized after behavioral (rotarod test analyzed weekly, and clasping analyzed at 10 weeks) and in vivo PET imaging (at 10 weeks) analysis. Their brains were rapidly removed and the two striata dissected and stored immediately at −80 °C for subsequent $H^+$-MRS analysis. It would have been interesting to detect any possible benefit of the Sativex-like combination of phytocannabinoids on animal survival, but this would have demanded a separate experiment, which was not approved due to ethical concerns. In all experiments, at least six animals were used per experimental group.

### *4.3. Behavioral Recording*

All behavioral tests were conducted prior to drug injections to avoid acute effects of the compounds under investigation, and they were carried out by researchers blinded to the treatment in each animal.

#### 4.3.1. Rotarod Test

We used a LE8200 device (Panlab, Barcelona, Spain), with acceleration from 4 to 40 rpm. over a period of 600 s. After a period of acclimation and training (first session: 0 rpm for 30 s; second and third sessions: 4 rpm for 60 s, with periods of 10 min between sessions) mice were tested on one day every week from week 4 of age, for four consecutive trials, with a rest period of approximately 20 min between trials. The average of the last three trials per day was used for the statistical analysis.

#### 4.3.2. Hindlimb Clasping

Mice were suspended by the tail, so that their body dangled in the air facing downward. Hindlimb position was observed with the mice lifted by the tail for 30 s and animals were scored according to the following scale: (i) score = 0 if the hindlimbs are consistently splayed outward, away from the abdomen; (ii) score = 1 if one hindlimb is retracted toward the abdomen; (iii) score = 2 if both hindlimbs are partially retracted toward the abdomen; and (iv) score = 3 if both hindlimbs are entirely retracted and touching the abdomen.

### *4.4. In Vivo Analysis of Glucose Metabolism: [$^{18}$F]FDG PET Imaging*

To evaluate the regional brain metabolic activity, PET with the radiotracer [$^{18}$F]-fluoro-deoxy-glucose ([$^{18}$F]FDG) was performed. Briefly, mice were fasted, to minimize the influence of glycemia, for at least 12 h previous to be i.p. injected with approximately 11.1 MBq of [$^{18}$F]FDG (Instituto Tecnológico PET, Madrid, Spain). After an uptake period of 45 min, mice were scanned with a small-animal hybrid PET/CT (computed tomography) device (Albira ARS scanner, Oncovision, Valencia, Spain) under 2% isoflurane anesthesia. PET acquisition time was 30 min and it was immediately followed by CT scanning. After reconstruction of the PET and CT images, these were co-registered to a magnetic resonance image (MRI) template for the mouse brain, in which the brain regions were delineated. To this aim, the CT image was first co-registered to the MRI template and the mathematical transformation was saved. Then, this transformation was applied to its own fused PET image. This step allows the right matching of the PET image with the mouse brain MRI template. All these steps were carried out using PMOD 3.0 software (PMOD Technologies Ltd., Zurich, Switzerland). As an index of regional metabolic activity, we used the standard uptake value (SUV), which is currently the most used quantification index using [$^{18}$F]FDG PET imaging

for small animals [40]. It represents the ratio of the regional radioactivity concentration measured by the PET scanner and the actual injected dose (corrected for radiotracer decay at the time of the injection) divided by the animal weight.

### *4.5. Proton Magnetic Resonance Spectroscopy ($H^+$-MRS)*

Ex vivo $H^+$-MRS analysis has been demonstrated to provide metabolic information with higher sensitivity and spectral resolution than in vivo magnetic resonance spectroscopy [21]. $H^+$-MRS was performed in the MRI Unit of the Instituto Pluridisciplinar (Universidad Complutense, Madrid, Spain) using a Bruker AMX500 spectrometer and following previously described procedures [21,22]. The spectra generated were analyzed for Lac, NAA, Glu, GSH, Cre, Tau, and Cho, and several ratios were calculated. They are relatively predictive of excitotoxicity (Glu/NAA), energy deficits (Lac/NAA, NAA/Cho), oxidative damage (GSH/Cre), and other HD-acting neurotoxic events (Tau/Cre, Tau/NAA), all being prognostic for brain integrity [21–26].

### *4.6. Statistics*

Data were assessed by one- or two-way ANOVA, followed by the Student-Newman-Keuls, Bonferroni, or Tukey tests as post-hoc assays, as required, using the GraphPad software (version 5.0, GraphPad Software, Inc., La Jolla, CA, USA).

## 5. Conclusions

In summary, in line with the promising prospects generated by its beneficial effects in neurotoxin-based models of HD, this study demonstrated that the Sativex-like combination of phytocannabinoids improved some behavioral and neurochemical parameters, but not all, related to the progression of striatal deterioration in R6/2 mice. This provides more evidence in support of its potential for developing a future disease modifying therapy for HD patients, despite the recently failed clinical evaluation of Sativex in a small population of patients. Higher dosage and longer treatments periods may be important to reveal such potentialities in patients.

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**Author Contributions:** Study design: Onintza Sagredo, Miguel A. Pozo, and Javier Fernández-Ruiz; Sample analysis: Sara Valdeolivas, Onintza Sagredo, and Mercedes Delgado (PET analysis); Data interpretation (including statistical assessment): Sara Valdeolivas, Onintza Sagredo, and Javier Fernández-Ruiz; Manuscript draft: Javier Fernández-Ruiz (revised, corrected, and approved by all authors).

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ORIGINAL ARTICLE

# Neuroprotective Properties of Cannabigerol in Huntington's Disease: Studies in R6/2 Mice and 3-Nitropropionate-lesioned Mice

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**Abstract** Different plant-derived and synthetic cannabinoids have shown to be neuroprotective in experimental models of Huntington's disease (HD) through cannabinoid receptor-dependent and/or independent mechanisms. Herein, we studied the effects of cannabigerol (CBG), a nonpsychotropic phytocannabinoid, in 2 different *in vivo* models of HD. CBG was extremely active as neuroprotectant in mice intoxicated with 3-nitropropionate (3NP), improving motor deficits and preserving striatal neurons against 3NP toxicity. In addition, CBG attenuated the reactive microgliosis and the upregulation of proinflammatory markers induced by 3NP, and improved the levels of antioxidant defenses that were also significantly reduced by 3NP. We also investigated the neuroprotective properties of CBG in R6/2 mice. Treatment with this phytocannabinoid produced a much lower, but significant, recovery in the deteriorated rotarod performance typical of R6/2 mice. Using HD array analysis, we were able to identify a series of genes linked to this disease (e.g., symplekin, Sin3a, Rcor1, histone deacetylase 2, huntingtin-associated protein 1, δ subunit of the gamma-aminobutyric acid-A receptor (GABA-A), and hippocalcin), whose expression was altered in R6/2 mice but partially normalized by CBG treatment. We also observed a modest improvement in the gene expression for brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), and peroxisome proliferator-activated receptor-γ (PPARγ), which is altered in these mice, as well as a small, but significant, reduction in the aggregation of mutant huntingtin in the striatal parenchyma in CBG-treated animals. In conclusion, our results open new research avenues for the use of CBG, alone or in combination with other phytocannabinoids or therapies, for the treatment of neurodegenerative diseases such as HD.

**Key Words** Phytocannabinoids · cannabigerol · Huntington's disease · inflammation · neuroprotection

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## Introduction

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by motor abnormalities, cognitive dysfunction, and psychiatric symptoms [1]. The primary cause of the disease is a mutation in the huntingtin gene, which consists of a CAG triplet repeat expansion translated into an abnormal polyglutamine tract in the amino-terminal portion of this protein that becomes toxic for striatal and cortical neuronal subpopulations [2]. At present, there is no specific pharmacotherapy to alleviate motor and cognitive symptoms and/or to arrest/delay disease progression in HD. Thus, even though a

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few compounds have produced encouraging results in preclinical studies (i.e., unsaturated fatty acids, inhibitors of histone deacetylases, coenzyme Q10, minocycline), none of the findings obtained in these studies have yet led to the development of an effective medicine [3]. Importantly, following on from an extensive preclinical evaluation using different experimental models of HD (reviewed in [4] and [5]), clinical tests are now being performed with cannabinoids. Using experimental models that reproduce different cytotoxic stimuli that operate in HD pathogenesis, for example rodents treated with mitochondrial toxins (e.g., inhibitors of mitochondrial complex II), quinolinate-lesioned mice, or transgenic mice bearing mutated forms of human huntingtin, preclinical studies with cannabinoids demonstrated preservation of striatal neurons [6–11]. The beneficial effects of cannabinoids were exerted through multiple mechanisms of action, including cannabinoid receptor type 1 ($CB_1$) activation (e.g., excitotoxic models [8, 11]), cannabinoid receptor type 2 ($CB_2$) activation (e.g., inflammatory models [10]), and $CB_1$/$CB_2$-independent mechanisms. For example, cannabinoid receptor-independent effects were induced by compounds with antioxidant profiles, such as $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and cannabidiol (CBD), and were particularly evident against the toxicity caused by the mitochondrial complex II inhibitor 3-nitropropionic acid (3NP), which primes oxidative injury [6, 7]. The combination of botanical extracts enriched in these 2 phytocannabinoids, similar to the formulation of the already approved cannabis-based medicine, Sativex® (GW Pharmaceuticals, Salisbury, UK) [12, 13], also preserved striatal neurons in rats subjected to 3NP intoxication [14], a model priming, as mentioned above, calpain activation and oxidative injury as major cytotoxic mechanisms and in which, as mentioned above, both $\Delta^9$-THC [6] and CBD [7], administered separately, have already been found to display neuroprotective properties. These effects of the Sativex®-like combination of phytocannabinoids were also, as expected, independent of $CB_1$ and $CB_2$ receptors [14], and attributed to the inherent antioxidant properties of both phytocannabinoids that may depend on their particular chemical structure (reviewed in [15]). In addition, it has been suggested that $\Delta^9$-THC, and especially CBD, may exert its neuroprotective effects by regulating the activity of inducible transcription factors such as nuclear factor kappa B (NF-κB), nuclear factor erythroid 2-related factor 2 (Nrf-2), and peroxisome proliferator-activated receptor (PPARγ) [16].

Cannabigerol (CBG) is another major phytocannabinoid having a pharmacological profile relatively similar to $\Delta^9$-THC and CBD in relation to its CB-independent activities. CBG is nonpsychotropic and does not bind or activate $CB_1$ or $CB_2$ [17, 18]. However, CBG is antioxidant [17, 18], as well as anti-inflammatory, as it inhibits lipopolysaccharide-induced (LPS) release of proinflammatory cytokines and prostaglandin E2 in primary microglial cells [19], activates PPARγ [19], and attenuates murine colitis induced by intracolonic administration of dinitrobenzene sulfonic acid [20]. CBG also targets α-2 adrenergic receptors [21], and serves as an antagonist of serotonin 5-hydroxytryptamine 1A receptors ($5HT_{1A}$) [21]. CBG has not been studied in HD but its pharmacological profile (e.g., antioxidant and activator of PPARγ) presents certain interest as a potential disease-modifying agent in this disorder. This possibility prompted us to extend our preclinical work with classic phytocannabinoids in HD to CBG using first a neurotoxin-based model, 3NP-lesioned mice. This is a model in which the death of striatal neurons is predominantly associated with the occurrence of mitochondrial dysfunction, calpain activation, and oxidative stress [14, 22]; therefore, a model in which CBG may be beneficial owing to its antioxidant and anti-inflammatory activities. Moreover, we also wanted to study CBG in R6/2 mice, a transgenic model of HD that reproduces a very aggressive pathological phenotype and that is frequently used in preclinical evaluation of potential neuroprotective compounds. Cannabinoids other than CBG are particularly active in this murine model [8, 9]. To this end, we subjected 3NP-lesioned or R6/2 mice, and their respective controls, in two separate experiments to daily treatments with CBG at a dose of 10 mg/kg. In the case of 3NP-lesioned mice, the treatments were prolonged for 4 days before the animals were euthanized and their brains collected for further analysis. In the case of R6/2 mice, treatments began at 4 weeks and were prolonged up to 10 weeks after birth, the age at which animals were also euthanized and their brains collected for further analysis. The progression of neurological deficits (e.g., failed rotarod performance, altered locomotor activity, clasping, dystonia) was recorded before (only in R6/2 mice) and/or during (in both experiments) the treatment period, whereas for the extent of the striatal damage, the brains were analyzed using different histological parameters [e.g., Nissl staining; NeuN or dopamine- and cyclic adenosine monophosphate-regulated phosphoprotein, Mr 32 kDA (DARPP-32) immunostaining; reactive microgliosis determined with Iba-1 immunohistochemistry; astrogliosis labelled with glial fibrillary acidic protein (GFAP) immunostaining], and the expression of various biochemical markers related to the endocannabinoid signaling system [e.g., $CB_1$ and $CB_2$, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) enzymes] or that have been found previously to be altered in different HD models [e.g., the neurotrophins brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1), the glutamate transporters GLT-1 and glutamate aspartate transporter (GLAST), DARPP-32, some antioxidant enzymes, and proinflammatory enzymes and cytokines] [2, 8–10, 14, 23]. The striatal samples from both experiments were also analyzed with a specific HD polymerase chain reaction (PCR) array system. We performed an additional experiment conducted with CBG in striatal progenitor cell lines $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ expressing

endogenous wild-type and mutant huntingtin, respectively, which was aimed at determining the effects of this phytocannabinoid on PPARγ receptors (see Supplementary Information).

## Materials and Methods

### Animals and Treatments

All animals used in this study were housed in a room with a 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 (European Union directive 86/609/EEC) and approved by the respective animal research ethic committees of our universities (Universidad de Córdoba and Universidad Complutense). To induce lesions of the striatum, 16-week-old C57BL/6 male mice (Harlan Ibérica, Barcelona, Spain) were subjected to seven intraperitoneal (i.p.) injections of 50 mg/kg 3NP [Sigma-Aldrich, St. Louis, MO, USA; one injection each every 12 h prepared in phosphate-buffered saline (PBS)]. 3NP-treated animals and their respective nonlesioned controls (injected with PBS) were used for pharmacological studies with CBG (THC Pharm GmbH, Frankfurt, Germany). Treatments consisted of 4 i.p. injections every 24 h with CBG at a dose of 10 mg/kg, a dose within the range of effective doses for phytocannabinoids in HD and other disorders when they were administered in pure form [8–11, 14] or vehicle (0.2 % dimethyl sulfoxide plus 5 % bovine serum albumin in PBS), with the first and the last injections 30 min before the first and the last injections of 3NP, respectively. All animals were euthanized 12 h after the last injection of 3NP. In a second experimental approach, we used a colony of R6/2 and wild-type mice generated from initial breeders obtained from Jackson Laboratories (Bar Harbor, ME, USA; code: B6CBA-Tg(HDexon1)62Gbp/1 J; 160±5 repeat expansions), which is presently available in our animal facilities. The colony was maintained by back-crossing R6/2 males with B6CBAF1/J females. Animals were subjected to genotyping to confirm the presence of the transgene with the mutated huntingtin (see details in [8] and [9]). Male R6/2 and wild-type animals were used at the age of 4 weeks after birth, an age at which motor symptoms have not appeared yet [8, 9]. Mice were treated daily with i.p. injections of CBG (10 mg/kg) or vehicle (Tween 80-saline, 1:16) up to the age of 10 weeks, at which point the animals were euthanized (always 24 ho after the last injection of CBG).

### Sampling

Once euthanized, mice were dissected and their brains removed. The right hemisphere was used to dissect the striatum, which was frozen in 2-methylbutane cooled in dry ice and stored at −80 °C for biochemical analyses [quantitative reverse transcription (qRT)-PCR, enzyme activities, array system]. The left hemisphere was fixed in fresh 4 % paraformaldehyde (in 0.1 M PBS) for 36 h at 4 °C, followed by cryoprotection in 30 % sucrose and storage at −80 °C for staining and immunohistochemical analysis. In all experiments, at least 6–8 animals were used per experimental group.

### Evaluation of the Neurological Deterioration

Mice were subjected to different behavioral tests for determining their neurological status. In R6/2 mice, we evaluated motor coordination in the RotaRod test, using a LE8200 device (Harvard Apparatus, Barcelona, Spain), with acceleration from 4 to 40 r.p.m. over a period of 600 s. Mice were tested on 1 day every week from week 4 of age, for 4 consecutive trials, with a rest period of approximately 20 min between trials. Data from the first trial were not used in the statistical analyses. The first RotaRod performance, at week 4, was established as basal performance. In 3NP-lesioned mice, we evaluated the motor activity (ambulation in an automated actimeter), the hindlimb clasping and dystonia, and the truncal dystonia, following previously-described procedures [24]. All behavioral tests were conducted prior to drug injections to avoid acute effects of the compounds under investigation.

### Real-time qRT-PCR Analysis

Total RNA was isolated from striata using either SurePrep RNA/Protein Purification Kit (Fisher Bioreagents, Madrid, Spain) or RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). 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. After genomic DNA was removed (to eliminate DNA contamination), single-stranded complementary DNA was synthesized from up to 1 μg of total RNA using Rneasy Mini Quantitect Reverse Transcription (Qiagen) and the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The reaction mixture was kept at −20 °C until enzymatic amplification. In the case of samples from R6/2 mice, quantitative RT-PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) to quantify mRNA levels for $CB_1$ (ref. Mm01212171_s1), $CB_2$ (ref. Mm02620087_s1), FAAH (ref. Mm00515684_m1), MAGL (ref. Mm00449274_m1), IGF-1 (ref. Mm00439560_m1), BDNF (ref. Mm04230607_s1), DARPP-32 (ref. Mm00454892_m1), GLT-1 (ref. Mm00441457_m1), GLAST (ref. Mm00600697_m1), tumor necrosis factor (TNF)-α (ref. Mm00443260_g1), and PPARγ (ref. Mm01184322_m1) using glyceraldehyde 3-phosphate dehydrogenase (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). The iQ$^{TM}$ SYBR Green Supermix (Bio-Rad) was used to quantify mRNA levels, in samples from 3NP-lesioned mice, for cyclooxygenase (COX)-2, TNF-α, interleukin (IL)-6, and inducible nitric oxide synthase (iNOS). Real-time PCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad). The GAPDH housekeeping gene was used to standardize the mRNA expression levels in every sample. Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Sequences of oligonucleotide primers are given in Table 1. The complementary DNA samples were also used for an array analysis using the RT$^2$ Profiler PCR Array Mouse HD (PAMM-123Z; Qiagen), which has been designed with 84 HD-related genes, following the manufacturer's instructions.

Measurement of Oxidative Stress Parameters

To determine different parameters related to oxidative stress and antioxidant systems, the right striatum obtained from 3NP-lesioned and control mice was homogenized in 0.1 M phosphate buffer (pH 7.4) using an Ultra-Turrax (IKA, Staufen im Breisgau, Germany) for 30 s on ice. Homogenates were cleared by centrifugation at 13,400 g at 4 °C and the supernatants were collected. The protein content of the samples was determined using the Bradford assay. Catalase activity was assayed by the method described by Luck [25] in which the breakdown of hydrogen peroxide ($H_2O_2$) is measured at 240 nm. Briefly, the assay mixture consisted of 12.5 mM $H_2O_2$ in 50 mM phosphate buffer (pH 7.0) and 0.05 ml of the supernatant of each striatal homogenate (10 %), and the change in absorbance was recorded at 240 nm. Enzyme activity was calculated using the millimolar extinction coefficient of $H_2O_2$ (0.07). The results are expressed as percentage of the mean activity in the control group. Superoxide dismutase (SOD) activity was assayed according to the method described by Kono [26], wherein the reduction of nitroblue tetrazolium chloride (NBT) was inhibited by the SOD that was measured at 560 nm spectrophotometrically. Briefly, the reaction was initiated by the addition of 20 mM of hydroxylamine hydrochloride to the mixture containing 1 mM of NBT and 0.1 ml of each striatal homogenate. The results are expressed as percentage of the mean activity in the control group. Reduced glutathione (GSH) levels in each striatal homogenate were estimated according to the method described by Ellman [27]. The tissue samples (0.1 ml) were treated with trichloroacetic acid (10 %) during 30 min at room temperature and then centrifuged at 600 g for 15 min. The supernatants were collected and incubated with 6 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow-colored product, its determination at 412 nm is used as an index of GSH concentrations, which are represented as percentage of mean levels found in the control group.

Histological Analyses

Brains fixed in 4 % paraformaldehyde and cryoprotected in 30 % sucrose were sliced (30-μm thick) with a cryostat at the level of the caudate-putamen and collected on gelatin-coated slides for immunohistochemical analysis of 1) EM48, a marker of mutant huntingtin aggregates, using a monoclonal anti-mouse EM48 antibody (Millipore, Billerica, MA, USA) used at 1/400 (see details in [28]); and 2) DARPP-32, a marker of striatal projection neurons, using a monoclonal anti-mouse DARPP-32 antibody (Cell Signaling Technology, Danvers, MA, USA) used at 1/300. In the case of 3NP model, 5-μm-thick sections were used for Cresyl-violet staining (see details in [29]) and for immunohistochemical analysis of 1) NeuN, a marker of neurons, using a monoclonal antimouse NeuN antibody (Millipore) used at 1/100; 2) Iba-1, a marker of microglial cells, using a monoclonal anti-mouse Iba-1 antibody (Millipore) used at 1/50; and 3) GFAP, a marker of astrocytes, monoclonal antimouse GFAP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at 1/50. In all cases, sections were incubated overnight at 4 °C. 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 antirat (1:500; Millipore), biotin-conjugated antirabbit (1:300; Sigma/Aldrich, Madrid, Spain) or goat antimouse (Millipore) secondary antibodies. Reaction was revealed with the Vectastain® Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Negative

**Table 1** List of mouse primer sequences used in quantitative polymerase chain reaction

| Gene | Forward | Reverse |
|---|---|---|
| *IL6* | 5' -GAACAACGATGATGCACTTGC- 3' | 5' -TCCAGGTAGCTATGGTACTCC- 3' |
| *iNOS* | 5' -AACGGAGAACGTTGGATTTG-3′ | 5′-CAGCACAAGGGGTTTTCTTC-3′ |
| *COX-2* | 5′-TGAGCAACTATTCCAAACCAGC-3′ | 5′-GCACGTAGTCTTCGATCACTATC-3′ |
| *TNFα* | 5' -AGAGGCACTCCCCCAAAAGA-3' | 5' -CGATCACCCCGAAGTTCCCATT-3' |
| *GAPDH* | 5′-TGGCAAAGTGGAGATTGTTGCC-3′ | 5′-AAGATGGTGATGGGCTTCCCG-3′ |

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 1200 F camera (Nikon, Tokyo, Japan) were used for slide observation and photography, and all image processing was done using ImageJ (National Institutes of Health, Bethesda. MD, USA). For this purpose, multiple sections, selected from levels located approximately 200 μm from the middle of the lesion, were obtained from each brain and used to generate a mean value per subject. In the case of the quantification of EM48 and DARPP-32 immunostaining, three 30-μm-thick slices (at a distance of 360 μm each other) per animal and 4 images at a magnification of 40× per each slice were used. The number of aggregates, or of DARPP-32-stained cells, was expressed as the mean value per field.

### Cell Cultures and PPARγ Transcriptional Activity

Additional experiments were conducted in conditionally immortalized striatal progenitor cell lines $STHdh^{Q7/Q7}$ expressing endogenous wild-type huntingtin, and $STHdh^{Q111/Q111}$ expressing comparable levels of mutant huntingtin with 111 glutamines [30]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza Ibérica, Barcelona, Spain) supplemented with 4 % fetal bovine serum and 4 % bovine growth serum, 2 mM L-glutamine, and antibiotics. The cells were grown at 33 °C in a humidified atmosphere containing 5 % $CO_2$. In 24-well plates, $10^5$ cells/ml were cultured and transiently co-transfected with the expression vector GAL4-PPARγ and the luciferase reporter vector GAL4-luc using Roti®-Fect (Carl Roth, Karlsruhe, Germany) following the manufacturer's instructions. Twenty-four hours post-transfection, cells were treated with increasing concentrations of CBG for 6 h. Then, the cells were lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM dithiothreitol, 1 % Triton X-100, and 7 % glycerol. Luciferase activity was measured in the cell lysate using a TriStar LB 941 multimode microplate reader (Berthold, Bad Wildbad, Germany) and following the instructions of the Luciferase Assay Kit (Promega, Madison, WI, USA). Protein concentration was measured by the Bradford assay (Bio-Rad). The background obtained with the lysis buffer was subtracted in each experimental value and the specific transactivation expressed as a fold induction over untreated cells. All the experiments were repeated at least 3 times. Rosiglitazone was purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and used as a positive control in these *in vitro* experiments.

### Statistics

Data were assessed by one- or two-way analysis of variance, as required, followed by the Student–Newman–Keuls test.

## Results

### Study of Neuroprotective Effects of CBG in 3NP-lesioned Mice

The intoxication of mice with 3NP resulted in a myriad of neurological, biochemical, and histological effects that were reminiscent of some aspects of HD pathology (reviewed in [22]). For example, 3NP-treated mice exhibited high scores in hindlimb clasping [$F(3,26)=13.87$; $P<0.01$], dystonia [$F(3,26)=10.55$; $P<0.01$], and in general locomotor activity [$F(3,26)=18.742$; $P<0.01$] compared with control animals (Fig. 1). A similar tendency was seen for truncal dystonia, but it was not statistically significant (Fig. 1). In these animals, the administration of CBG improved the motor deficits typical of 3NP-lesioned mice by reducing hindlimb clasping and dystonia and general locomotor activity, but had no effect on trunkal dystonia (Fig. 1).

The striatal parenchyma of these 3NP-treated animals showed an important reduction in Nissl-stained cells [>50 %; $F(3,8)=96.89$, $P<0.01$], which indicates an important degree of neuronal death caused by 3NP that was clearly prevented by treatment with CBG (Fig. 2). Neuronal cell death was confirmed by NeuN immunohistochemistry, which proved a reduction of about 50 % in the immunolabelling for this neuronal marker in the striatal parenchyma of 3NP-lesioned mice [$F(3,8)=92.46$; $P<0.01$], which was paralleled by a notable increase in GFAP [$F(3,8)=83.984$; $P<0.01$] and Iba-1 [$F(3,8)=112.267$; $P<0.01$] immunostainings, indicating the occurrence of astrogliosis and reactive microgliosis, respectively (Fig. 3). NeuN immunostaining confirmed that CBG treatment prevented 3NP-induced neuronal loss (Fig. 3A). However, this treatment did not reduce the number of GFAP-positive cells, which, in fact, experienced an increase (Fig. 3B), and produced only a modest reduction in the reactive microgliosis (Iba-1-positive cells) (Fig. 3C). These observations were not unexpected as glial activation has been reported to be very poor after 3NP intoxication in rodents [6, 7, 14], oxidative injury and calpain activation being the key cytotoxic events underlying neuronal death in this experimental model [22].

As microglia activation and astrogliosis were barely or not affected by CBG, we were interested to investigate the mRNA expression of specific proinflammatory markers. We show that the expression of inflammatory enzymes, for example COX-2 [$F(3,8)=29.071$; $P<0.01$ (Fig. 4A)] and iNOS [$F(3,8)=34.051$; $P<0.01$ (Fig. 4D)], and the expression of proinflammatory cytokines, for example TNF-$\alpha$ [$F(3,8)=53.869$; $P<0.01$ (Fig. 4B)] and IL-6 [$F(3,8)=6.091$; $P<0.05$ (Fig. 4C)] were significantly upregulated in 3NP-lesioned mice. CBG significantly attenuated the upregulation of all the proinflammatory markers induced by 3NP (Fig. 4). Taken together, these results suggest that CBG mediates its anti-

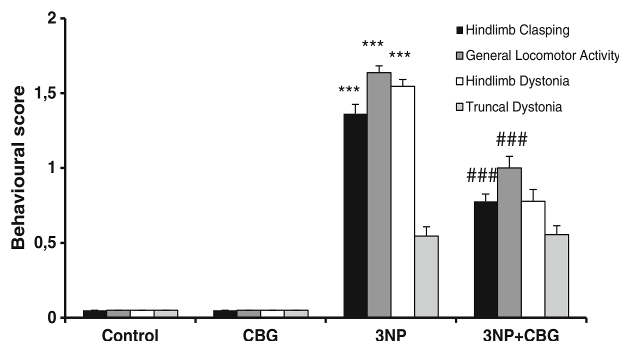


**Fig. 1** Behavioral score after 3-nitropropionic acid (3NP) intoxication. Hindlimb clasping, general locomotor activity, hindlimb dystonia, and truncal dystonia were rated from 0 to 2 based on severity: a score of 0 typically indicates normal function and 2 indicates seriously affected function. Values are expressed as means ± SEM for 6–8 animals per group. Data were subjected to one-way analysis of variance followed by the Student–Newman–Keuls test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ when comparing the control group with the 3NP group. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ when comparing 3NP group with 3NP + cannabigerol (CBG) group

inflammatory activity on activated microglia and not in the signaling pathways that drive microglia cells from a resting state to an activated state.

Next, we used the striatum of 3NP-lesioned mice for analysis of some biochemical markers related to oxidative stress (e.g., catalase activity, SOD activity and GSH levels), which are particularly affected in this model [22]. As expected, there was a marked reduction in the activities of catalase [F(3,8)= 12.593; $P<0.01$ (Fig. 5A)] and, in particular, SOD [F(3,8)= 20.262; $P<0.01$ (Fig. 5C)], as well as in the levels of GSH [F(3,8)=4.487; $P<0.05$ (Fig. 5B)] compared with controls. CBG treatment recovered the activities of antioxidant enzymes, for example catalase, SOD-1, and the levels of GSH were reduced in the striatum of 3NP-intoxicated mice (Fig. 5). Collectively, these effects were compatible with an important neuroprotective effect exerted by CBG against the striatal

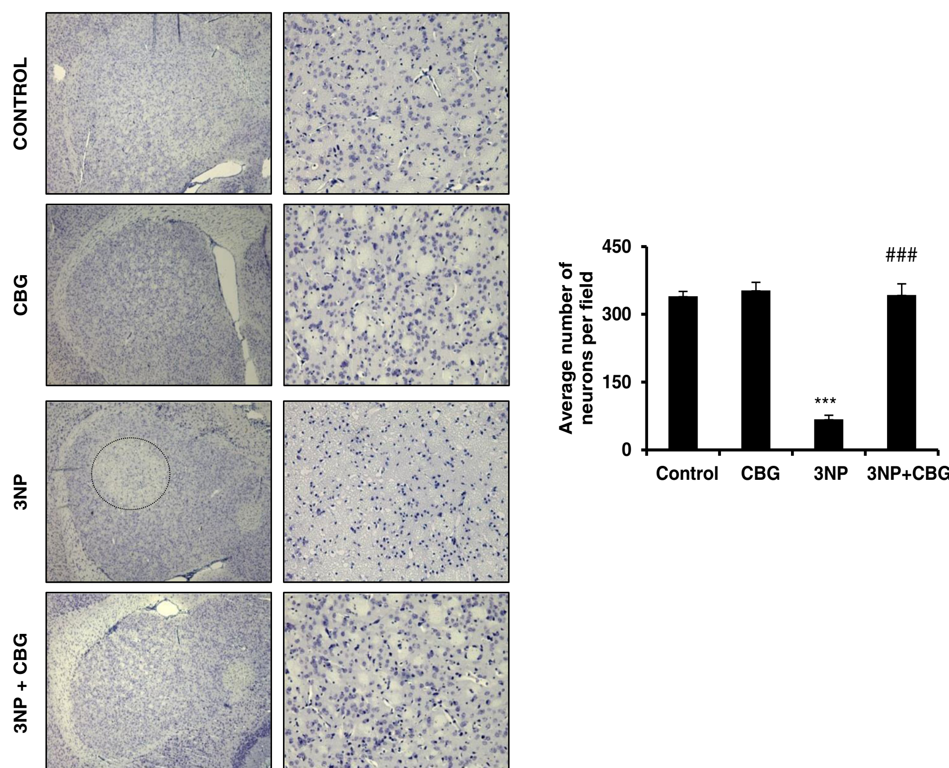


**Fig. 2** Systemic administration of 3-nitropropionic acid (3NP) leads to a progressive and selective degeneration in the striatum. (Left) Cresyl violet staining was performed on brain sections from control, cannabigerol (CBG)-, 3NP-, and 3NP + CBG-treated mice. Low (left column) and high magnification (right column) showing the selective loss of cells in the striatum at day 5 (pale region, outlined). This lesion was not detectable in the group that received CBG. Images were acquired by using light microscopy. (Right) Quantification of Nissl-positive cells in the mouse striatum. Total average number of neurons (100× magnification) is shown. Values are expressed as means ± SEM for 6–8 animals per group. Data were subjected to one-way analysis of variance followed by the Student–Newman–Keuls test. ***$P<0.001$ when comparing the control group with the 3NP and CBG group. $^{\#\#\#}P<0.001$ when comparing the 3NP group with the 3NP + CBG group

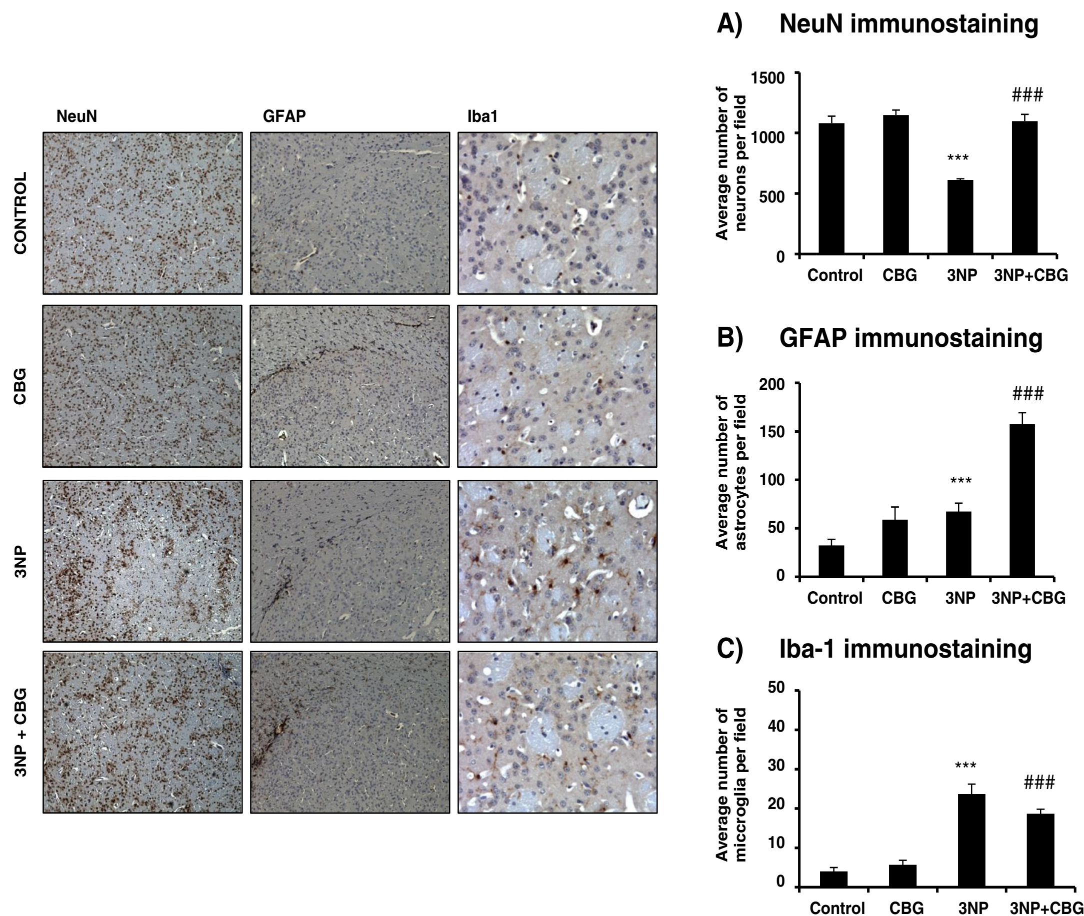


**Fig. 3** (Left panels) Photomicrographs of NeuN (10×)-, glial fibrillary acidic protein (GFAP) (10×)-, and Iba1 (20×)-immunostained sections through the coronal section of striatum of control and 3-nitropropionic acid (3NP)-lesioned mice treated with vehicle or cannabigerol (CBG). They show a significant loss of NeuN-positive cells in the striatum of 3NP-treated mice compared with controls. CBG treatment significantly reduced 3NP-induced loss of striatal NeuN-positive cells. (Right panels) Quantification of (**A**) NeuN-, (**B**) GFAP-, and (**C**) Iba1-positive cells in the mouse striatum. Total average number of neurons, astrocytes (100× magnification) and microglia (200× magnification) is shown. Values are expressed as means ± SEM for 6–8 animals per group. Data were subjected to one-way analysis of variance followed by the Student–Newman–Keuls test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ when comparing the control group with the 3NP and CBG group. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ when comparing the 3NP group with the 3NP + CBG group

damage caused by 3NP, which is also supported by the fact that the phytocannabinoid was mostly inactive on these parameters when it was administered to nonlesioned control animals (Figs 1–5).

Lastly, cDNA samples obtained from the striatum of animals lesioned with 3NP, their controls, and the 3NP-lesioned mice that were treated with CBG were analyzed with a specific HD array that measured the mRNA expression of 84 genes related to HD (Table S1; see Supplementary Information). Our purpose was to identify possible genes that, being affected by 3NP insult reproducing the changes observed in the human pathology, may be normalized by treatment with CBG. Using this HD RT-PCR panel, we found only 2 genes, *Cd44* and *Sgk1*, to be significantly upregulated (>2-fold induction or repression). *Cd44* was induced in 3NP mice compared with control mice (3.0-fold induction) and was significantly downregulated in 3NP mice treated with CBG (−1.3-fold repression). *Sgk1* was induced in 3NP mice compared with control mice (2.7-fold induction) and was also significantly downregulated in 3NP mice treated with CBG (−1.41-fold repression). Although, in general, these results confirm that the molecular mechanisms underling HD physiopathology are quite different in 3NP and R6/2 murine models (see below), previous studies have confirmed the relation of these two genes with HD and other neuroinflammatory disorders [31, 32].

## Study of Neuroprotective Effects of CBG in R6/2 Mice

We also investigated the neuroprotective properties of CBG in a transgenic murine model of HD, the R6/2 mice, which exhibit a very aggressive pathological phenotype that recapitulates most of the cytotoxic mechanisms that operate in the human pathology. Thus, compared with wild-type animals, R6/2 mice exhibited a characteristic loss of weight that was initiated at 8 weeks of age and that worsened at 10 weeks of

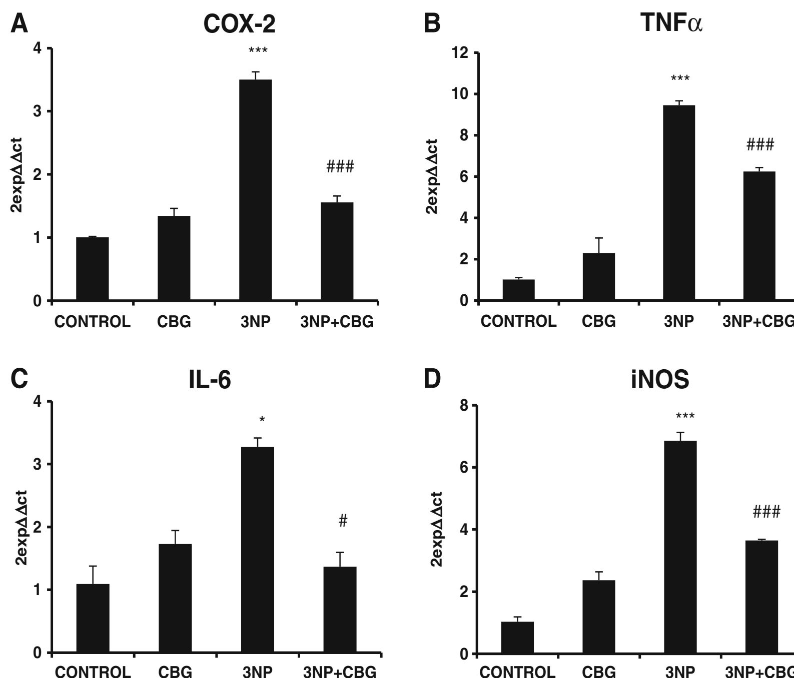


**Fig. 4** Gene expression of inflammatory markers including (**A**) cyclooxygenase (COX)-2, (**B**) tumor necrosis factor (TNF)-α, (**C**) interleukin (IL)-6, and (**D**) inducible nitric oxide synthase (iNOS) was significantly downregulated in 3-nitropropionic acid (3NP) + cannabigerol (CBG)-treated mice compared with 3NP mice. Values are expressed as means ± SEM for 6–8 animals per group. Data were subjected to one-way analysis of variance followed by the Student–Newman–Keuls test. **P*<0.05, ***P*<0.01, ****P*<0.001 indicates significant changes between controls and 3NP mice. #*P*<0.05, ##*P*<0.01 indicates significant changes between 3NP- and 3NP + CBG-treated mice

age when the animals were euthanized [F(15,180)=12.13; *P*<0.01 (Fig. 6A)]. This loss of weight has been largely reported in R6/2 mice and also in other transgenic models of HD [7–9, 14]. Treatment with CBG was not effective in recovering the characteristic loss of weight of R6/2 mice; it produced an apparent worsening effect (Fig. 6A). In parallel to the weight loss, R6/2 mice exhibited a deterioration in rotarod performance that was already evident at the age of 5 weeks, then occurring before the loss of weight, and that reached a maximum at 9 weeks after birth [F(15,180)=4.208; *P*<0.01), 1 week before animals were euthanized (Fig. 6B). The administration of CBG produced a modest recovery in this deteriorated rotarod performance, as revealed the fact that the time in the rod of R6/2 mice treated with CBG was always higher at all time points analyzed compared with R6/2 mice treated with vehicle (Fig. 6B). However, these differences were not statistically significant, being evident only because of a different probability level for both groups compared with wild-type animals at specific time points, for example 8 weeks (Fig. 6B).

The analysis of the postmortem striatum of R6/2 animals at 10 weeks after birth proved important changes in specific markers of HD pathology, in particular, a profound reduction in the expression of $CB_1$ [F(3,29)=22.50; *P*<0.01 (Fig. 7A)], as has been largely demonstrated in patients with HD [33], and in this and other experimental models of HD [8, 14]. Upregulatory responses of $CB_2$ have been also found in previous studies [9, 10], and were also evident here, but to a significantly lesser extent [F(3,26)=3.079; *P*<0.05 (Fig. 7B)], and the same happened with expression of FAAH, which only showed a nonsignificant trend towards a decrease [F(3,29)= 1.208; Fig. 7C), and therefore a much more moderate reduction than in previous studies in patients with HD [34]. However, the expression of MAGL had not previously been studied in R6/2 mice or other HD models, and proved to have a marked downregulatory response [F(3,29)=23.02; *P*<0.01

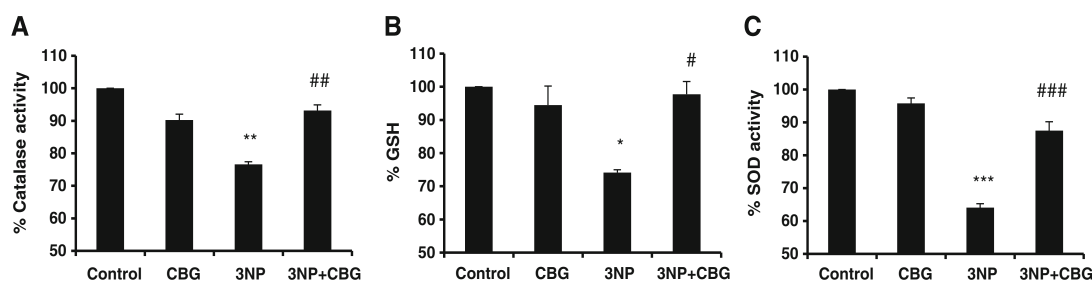


**Fig. 5** Effect of cannabigerol (CBG) on antioxidant defenses in the striatum of 3-nitropropionic acid (3NP)-treated and control mice. Estimation of (**A**) catalase activity, (**B**) reduced glutathione (GSH) levels, and (**C**) superoxide dismutase (SOD) activity. Data presented are the percentage of the vehicle-treated control group and are expressed as means ± SEM for 6–8 animals per group. Data were subjected to one-way analysis of variance followed by the Student–Newman–Keuls test. **P*<0.05, ***P*<0.01, ****P*<0.001 when comparing the control group with the 3NP and CBG group. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 when comparing the 3NP group with the 3NP + CBG group

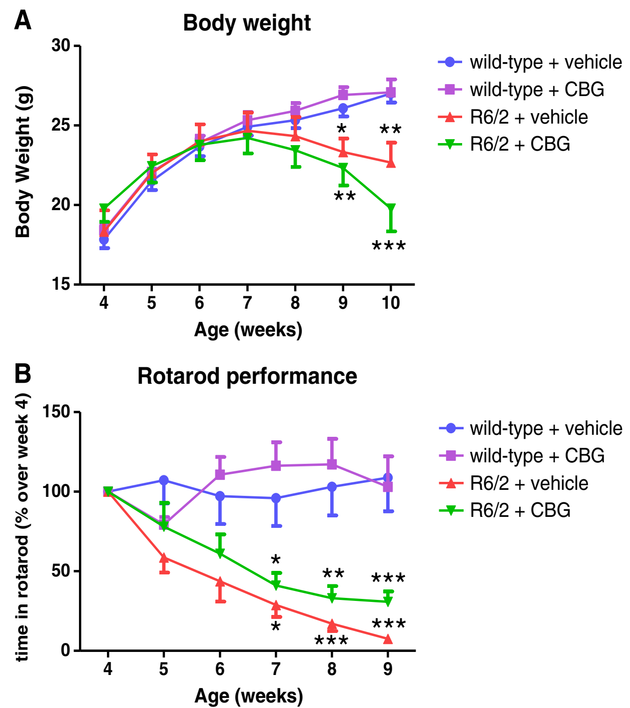


**Fig. 6** (**A**) Weight gain and (**B**) rotarod performance in R6/2 mice treated from the age of 4 weeks with cannabigerol (CBG) or vehicle (Tween 80-saline). Values are expressed as means ± SEM for 6–8 animals per group. Data were subjected to one-way analysis of variance followed by the Student–Newman–Keuls test (*$P$<0.05, **$P$<0.01, ***$P$<0.005 compared with wild-type animals treated with vehicle)

(Fig. 7D)]. Treatment with CBG only affected the gene expression for $CB_2$ with a small effect, which is reflected in the loss of statistical significance for the differences observed between wild-type animals and R6/2 mice when these mice were treated with CBG (Fig. 7B).

We also found the expected responses in R6/2 for other biochemical markers according to previous studies [8, 9, 35], for example in the neurotrophins BDNF and IGF-1, the expression of which experienced in both cases showed a dramatic reduction [BDNF: $F(3,28)=5.618$ ($P<0.05$); IGF-1: $F(3,29)=6.42$ ($P<0.05$) (Fig. 8A, B)], as previously described [2, 8]. Treatment with CBG partially improved deficits in both BDNF (Fig. 8A) and IGF-1 (Fig. 8B), but the effects were modest and similar to the case of $CB_2$ (e.g., loss of statistical significance of the differences observed between wild-type animals and R6/2 mice). R6/2 mice also exhibited deficits in the glial glutamate transporters GLAST [$F(3,31)=11.44$; $P<0.01$ (Fig. 8D)] and, in particular, GLT-1 [$F(3,31)=27.85$; $P<0.01$ (Fig. 8C)], also in accordance with previous data [36], but, in this case, there was no recovery by the treatment with CBG (Fig. 8C, D).

We also quantified DARPP-32, a protein involved in dopaminergic receptor signaling in striatal projection neurons, which experienced a dramatic reduction in gene expression [$F(3,31)=18.79$; $P<0.01$ (Fig. 9A)] and immunostaining [$F(3,18)=11.09$; $P<0.05$; Fig. 9B)] in R6/2 mice, concordant with the expected death of these neurons, and similar to previous studies [37]. None of these reductions was reversed by the treatment with CBG (Fig. 9A, B). We also found changes in inflammation-related markers such as the PPARγ receptors, which were downregulated in R6/2 mice [$F(3,25)=4.32$; $P<0.05$ (Fig. 9C)], and the cytokine TNF-α, which experienced a marked increase [$F(3,23)=5.716$; $P<0.01$ (Fig. 9D)], in agreement with previous studies [38]. However, we did not find any changes in other proinflammatory parameters, such as the enzymes COX-2 or iNOS, and the cytokine IL-1β (data not shown), although this lack of response has been documented in the literature [39]. Treatment with CBG partially reversed the downregulation of PPARγ in R6/2 mice (Fig. 9C), being less effective against the increase in TNF-α, although a certain trend towards an attenuation may be appreciated in this parameter (Fig. 9D). In parallel to the increase in PPARγ expression by CBG in these mice, we also found that this phytocannabinoid dose-dependently activated PPARγ in cultured striatal cells expressing endogenous wild-type and mutant huntingtins (Figure S1; see Supplementary Information).

We were able to detect the presence of immunoreactivity for mutant huntingtin aggregates, labeled with EM48 antibody, in the striatal parenchyma of R6/2 mice (Fig. 10), although our immunohistochemical analysis with GFAP and Iba-1 did not reveal a marked glial activation (data not shown), as found in some studies [40]. Interestingly, EM48 immunostaining was slightly, but significantly, reduced in R6/2 mice treated with CBG (Fig. 10). Given that the number of striatal neurons does not appear to be significantly altered by treatment with CBG in R6/2 mice, according to the data obtained for DARPP-32 immunostaining (Fig. 9b), we believe that the reduction in EM48 immunostaining by CBG does not reflect a reduction in the number of neurons but a decrease in the number of mutant huntingtin aggregates in surviving neurons, which may be a beneficial event.

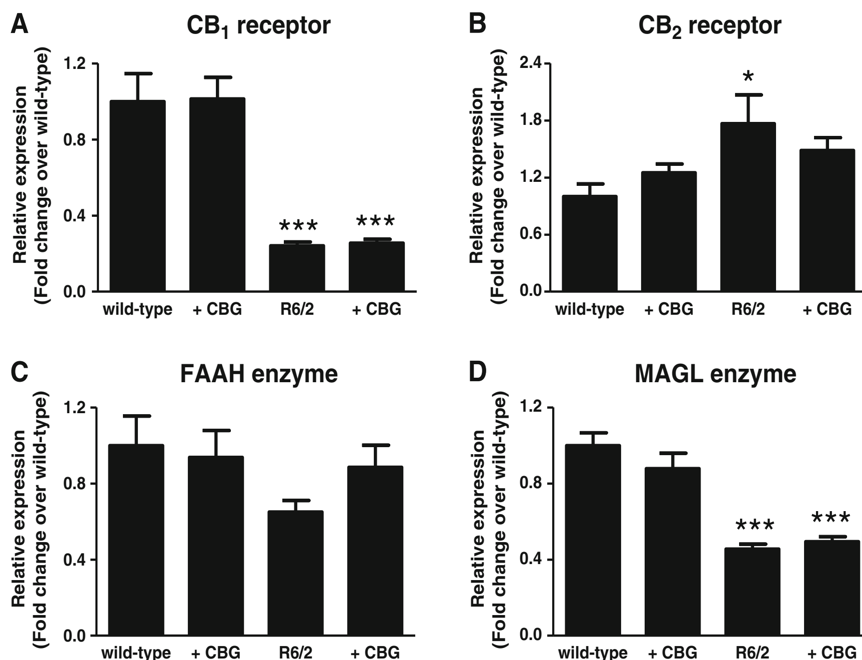


**Fig. 7** Gene expression for (**A**) cannabinoid receptor type 1 ($CB_1$) and (**B**) cannabinoid receptor type 2 ($CB_2$) receptors, and (**C**) fatty acid hydrolase (FAAH) and (**D**) monoacylglycerol lipase (MAGL) measured in the striatum of R6/2 mice (10 weeks after birth) treated from the age of 4 weeks with cannabigerol (CBG) or vehicle (Tween 80-saline). Values correspond to fold of change over wild-type animals and are expressed as means ± SEM for 6–8 animals per group. Data were subjected to one-way analysis of variance followed by the Student–Newman–Keuls test (*$P$<0.05, **$P$<0.01, ***$P$<0.005 compared with wild-type animals treated with vehicle)

We also used the cDNA samples obtained from the striatum of R6/2 mice, their controls, and the transgenic mice that were treated with CBG for analysis with a specific HD array. Again, our purpose was to identify possible genes that, being affected in R6/2 mice reproducing more accurately the changes observed in the human pathology, may be normalized by treatment with CBG. We found numerous genes up- or down-regulated in R6/2 mice (Table S2; see Supplementary Information), but 7 that have been frequently linked to this disease given their role in the regulation of gene transcription (e.g., symplekin, Sin3a, Rcor1, histone deacetylase 2, and huntingtin-associated protein 1), GABA transmission (e.g. δ subunit of the GABA-A receptor), and calcium homeostasis (e.g. hippocalcin), were found to be up- or down-regulated in R6/2 mice (Fig. 11A), as has previously been found in patients with HD or experimental models [41–44]. Importantly, the expression of these 7 genes was partially normalized by treatment with CBG (Fig. 11B), with some cases, for example huntingtin-associated protein 1, the increase found in R6/2 mice being completely reversed by CBG and exhibiting even lower expression levels than wild-type animals (Fig. 11B).

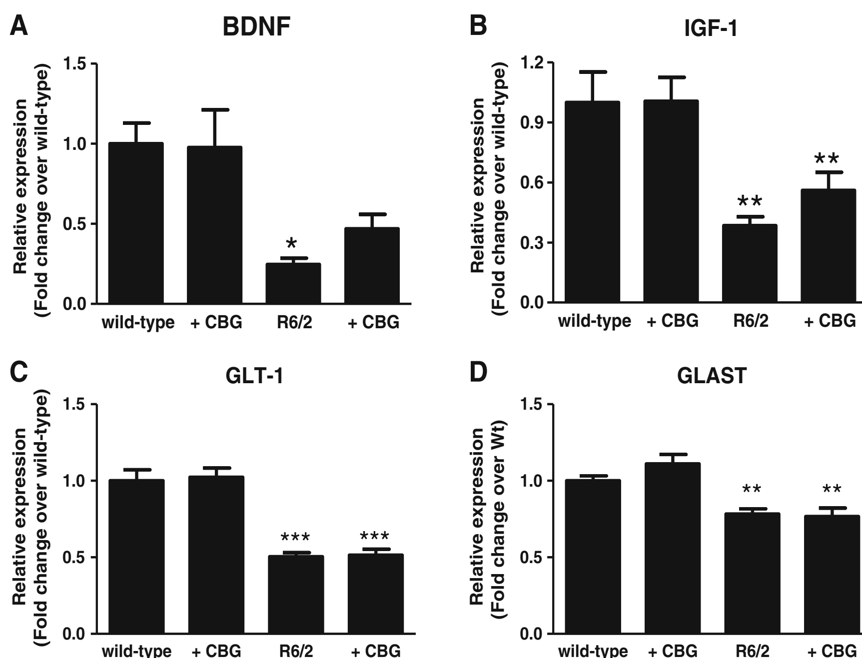


**Fig. 8** Gene expression for (**A**) brain-derived neurotrophic factor (BDNF), (**B**) insulin-like growth factor (IGF)-1, (**C**) glutamate transporter (GLT)-1, and (**D**) glutamate aspartate transporter (GLAST) measured in the striatum of R6/2 mice (10 weeks after birth) treated from the age of 4 weeks with cannabigerol (CBG) or vehicle (Tween 80-saline). Values correspond to fold of change over wild-type animals and are expressed as means ± SEM for 6–8 animals per group. Data were subjected to one-way analysis of variance followed by the Student–Newman–Keuls test (*$P$<0.05, **$P$<0.01, ***$P$<0.005 compared with wild-type animals treated with vehicle)

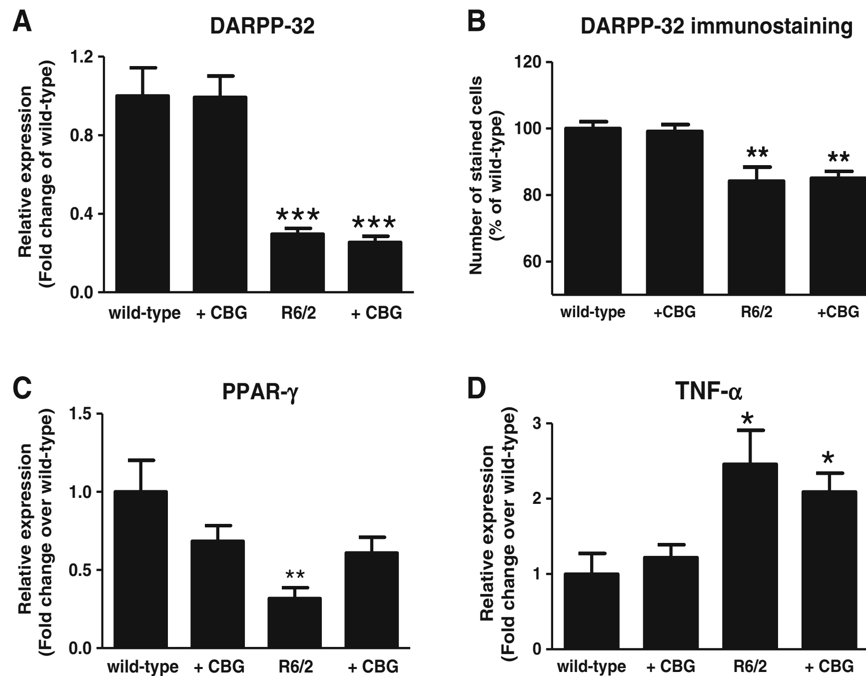


**Fig. 9** Gene expression for (**A**) dopamine- and cyclic adenosine monophosphate-regulated phosphoprotein, Mr 32 kDA (DARPP-32), (**C**) peroxisome proliferator-activated receptor (PPAR)γ, and (**D**) tumour necrosis factor (TNF)-α, and (**B**) DARPP-32 immunostaining measured in the striatum of R6/2 mice (10 weeks after birth) treated from the age of 4 weeks with cannabigerol (CBG) or vehicle (Tween 80-saline). Values correspond to fold of change (gene expression) or percentage (immunostaining) over wild-type animals and are expressed as means ± SEM for 6–8 animals per group. Data were subjected to one-way analysis of variance followed by the Student–Newman–Keuls test (**P*<0.05, ***P*<0.01, ****P*<0.005 compared with wild-type animals treated with vehicle)

## Discussion

Only a few clinical studies have been performed to determine whether cannabinoid compounds are efficacious in HD, and the results they have yielded are rather controversial [45–48]. Possibly, the reason for such controversy is that these clinical studies concentrated more on HD symptoms rather than on disease progression. However, recent animal studies have demonstrated that combinations of different cannabinoids or the use of a broad-spectrum cannabinoid may delay disease progression by preserving striatal neurons from death in different animal models of HD, thus stressing the need for new clinical studies directed at testing whether the neuroprotective effects that certain cannabinoids induce in experimental models of HD are also reproduced in patients with HD [7–11]. This includes the evaluation of $\Delta^9$-THC and CBD, separately or in a combination [6–9], Sativex®, which has been found to be neuroprotective in different animal models of HD [14, 49], and additional phytocannabinoids.

We were particularly interested in investigating whether CBG, another major phytocannabinoid present in *Cannabis sativa*, could be as effective a disease-modifying agent in HD. We studied its effects in 2 experimental models of HD, and found particularly positive results in the 3NP-lesioned mice model, a model in which the death of striatal neurons is dependent on mitochondrial damage, activation of calpain,

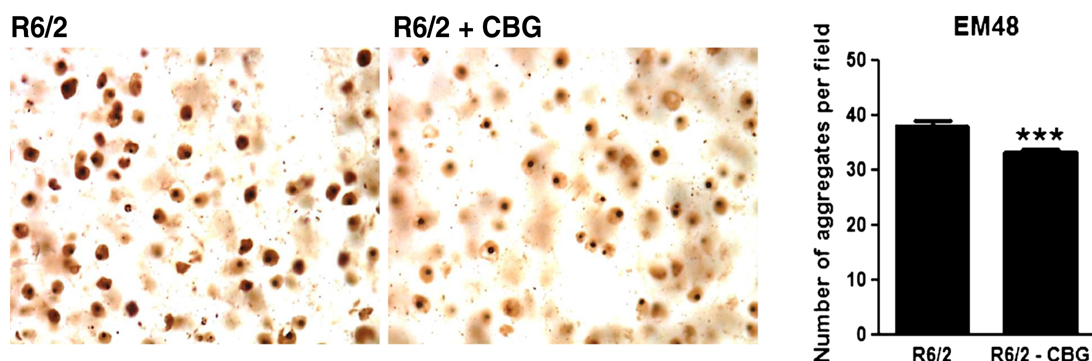


**Fig. 10** EM48 immunostaining (representative of mutant huntingtin aggregates) in the striatum of R6/2 mice (at 10 weeks after birth) treated from the age of 4 weeks with cannabigerol (CBG) or vehicle (Tween 80-saline). The stainings were repeated in 5–6 animals per group. Magnification=40×. Data were subjected to Student's *t* test (****P*<0.005 compared with R6/2 mice treated with vehicle)

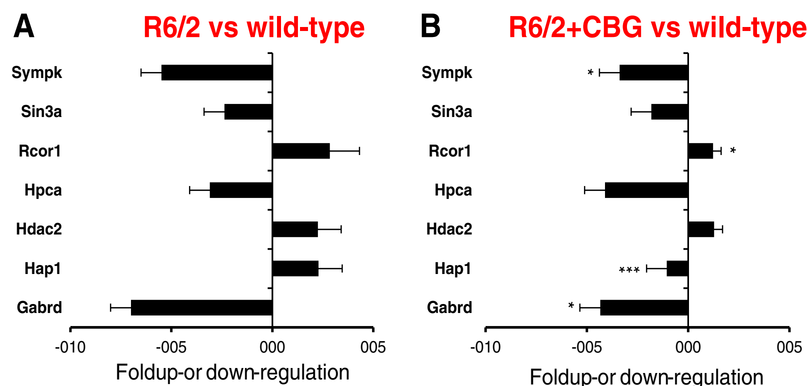


**Fig. 11** Huntingdon's disease (HD) array analysis showing the up- or downregulatory responses of some genes specifically affected in the striatum of (**A**) R6/2 compared with wild-type mice or (**B**) R6/2 mice treated with CBG compared with wild-type mice. One microgram of RNA was retrotranscribed and the resulting cDNA was analyzed in a mouse HD polymerase chain reaction array. Five housekeeping genes contained on the experimental system were used to standardize the mRNA expression levels in every sample. Values correspond to number of folds that a specific gene is up- or downregulated. Data were assessed by the unpaired two-tailed Student's *t* test. The 7 genes presented in (A) were selected because the up- and downregulatory responses found between R6/2 and wild-type mice were more than 2-fold higher, and these differences were statistically significant in all cases, and also because these differences were reversed by the treatment with cannabigerol (CBG) (*$P<0.05$, **$P<0.01$, ***$P<0.005$)

and the generation of reactive oxygen species [14, 22]. In this model, subchronic administration of CBG preserved striatal neurons from 3NP-induced death presumably by regulating pro-oxidant and proinflammatory responses induced by 3NP intoxication. Importantly, the preservation of striatal neurons and the improvement of their homeostasis by CBG resulted in a significant improvement in the neurological deterioration typical of this model. These positive effects found in the 3NP model of HD were also investigated in a transgenic model of this disorder, the R6/2 mice, although the effects of CBG were much more modest in this model compared with the 3NP-lesioned mice. The most important observations in R6/2 mice were obtained from the analysis of the neurotrophins BDNF and IGF-1, and the PPARγ receptors, the deficits of which are strongly indicative of the deterioration in the striatal function—deficits that were partially reversed by CBG. We also collected some evidence from the data obtained with a specific HD array analysis, which showed some important HD-related genes, the up- or downregulatory responses of which were significantly attenuated by the treatment with CBG. Both types of responses are strongly associated with patients with HD in whom BDNF deficiency and alterations in symplekin [2], Sin3a, Rcor1, histone deacetylase 2, huntingtin-associated protein 1, and other genes related to the regulation of gene transcription have been found to play a key role in HD pathogenesis [41–44]. The presence of mutant huntingtin aggregates in the striatal parenchyma of R6/2 mice, another key event in human HD pathogenesis, was slightly reduced by CBG in these mice. By contrast, we did not obtain any evidence that CBG may preserve striatal neurons from death in R6/2 mice, as revealed by DARPP-32 immunostaining, but this fact appears to support the fact that the reduction in the number of mutant huntingtin aggregates reflects a lower presence of these aggregates in surviving neurons rather than a reduction due to lower number of striatal neurons. Reduction in huntingtin aggregation has been associated with a better striatal function, and has been proposed as a therapeutic objective in this disease [3].

In this study, we have not addressed the potential mechanisms involved in the beneficial effects of CBG in both HD models, something that will be done in follow-up studies. It is important to note that the profile of CBG has been poorly studied in relation to its therapeutic and pharmacological effects and, in particular, to its mechanisms of action, which makes it difficult to relate the effects found to specific targets and signaling pathways. However, we assume that the effects are not dependent on the activation of $CB_1$ and/or $CB_2$, given the poor affinity of CBG for these classic cannabinoid receptors [17, 18]. Like $\Delta^9$-THC and CBD, CBG also penetrates the blood–brain barrier after i.p. delivery [50], and may target α-2 adrenergic receptors and PPARγ receptors in the brain [19, 21]. It has been shown that the expression of α-2 adrenergic receptors is increased in the hypothalamus of transgenic rats for HD [51], but antagonism rather than agonism is involved α-2 adrenergic receptor-mediated neuroprotection [52], and therefore it is unlikely that CBG alleviates HD symptoms and disease markers by targeting this receptor. On the contrary, PPARγ agonists such as thioglitazones have been shown to have a neuroprotective effect in *in vitro* and *in vivo* models of HD [53–55]. There is strong evidence that mitochondrial dysfunction results in neurodegeneration and may contribute to the pathogenesis of HD. Studies over the last few years have implicated an impaired function of PPARγ co-activator-1α (PGC-1α), a master co-regulator of mitochondrial biogenesis, metabolism, and antioxidant defenses, in causing mitochondrial dysfunction in HD. PPARγ agonists are neuroprotective, increase oxidative phosphorylation capacity in mouse and human cells, and

enhance mitochondrial function [56]. In addition, it has also been demonstrated that oral treatment with rosiglitazone induced mitochondrial biogenesis in mouse brain [57]. Therefore, we assume that the activation of PPARγ receptors may be certainly the most feasible mechanism for CBG effects in HD, in particular in 3NP-lesioned mice, although this would need to be demonstrated in follow-up studies. We also consider that these nuclear receptors may be also involved in the effects of CBG in R6/2 mice. In support of this hypothesis, we have found that CBG activated PPARγ in striatal cells expressing endogenous wild-type and mutant huntingtins, an experimental condition relatively comparable with the case of R6/2 mice. However, low levels of PPARγ receptors are expected in the striatum of these mice given their low levels of gene expression found in our study. In fact, these presumably reduced PPARγ receptor levels, together with the fact that CBG does not activate $CB_1$ and $CB_2$ and that, in R6/2 mice, the role of these receptors in mediating the neuroprotective effects of cannabinoids is much more relevant [8, 9], might explain that the effects found with CBG in R6/2 mice have been much more modest compared with 3NP-lesioned mice, suggesting the need to continue the studies in R6/2 mice using combinations of CBG with other phytocannabinoids.

However, substantial literature indicates that HD pathology develops via multiple pathways that may act synergistically. A recent report has investigated the gene expression profile in different animal models of HD compared with striatal gene expression phenotype of human HD [58]. In such a study, the authors found differences between the R6/2 and the 3NP models, showing that the 3NP profile is consistent with neuroinflammation, with changes in genes involved in immune response, response to wounding, defense response, and inflammatory response. In our study, with a limited number of HD-related genes, we also found clear differences between R6/2 (significant changes in 21 of 84 genes) and 3NP (significant changes in 3 of 84 genes) (see Supplementary Information) models. This result may be explained by the rapid induction of cell death mechanisms mediated by 3NP, and the array data may reflect only a small fraction of dysfunctional neurons present at one time. Thus, it will be interesting to study the effect of CBG on the profile of gene expression in 3NP-lesioned animals before the loss of striatal cells.

Finally, given that the improvement of antioxidant defenses by CBG, reflected in increased activities of catalase and SOD-1 and higher levels of GSH, appears to be a key mechanism for the beneficial effects of this phytocannabinoid in the 3NP model, we were also interested in investigating whether the neuroprotective effects of CBG in this pro-oxidant model may be associated with specific effects on Nrf-2 signaling and/or on the target genes for this transcription factor, a fact also proposed for the antioxidant effects of cannabidiol [15]. However, preliminary immunostaining of Nrf-2 and qRT-PCR analysis of Nrf-2-dependent genes failed to prove any effect of CBG at this level (data not shown).

In summary, CBG appears to have a promising neuroprotective profile for the treatment of HD, a fact already investigated with other phytocannabinoids separately or in combination [6–9, 14, 49]. CBG appears to be particularly active against the mitochondrial dysfunction, calpain activation and oxidative injury caused by 3NP. It was also active in R6/2 mice, but with only modest effect, so it is possible that it needs to be combined with another phytocannabinoid in these mice to enhance its therapeutic effects, in particular with cannabinoids having activity at $CB_1$ and/or $CB_2$ receptors, which have been found to serve as therapeutic targets in these mice [8, 9]. This additional investigation will be critical before proceeding to clinical studies with CBG in patients with HD.

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**Required Author Forms** Disclosure forms provided by the authors are available with the online version of this article.

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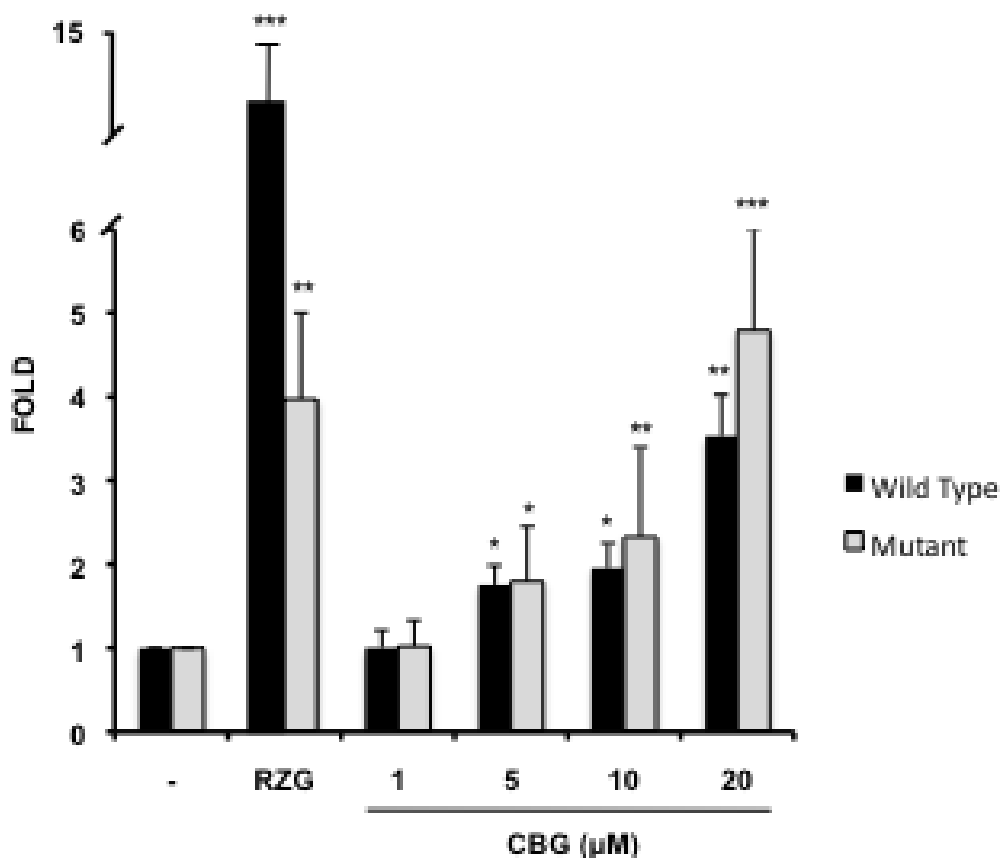
**Supplementary Figure 1**




**Effect of CBG on PPARγ transcriptional activity.** STHdh$^{Q7/Q7}$ (wild-type) and STHdh$^{Q111/Q111}$ (mutant) cells were co-transfected with GAL4-PPARγ and GAL4-luc. Cells were treated with of CBG at the indicated concentrations for 12 h and luciferase activity measured in the cell lysates. Fold activation level was calculated, taking the control sample as reference. Data are expressed as mean ± S.D. of at least three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.005 in an unpaired two-tailed Student's *t* test.

**Table 1S.** HD array analysis (84 genes) showing their up- or down-regulatory responses in the striatum of control and 3NP-lesioned mice treated with CBG or vehicle. Values correspond to number of folds that a specific gene is up- (positive value) or down-regulated (negative value).

**3NP vs control**

| Gene | Fold Up- or Down-Regulation |
|---|---|
| Akt1 | -1,0396 |
| Apoe | -1,4804 |
| Aqp1 | 1,0898 |
| Arfip2 | 1,2008 |
| Atp2b2 | -1,2025 |
| Bax | -1,0253 |
| Bbox1 | -1,0762 |
| Bdnf | 1,5845 |
| C3 | 1,1761 |
| Calb1 | -1,1376 |
| Casp3 | 1,1925 |
| Casp8 | 1,3890 |
| Cd44 | **3,0189** |
| Cltc | 1,1519 |
| Cnr1 | -1,0042 |
| Creb1 | -1,1615 |
| Crebbp | 1,1680 |
| Dctn1 | 1,1127 |
| Dlg4 | -1,2449 |
| Eef1a2 | -1,0112 |
| Egfr | -1,2277 |
| Elmo1 | 1,2781 |
| Fgf12 | 1,2260 |
| Gabrd | -1,1696 |
| Gja1 | -1,6088 |
| Gjb6 | 1,2260 |
| Gnaq | 1,0526 |
| Gpx1 | 1,2092 |
| Grb2 | 1,1599 |
| Grin2a | 1,3794 |
| Grin2b | 1,2431 |
| Grm5 | -1,0614 |
| Hap1 | 1,0098 |
| Hdac1 | 1,2176 |
| Hdac2 | -1,1142 |
| Hip1 | 1,1599 |
| Homer1 | -1,1455 |
| Hpca | 1,1127 |
| Htt | 1,1050 |
| Ift57 | 1,0526 |
| Igf1 | 1,3416 |
| Itpr1 | -1,0541 |
| Kcnab1 | -1,1142 |
| Kcnab2 | 1,0238 |

| Gene | Fold Up- or Down-Regulation |
|---|---|
| Kcnc3 | 1,0098 |
| Lpl | 1,0454 |
| Map3k10 | -1,0989 |
| Ncor1 | 1,1204 |
| Nefl | -1,5326 |
| Ngef | -1,0468 |
| Ntrk2 | 1,2431 |
| Pacsin1 | -1,0324 |
| Pgk1 | 1,0454 |
| Plcb4 | 1,0238 |
| Plod2 | -1,0837 |
| Ppargc1a | 1,3794 |
| Ppp3ca | -1,0253 |
| Prkcb | 1,1050 |
| Prpf40a | 1,2518 |
| Ptpn11 | 1,4083 |
| Rab6 | 1,0673 |
| Rcor1 | 1,0168 |
| Rest | 1,5845 |
| Rgs4 | -1,2449 |
| Rilp | 1,9106 |
| Rph3a | -1,3717 |
| Rxrb | -1,0042 |
| Sgk1 | 2,7207 |
| Sin3a | 1,0028 |
| Slc14a1 | 1,8583 |
| Slc25a4 | 1,0822 |
| Snap25 | 1,0168 |
| Sod1 | 1,1127 |
| Sox2 | -1,1142 |
| Sp1 | -1,1535 |
| Sympk | -1,0989 |
| Syn1 | 1,0098 |
| Tac1 | 1,1439 |
| Tbp | 1,1761 |
| Tgm2 | 2,0907 |
| Tollip | 1,3324 |
| Trp53 | 1,1599 |
| Tubb5 | -1,1297 |
| Zbtb16 | 1,6178 |
| Actb | 1,1439 |
| B2m | 1,0310 |
| Gapdh | -1,1065 |
| Gusb | -1,0253 |
| Hsp90ab1 | -1,0396 |

**3NP+CBG vs control**

| Gene | Fold Up- or Down-Regulation |
|---|---|
| Akt1 | -1,1810 |
| Apoe | -1,1892 |
| Aqp1 | 1,1096 |
| Arfip2 | -1,0570 |
| Atp2b2 | -1,0792 |
| Bax | -1,0425 |
| Bbox1 | -1,1647 |
| Bdnf | 1,0644 |
| C3 | 1,0792 |
| Calb1 | -1,2226 |
| Casp3 | -1,3195 |
| Casp8 | 1,3566 |
| Cd44 | -1,3104 |
| Cltc | -1,1096 |
| Cnr1 | -1,1173 |
| Creb1 | 1,1567 |
| Crebbp | 1,0570 |
| Dctn1 | 1,1487 |
| Dlg4 | -1,1408 |
| Eef1a2 | 1,0070 |
| Egfr | -1,1647 |
| Elmo1 | 1,1810 |
| Fgf12 | 1,0353 |
| Gabrd | -1,0570 |
| Gja1 | -1,0353 |
| Gjb6 | 1,1567 |
| Gnaq | -1,0353 |
| Gpx1 | 1,2746 |
| Grb2 | 1,0353 |
| Grin2a | 1,1892 |
| Grin2b | 1,2058 |
| Grm5 | 1,0070 |
| Hap1 | -1,0497 |
| Hdac1 | 1,0353 |
| Hdac2 | -1,1567 |
| Hip1 | 1,1408 |
| Homer1 | 1,0281 |
| Hpca | 1,0210 |
| Htt | -1,0000 |
| Ift57 | 1,1329 |
| Igf1 | 1,1251 |
| Itpr1 | 1,1892 |
| Kcnab1 | -1,1647 |
| Kcnab2 | 1,0353 |

| Gene | Fold Up- or Down-Regulation |
|---|---|
| Kcnc3 | 1,0943 |
| Lpl | -1,0140 |
| Map3k10 | 1,0210 |
| Ncor1 | 1,0718 |
| Nefl | -1,2142 |
| Ngef | -1,1096 |
| Ntrk2 | 1,0570 |
| Pacsin1 | -1,0425 |
| Pgk1 | -1,1408 |
| Plcb4 | 1,1408 |
| Plod2 | -1,1647 |
| Ppargc1a | 1,1728 |
| Ppp3ca | 1,0353 |
| Prkcb | 1,0644 |
| Prpf40a | 1,0281 |
| Ptpn11 | 1,0792 |
| Rab6 | 1,1647 |
| Rcor1 | -1,0281 |
| Rest | 1,0210 |
| Rgs4 | -1,1647 |
| Rilp | -1,0570 |
| Rph3a | -1,0792 |
| Rxrb | 1,1567 |
| Sgk1 | -1,4142 |
| Sin3a | 1,1019 |
| Slc14a1 | 1,2311 |
| Slc25a4 | -1,1096 |
| Snap25 | -1,0140 |
| Sod1 | 1,0210 |
| Sox2 | -1,1019 |
| Sp1 | -1,0867 |
| Sympk | 1,0425 |
| Syn1 | 1,0570 |
| Tac1 | 1,3472 |
| Tbp | 1,1975 |
| Tgm2 | 1,5911 |
| Tollip | 1,0718 |
| Trp53 | 1,3851 |
| Tubb5 | -1,0718 |
| Zbtb16 | 1,0943 |
| Actb | -1,0644 |
| B2m | 1,1728 |
| Gapdh | -1,2483 |
| Gusb | 1,0867 |
| Hsp90ab1 | 1,0425 |

**Table 2S.** HD array analysis (84 genes) showing their up- or down-regulatory responses in the striatum of wild-type and R6/2 mice treated with CBG or vehicle. Values correspond to number of folds that a specific gene is up- (positive value) or down-regulated (negative value).

## R6/2 vs wild-type

| Gene | Fold Up- or Down-Regulation |
|---|---|
| Akt1 | 1,0733 |
| Apoe | -2,3424 |
| Aqp1 | -1,4025 |
| Arfip2 | -1,0629 |
| Atp2b2 | -2,5633 |
| Bax | 1,1503 |
| Bbox1 | 2,5883 |
| Bdnf | -1,2209 |
| C3 | -2,6537 |
| Calb1 | 1,4969 |
| Casp3 | 1,3491 |
| Casp8 | 1,3398 |
| Cd44 | **-3,3589** |
| Cltc | 1,0084 |
| Cnr1 | **-3,9945** |
| Creb1 | 1,1345 |
| Crebbp | 1,6609 |
| Dctn1 | -1,8125 |
| Dlg4 | -2,4083 |
| Eef1a2 | -1,8895 |
| Egfr | 1,3122 |
| Elmo1 | -1,0852 |
| Fgf12 | -1,1157 |
| Gabrd | **-7,0031** |
| Gja1 | **-3,3823** |
| Gjb6 | -1,2041 |
| Gnaq | 1,6044 |
| Gpx1 | 1,5178 |
| Grb2 | 1,8050 |
| Grin2a | -1,1958 |
| Grin2b | -2,1555 |
| Grm5 | 1,0367 |
| Hap1 | 2,2847 |
| Hdac1 | 1,7195 |
| Hdac2 | 2,2532 |
| Hip1 | -1,0338 |
| Homer1 | -1,9026 |
| Hpca | **-3,0908** |
| Htt | -1,6223 |
| Ift57 | 1,0733 |
| Igf1 | -1,8506 |
| Itpr1 | -2,7856 |
| Kcnab1 | -1,5670 |
| Kcnab2 | 1,1503 |

| Gene | Fold Up- or Down-Regulation |
|---|---|
| Kcnc3 | -1,1313 |
| Lpl | -1,4722 |
| Map3k10 | 1,0807 |
| Ncor1 | 1,3967 |
| Nefl | 1,4459 |
| Ngef | -1,8251 |
| Ntrk2 | 1,0958 |
| Pacsin1 | -1,6795 |
| Pgk1 | 1,1423 |
| Plcb4 | -1,5562 |
| Plod2 | 1,7435 |
| Ppargc1a | 1,0014 |
| Ppp3ca | **-3,0063** |
| Prkcb | -1,1631 |
| Prpf40a | 1,3491 |
| Ptpn11 | 1,1663 |
| Rab6 | 1,3122 |
| Rcor1 | 2,8521 |
| Rest | -1,4221 |
| Rgs4 | **-3,2898** |
| Rilp | -1,2816 |
| Rph3a | -1,5347 |
| Rxrb | **-3,8053** |
| Sgk1 | -1,5889 |
| Sin3a | -2,3751 |
| Slc14a1 | -1,3268 |
| Slc25a4 | 1,5497 |
| Snap25 | -1,0777 |
| Sod1 | 1,9480 |
| Sox2 | -1,1080 |
| Sp1 | 1,6609 |
| Sympk | **-5,4945** |
| Syn1 | 1,0882 |
| Tac1 | 1,2159 |
| Tbp | 1,5284 |
| Tgm2 | 1,6609 |
| Tollip | 1,4459 |
| Trp53 | 1,2159 |
| Tubb5 | -1,1004 |
| Zbtb16 | -1,4722 |
| Actb | -1,9697 |
| B2m | 1,5497 |
| Gapdh | 1,0367 |
| Gusb | 1,1583 |
| Hsp90ab1 | 1,0585 |

## R6/2+CBG vs wild-type

| Gene | Fold Up- or Down-Regulation |
|---|---|
| Akt1 | -1,0483 |
| Apoe | -2,5105 |
| Aqp1 | -1,5999 |
| Arfip2 | 1,0439 |
| Atp2b2 | -3,0063 |
| Bax | -1,0556 |
| Bbox1 | -1,2995 |
| Bdnf | 1,2414 |
| C3 | -3,7529 |
| Calb1 | -1,5562 |
| Casp3 | 1,2675 |
| Casp8 | -1,4722 |
| Cd44 | -1,2728 |
| Cltc | -1,1080 |
| Cnr1 | -4,0784 |
| Creb1 | -1,1392 |
| Crebbp | 1,0585 |
| Dctn1 | -1,7630 |
| Dlg4 | -2,4419 |
| Eef1a2 | -1,6223 |
| Egfr | -1,2294 |
| Elmo1 | -1,8000 |
| Fgf12 | -1,1157 |
| Gabrd | -4,3409 |
| Gja1 | -3,3357 |
| Gjb6 | -1,1794 |
| Gnaq | 1,1266 |
| Gpx1 | 1,0439 |
| Grb2 | 1,2414 |
| Grin2a | -1,3454 |
| Grin2b | -1,8895 |
| Grm5 | -1,8251 |
| Hap1 | -1,0556 |
| Hdac1 | 1,2763 |
| Hdac2 | 1,2852 |
| Hip1 | -1,8378 |
| Homer1 | -2,2315 |
| Hpca | -4,1068 |
| Htt | -1,9159 |
| Ift57 | -1,1471 |
| Igf1 | -1,2294 |
| Itpr1 | -2,1705 |
| Kcnab1 | -2,5991 |
| Kcnab2 | -1,1004 |

| Gene | Fold Up- or Down-Regulation |
|---|---|
| Kcnc3 | -1,1313 |
| Lpl | -2,9856 |
| Map3k10 | -1,0852 |
| Ncor1 | 1,0154 |
| Nefl | 1,2414 |
| Ngef | -2,1856 |
| Ntrk2 | -1,1958 |
| Pacsin1 | -1,4025 |
| Pgk1 | 1,0295 |
| Plcb4 | -1,3268 |
| Plod2 | -1,1712 |
| Ppargc1a | -1,2041 |
| Ppp3ca | -2,5456 |
| Prkcb | -2,0820 |
| Prpf40a | 1,2243 |
| Ptpn11 | -1,1712 |
| Rab6 | 1,3305 |
| Rcor1 | 1,2329 |
| Rest | -2,0392 |
| Rgs4 | -3,2898 |
| Rilp | -1,4928 |
| Rph3a | -1,4520 |
| Rxrb | -1,8506 |
| Sgk1 | -1,4722 |
| Sin3a | -1,8125 |
| Slc14a1 | -1,7876 |
| Slc25a4 | 1,3775 |
| Snap25 | 1,0733 |
| Sod1 | 1,1034 |
| Sox2 | -1,2816 |
| Sp1 | 1,0439 |
| Sympk | -3,3823 |
| Syn1 | 1,0882 |
| Tac1 | -1,3177 |
| Tbp | 1,1423 |
| Tgm2 | 1,2763 |
| Tollip | 1,1663 |
| Trp53 | 1,1423 |
| Tubb5 | -1,0703 |
| Zbtb16 | 1,2588 |
| Actb | -1,5889 |
| B2m | 1,2329 |
| Gapdh | 1,1111 |
| Gusb | 1,1991 |
| Hsp90ab1 | -1,0338 |

# CHAPTER 2

## Evaluation of Sativex in a pilot clinical trial with patients of HD.

All the preclinical data obtained (here and previously) from the different studies exploring the potential of different cannabinoid compounds as disease-modifying treatments in animal and cellular models of HD supports the evaluation any cannabinoid formulation in the clinical field. The data generated so far support more the use of a broad-spectrum cannabinoid or a combination of cannabinoids with complementary pharmacological profiles, rather than using a very selective cannabinoid. In this Chapter, we present the results of a double-blind, randomized, cross-over, placebo-controlled pilot clinical trial with Sativex in patients with HD. Sativex is an already licenced medicine in Spain for the treatment of spasticity in patients with MS. The primary endpoint of this study was achieved, since the treatment with Sativex was safe and well tolerated. However, it did not cause any improvement in motor performance and cognitive and psychiatric measures. Besides, no change was observed in the biomarkers examined, except for an increase in $CB_2$ expression in the patients' lymphocytes during Sativex-treatment phase, which was interpreted as a possible cytoprotective and antiinflamatory response given the functions of this receptor. Additional studies, with more subjects and in particular, longer treatment duration are proposed to further evaluate if Sativex can produce a clinical benefit in HD patients.

This study was done in collaboration with the group led by Justo García de Yébenes at "Hospital Ramón y Cajal", and in collaboration with the groups led by Manuel Guzmán at "Universidad Complutense" and Julián Romero at "Hospital Universitario Fundación Alcorcón". We participated in the analysis of gene expression of different cannabinoid and HD markers in the lymphocytes of HD patients.

**Papers included in this Chapter:**

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ORIGINAL COMMUNICATION

# A double-blind, randomized, cross-over, placebo-controlled, pilot trial with Sativex in Huntington's disease

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**Abstract** Huntington's disease (HD) is a neurodegenerative disease for which there is no curative treatment available. Given that the endocannabinoid system is involved in the pathogenesis of HD mouse models, stimulation of specific targets within this signaling system has been investigated as a promising therapeutic agent in HD. We conducted a double-blind, randomized, placebo-controlled, cross-over pilot clinical trial with Sativex®, a botanical extract with an equimolecular combination of delta-9-tetrahydrocannabinol and cannabidiol. Both Sativex® and placebo were dispensed as an oral spray, to be administered up to 12 sprays/day for 12 weeks. The primary objective was safety, assessed by the absence of more severe adverse events (SAE) and no greater deterioration of motor, cognitive, behavioral and functional scales during the phase of active treatment. Secondary objectives were clinical improvement of Unified Huntington Disease Rating Scale scores. Twenty-six patients were randomized and 24 completed the trial. After ruling-out period and sequence effects, safety and tolerability were confirmed. No differences on motor ($p = 0.286$), cognitive ($p = 0.824$), behavioral ($p = 1.0$) and functional ($p = 0.581$) scores were detected during treatment with Sativex® as compared to placebo. No significant molecular effects were detected on the biomarker analysis. Sativex® is safe and well tolerated in patients with HD, with no SAE or clinical worsening. No significant symptomatic effects were detected at the prescribed dosage and for a 12-week period. Also, no significant molecular changes were observed on the biomarkers. Future study designs should consider higher doses, longer treatment periods and/or alternative cannabinoid combinations.

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**Keywords** Huntington's disease · Cannabinoid · Sativex · Clinical trial

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## Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder, that progressively affects cognition, behavior and movement. HD is caused by an unstable expansion of the CAG trinucleotide repeat in the *Huntingtin* (*HTT*) gene, producing generalized neuronal degeneration that specially affects the striatum [1–3].

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There is no known neuroprotective therapy available for HD and current symptomatic treatment is limited. Previous studies in animal models of HD suggest that cannabinoid-based treatments might have a neuroprotective effect. The endocannabinoid system has been proven to confer neuroprotection through its direct participation in cellular survival pathways and in neurogenesis control [4–7]. Given that the endocannabinoid system is involved in HD pathogenesis, stimulation of specific targets within this signaling system has been investigated as a promising therapeutic strategy that improves deficits in HD models [8–12]. Some cannabinoids are known to inhibit excitotoxicity events, oxidative stress and neuroinflammation, which makes them attractive for the treatment of not only of HD, but also other neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [13, 14].

There are two major cannabinoid receptor types: the CB1 receptor, which is widely distributed through many different regions of the central nervous system, particularly in the basal ganglia, cortex, hippocampus and cerebellum, and the CB2 receptor, which is expressed mainly in the immune system modulating inflammation. Other non-classic receptors, such as TRPV1 (transient receptor potential cation channel, subfamily V, member 1) have also been proposed as potential cannabinoid receptors [15–17]. CB1 receptors progressively lose their functionality from HD early stages, potentially increasing vulnerability to the different cytotoxic stimuli related to cellular damage [8, 18], although a recent study has unequivocally demonstrated that the pool of CB1 receptors that are neuroprotective in HD mouse models would be located in neurons that do not degenerate primarily in HD (i.e. corticostriatal neurons) [19]. In normal conditions, CB2 receptors are found at very small levels in the striatum, but an up regulation can be detected in response to cellular damage. CB2 activation in glial cells could improve homeostasis in HD by reducing the cytotoxic influence of reactive microglial cells and increasing the trophic support provided by astrocytes [20]. The reduction of oxidative stress caused by cannabinoids via cannabinoid receptor-independent mechanisms has also been described [9, 21].

Previous clinical trials with cannabinoids in HD were based on the change of symptoms, such as chorea and behavioral alterations. Nabilone, a synthetic cannabinoid receptor agonist, was investigated in two non-controlled trials with contradictory results [22, 23]. One of these trials showed an improvement in hyperkinesis and behavioral alterations [23], while in the other trial a worsening was observed [22]. Positive results were found with the same compound in a cross, double blinded, placebo controlled trial [24]. CBD (cannabidiol) effects were also studied in another trial with fifteen HD patients, and although it was well tolerated, no positive results were obtained [25].

Sativex® mouth spray solution is a standardized botanic extract with an approximately equimolar combination of Δ9-Tetrahydrocannabinol (THC) and cannabidiol (CBD) (specifically, 2.7 mg THC/2.5 mg CBD per spray) [7, 12, 26]. These two compounds were tested separately in animal models of striatal damage with positive results [27, 28], and further studies conducted with a Sativex-like combination of both phytocannabinoids enhanced these benefits [29]. Although the long-term effects of Sativex® use are still unknown, it is currently approved for the treatment of multiple-sclerosis associated spasticity in many countries as well as for neuropathic pain in Canada. CBD has shown good tolerance and low toxicity. Moreover, CBD could counteract possible adverse effects related to THC chronic use, such as psychotic alterations [26], a mayor concern in HD. For all these reasons, we decided to test whether Sativex® was an appropriate medicine for the experimental treatment of HD.

## Patients and methods

### Patient selection

The study was performed at Ramón y Cajal Hospital, Madrid, Spain. The protocol and consent forms were approved by the Spanish Agency of Medicines and the hospital's local Research Ethical Committee. GW Pharmaceuticals Ltd. supplied the Sativex® and identical placebo vials.

### Inclusion criteria

(1) HD patients, older than 18 years and able to understand the study protocol, to attend the study visits and to provide written informed consent, (2) Stable baseline medication for at least 6 weeks prior to randomization, (3) UHDRS-motor score ranging from 5 to 50, (4) Good cognitive status (MMSE >25) at the screening visit, with no evidence of major depression, and no evidence of psychosis.

### Exclusion criteria

(1) Pregnant or lactating women, (2) History of drug addiction, (3) History of psychosis or suicidal ideation, (4) Diseases of the oral cavity preventing the safe administration of the drug, (5) Drug administration contraindicated according to the summary of product characteristics (SmPC), (6) Active consumers of marijuana or other cannabis-derived products.

### Patients

Twenty-six patients were recruited from the Ramón y Cajal Hospital Huntington Disease clinics. A previous selection

from the Huntington's disease clinics patient database was performed according to the inclusion and exclusion criteria to optimize the screening. Written informed consent was obtained from all participants.

### Outcome measures

The main objective in the study was to assess Sativex® safety and tolerability administered to HD patients for 12 weeks. The primary outcome measurements were the absence of Severe Adverse Events (SAE) reported during a time frame of 8 months and the absence of worsening in clinical symptoms as measured by the motor, cognitive, psychiatric and functional Unified Huntington's Disease Rating Scale (UHDRs) scores, at the end of both treatment periods.

The mUHDRS scale ranges from 0 to 124 [30]. A change of three points or more in the total mUHDRS score was considered a clinically relevant effect. The motor component of UHDRS scale was sub-divided for analysis purposes in four items: eye movements, maximal dystonia, maximal chorea and a sum of the remaining motor items. Cognition was assessed through cUHDRS (Verbal Fluency, Categorical Fluency and Stroop Interference). Psychiatric changes were assessed with the Hospital Anxiety and Depression Scales (HADS), the neuropsychiatric inventory (NPI) and the behavioral UHDRS scale. Functional component changes were assessed with the fUHDRS. Relevant clinical worsening was defined as a change of 10 or more in the cognitive scales, change of 3 or more in the cUHDRS subscale and a change of 2 or more in the fUHDRS subscale.

To evaluate putative subclinical molecular effects of cannabinoids we have measured the following biomarkers measured according to already published standardized procedures: (1) cerebrospinal fluid (CSF) protein concentrations including Tau, p-Tau and amyloid β-42 peptide (aβ-42) [31]; (2) CSF monoamine levels including 3-methoxy-4-hydroxyphenylglycol (MHPG), 3,4-dihydroxyphenylacetic (DOPAC), tryptophan (Trp), 5-hydroxyindoleacetic acid (5HIAA) and homovalinic acid (HVA) [32]; (3) plasma miR-34b levels [33]; (4) CSF endocannabinoid levels (anandamide, AEA; palmitoylethanolamide, PEA; 2-arachidonoyglicerol, 2-AG) [34, 35]; (5) CSF Brain-derived neurotrophic factor (BDNF); (6) sensitivity to toxins ($H_2O_2$, rotenone, kainic acid, quinolinic acid, malonate) in skin fibroblasts [36] and (7) gene expression of endocannabinoid system elements in peripheral lymphocytes [37]. The election of this panel of analyzes was based on the previous evidence on their potential as HD activity biomarkers.

### Study design

Patients were randomized to either Sativex® followed by placebo (Group A) or placebo followed by Sativex® (Group B). The two treatment blocks lasted 12 weeks each, separated by a 4-week washout period. The first 4 weeks of each period were of titration, gradually increasing Sativex® or placebo doses (as specified in the SmPC). Six visits were carried out on weeks 0 (screening), 4 (randomization and beginning of the first period of treatment), 16 (end of the first phase of treatment and beginning of washout period), 20 (beginning of the second period of treatment), 32 (end of the second period of treatment) and 36 (end of the trial). Each visit included hematological and biochemical blood analysis, a 12-lead ECG and urine sampling determining cannabinoid concentration to check compliance. Patients were given the option to sign a second informed consent for CSF donation at visits 3 and 6, both after 12 weeks of placebo or Sativex® administration. Likewise, at the same visits, the patients were given the option of signing a third informed consent for donation of skin biopsies that were used to obtain cultures of fibroblasts to be used in pharmacological studies [36, 38] (Fig. 1).

### Randomization and statistical analysis

Randomization was carried out through a random number simple table, specifically developed by the Biostatistical Service at Hospital Ramón y Cajal. Patients and clinical raters were blind to treatment allocation. The placebo used was a solvent, packed in containers identic to those of the studied drug. GW Pharma Ltd (Porton Down Science Park, Salisbury, Wiltshire, SP4 OJQ, UK) provided both placebo and Sativex®. An independent safety committee monitored all the trial.

The number of patients to be included in the study was calculated rescaling biomarker variables so as the means would be 100, assuming that the mean after treatment would be 85, with a difference in standard deviation of 20. This would give an effect size of 0.75 with an alpha level of 0.05 and 10 % power, assuming a 10 % drop-out rate. According to these data, we estimated that 24 patients were enough for this proof of concept study. To prevent possible drop-outs, 2 additional subjects were included.

Changes in clinical scales were calculated comparing values obtained in the active drug phase with those of the placebo phase. Results in parametric variables are given as mean ± standard deviation. Comparison was performed through paired *t*. The significance level required was $p < 0.05$. The statistical analysis of adverse effects was carried out through Chi-square comparison of parameters corresponding to active drug phase and placebo phase. Results are given in frequency tables. Comparison of the responses between the different treatment groups was performed through paired Student's *t* or Wilcoxon test, depending on the respective variable distribution, and applying Bonferroni correction for multiple comparisons.

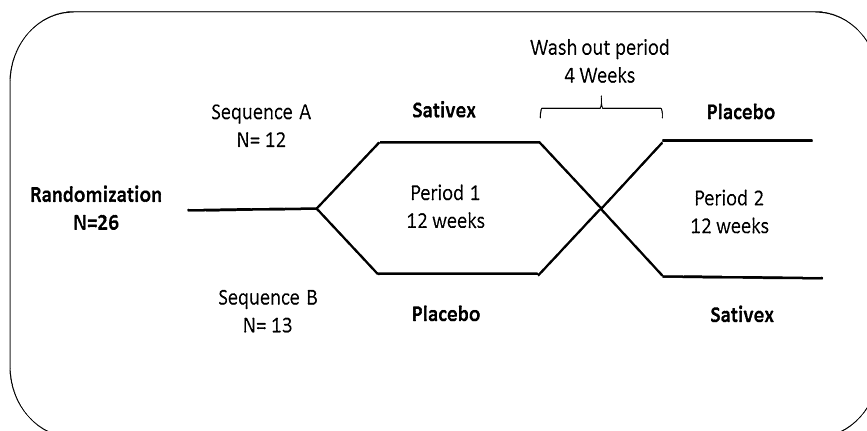


**Fig. 1** Study flow chart

Following protocol indications, only patients that had completed the two treatment phases (placebo and active drug) were included in the study. Sequence period and treatment effects were evaluated.

## Results

Twenty-six patients were recruited from the Ramón y Cajal Hospital Huntington's Disease clinics, in the disease initial phase. One patient withdrew from the study before randomization due to a cholelithiasis that required surgical intervention. Twenty-five patients (14 male) were thus allocated to treatment over a period of 3 months: 12 to Sativex® followed by placebo, and 13 to placebo followed by Sativex®. The mean age at randomization was 47.6 (SD 12.4; range 26–65 years). The mean age at disease onset was of 41 (SD 0.5) years. The mean time from disease onset was of 6.6 years (SD 4.3). The mean number of CAG repeats in the longest allele was almost 46. Most patients were under stable symptomatic treatment, with a great predominance of benzodiazepines (60 %) (Table 1).

The baseline characteristics of both groups were compared in both pre-treatment visits. In the first basal visit, group B (placebo-Sativex) presented significant lower scores in eye movements (7 versus 4, $p = 0.013$) and total mUHDRS scale (32 versus 20, $p = 0.004$). Values for maximal dystonia were on the limit of statistical significance (2 versus 1, $p = 0.048$). Differences between the two groups in the other assessed components was not statistically significant, except for interference Stroop which presented a lower value for the group A (Sativex-placebo) (18 versus 28, $p = 0.045$). In the second pre-treatment visit, significant differences were detected for eye movements (6.5 versus 2, $p = 0.013$), total mUHDRS (33.5 versus 22, $p = 0.003$), HADS (14 versus 10, $p = 0.006$) and NPI (12 versus 7; $p = 0.023$). In all cases, the most unfavorable scores were those belonging to the group treated with Sativex® in the first period and with placebo in the second one (see Table 1 supplementary material).

Treatment compliance, assessed checking the returned packages and through the urine test, reached 95.8 %. McNemar test for paired data confirmed the absence of statistically significant differences between drug and placebo tolerability ($p = 0.414$). A total of 20 patients (80 %) tolerated the 12 daily Sativex® sprays and 22 patients (88 %) tolerated the 12 daily placebo sprays.

### Biomarkers

*CSF protein concentrations* Changes of CSF protein concentrations are a well-documented marker of neurodegeneration and reflect neuropathological changes in Alzheimer's disease and other neurodegenerative diseases [39]. Also, Tau and p-Tau are validated markers of neuronal damage. However, we did not find any significant differences between the patients tretated with Sativex® and the patients who received placebo (Table 2; Fig. 2).

*CSF monoamine levels* Measurement of monoamine metabolites can be used to investigate serotonin and catecholamine metabolism, thus providing an indication of the activity of central monoaminergic neuronal pathways. Changes in the CSF monoamine metabolite concentrations have been linked to various disease phenotypes, including HD, and several psychiatric symptoms, such as depression and suicidal ideation. Also, it is known that certain medications have an impact on the CSF metabolite concentrations, reflecting an influence on the monoaminergic pathways [32]. However, we did not observe any significant differences between the placebo and Sativex® groups in any of the monoamine metabolites analyzed (Table 2; Fig. 2).

*Plasma miR-34b levels* MicroRNA formation is altered by mutant huntingtin and microRNAs are present in plasma and have been proposed as useful biomarkers in HD,

**Table 1** Patient demographic and baseline characteristics

| | Total, $N = 25$ | Group A (Sativex®-Placebo) $N = 12$ | Group B (Placebo-Sativex®) $N = 13$ |
|---|---|---|---|
| Mean ± SD age, years | 47.6 ± 12.4 | 49.2 ± 11.4 | 46.1 ± 13.6 |
| Male:female | 14:11 | 7:5 | 7:6 |
| Age at onset ± SD, years | 41.0 ± 10.5 | 40.7 ± 9.4 | 41.1 ± 11.8 |
| Caucasian:Latino | 24:1 | 12:0 | 12:1 |
| Mean ± SD duration, years | 6.6 ± 4.3 | 8.4 ± 4.7 | 4.8 ± 3.1 |
| Mean CAG ± SD | 45.7 ± 3.7 | 44.9 ± 3.4 | 46.4 ± 3.9 |
| BMI (kg/m$^2$) | 22.0 ± 3.4 | 21.9 ± 2.9 | 22.2 ± 4.1 |
| Other medication | | | |
| Dopamine blockers | 6 | 2 | 4 |
| Tetrabenazine | 6 | 4 | 2 |
| Amantadine | 1 | 1 | 0 |
| Antidepressants | 5 | 3 | 2 |
| Benzodiacepines | 15 | 8 | 7 |

Values are expressed in relation to order of drug administration (mean ± SD)

*BMI* body mass index, *SD* standard deviation

**Table 2** Biomarker determinations in patients after Sativex and placebo treatments

| | Sativex | Placebo | *P* value |
|---|---|---|---|
| CSF protein concentrations | | | |
| aβ-42 (ρg/mL) | 757.1 (318.9) | 655.3 (275.9) | 0.258 |
| Tau (ρg/mL) | 206.4 (91.3) | 202.4 (76.0) | 0.876 |
| p-Tau (ρg/mL) | 47.9 (14.9) | 50.2 (15.6) | 0.627 |
| CSF monoamine levels | | | |
| MHPG (ηg/mL) | 14.5 (3.7) | 14.5 (3.9) | 0.992 |
| DOPAC × 100 (ηg/mL) | 0.3 (0.1) × 100 | 0.3 (0.2) × 100 | 0.876 |
| Trp/10 (ηg/mL/10) | 588.6 (186.7)/10 | 621.0 (233.2)/10 | 0.618 |
| 5HIAA (ηg/mL) | 27.1 (9.3) | 27.8 (9.3) | 0.804 |
| HVA (ηg/mL) | 51.0 (19.1) | 53.1 (22.1) | 0.732 |
| Plasma miR-34b levels | 14.5 (1.0) | 14.3 (0.91) | 0.595 |
| CSF endocannabinoid levels | | | |
| AEA (pmol/mL) | <0.15 | <0.15 | – |
| PEA (pmol/mL) | 2.5 (0.6) | 2.8 (0.9) | 0.374 |
| 2-AG (pmol/mL) | <1 | <1 | – |
| CSF BDNF levels | 27.7 (8.1) | 30.2 (9.5) | 0.322 |
| Endocannabinoid gene expression in lymphocytes | | | |
| CB2 ($2^{-\Delta\Delta Ct}$) | 0.00046 (0.00006) | 0.00071 (0.00009) | <0.005 |
| FAAH ($2^{-\Delta\Delta Ct}$) | 0.00169 (0.00023) | 0.00227 (0.00049) | n.s. |
| MAGL ($2^{-\Delta\Delta Ct}$) | 0.00047 (0.00011) | 0.00061 (0.00017) | n.s. |
| ACO2 ($2^{-\Delta\Delta Ct}$) | 0.00821 (0.00140) | 0.00641 (0.00112) | n.s. |
| UCP2 ($2^{-\Delta\Delta Ct}$) | 0.126 (0.018) | 0.108 (0.013) | n.s. |

Values express as mean ± SD

*CSF* cerebrospinal fluid, *miR* MicroRNA, *BDNF* brain derived neurotrophic factor, *MHPG* 3-methoxy-4-hydroxyphenylglycol, *DOPAC* 3,4 dihydroxyphenylacetic acid, *Trp* tryptophan, *5HIAA* 5-hydroxyindolacetic acid, *HVA* homovanilic acid, *AEA* anandamide, *PEA* palmitoylethanolamide, *2-AG* 2-arachidonoyglycerol, *FAAH* fatty acid amide hydrolase, *MAGL* monoacylglycerol lipase, *ACO2* aconitase-2, *UCP2* uncoupling protein-2, *n.s.* non significant

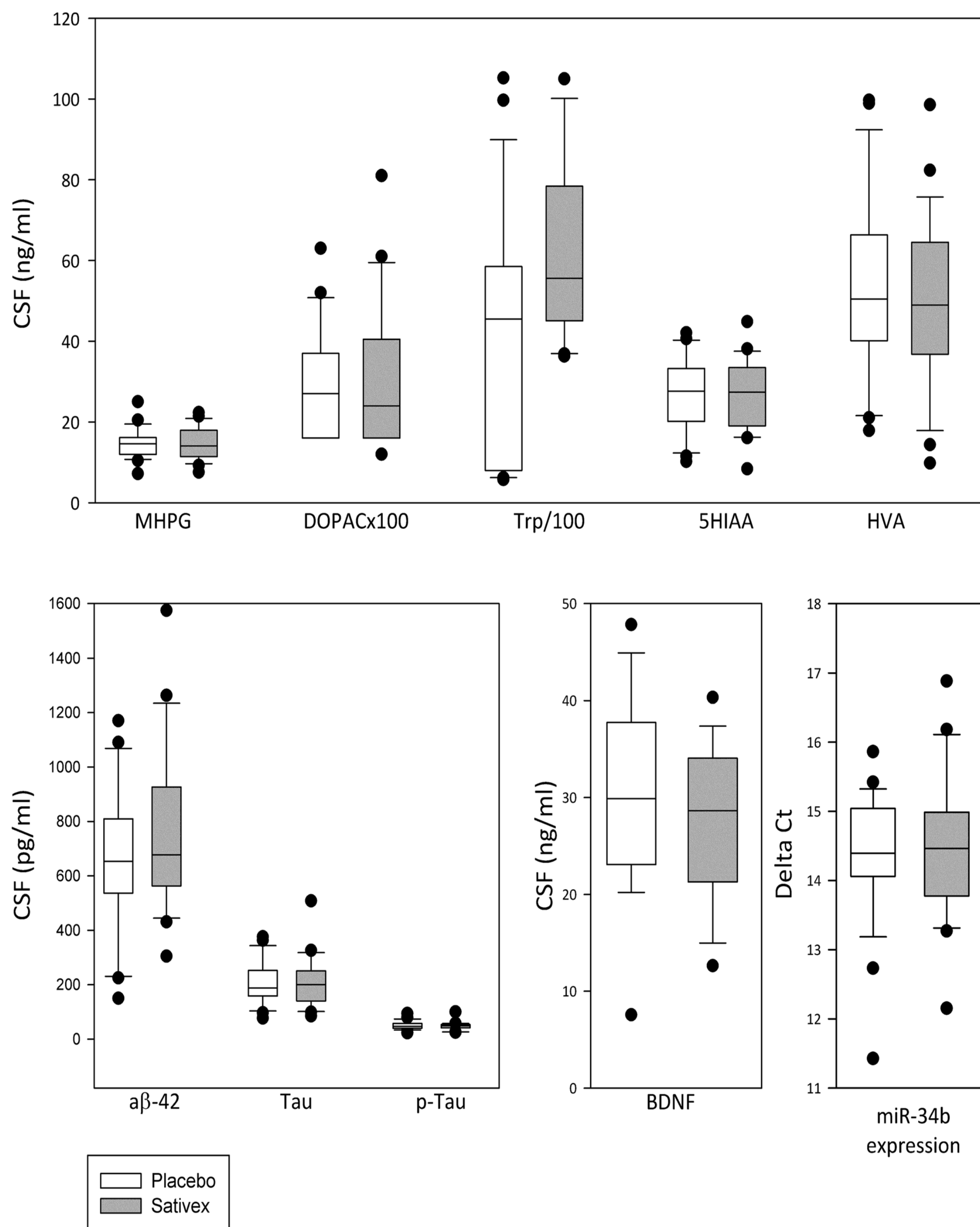


**Fig. 2** Total monoamine metabolites, aβ-42, Tau and p-Tau, BDNF and miR-34b expression levels in Placebo and Sativex® groups

particullary miR-34b, which is significantly elevated in HD gene carriers [33]. Hence, miR-34b expression was determined in plasma from the patients by qRT-PCR after RNA extraction. However, we did not find any significant difference between Sativex®-treated patients and placebo-treated patients (Table 2; Fig. 2).

*CSF endocannabinoid levels* CSF endocannabinoid levels were determined by liquid chromatography coupled to mass spectrometry as described previously [34, 35]. Endocannabinoid and PEA levels are known to change in the brain areas of transgenic mouse models of HD, reflecting an impaired endocannabinoid system, which is a hallmark of symptomatic HD [35]. Specifically, an increase in the CSF levels of putative neuroprotective substances, such as the endocannabinoid or endocannabinoid-like compounds, such as AEA, 2-AG and PEA could be considered as potentially benefitable to subjects treated with Sativex®. On the one hand, 2-AG and AEA levels were undetectable in all patients tested (less than 1 pmol/mL for 2-AG and less than 0.15 pmol/mL for AEA), thus

suggesting that HD may decrease 2-AG and AEA levels in CSF. This would be in line with the decrease in 2-AG and AEA levels found in the striatum of symptomatic R6/2 mice compared to wild-type animals [35]. On the other hand, PEA levels were readily detectable in all cases. However, no significant differences were observed between Sativex® and placebo-treated patients (Table 2).

*CSF BDNF concentration* The influence of BDNF, an key neurotrophin implicated in neuronal survival, has been widely investigated in HD and low levels of BDNF are considered key in the pathogenesis of HD in animal models [40, 41]. An increase in BDNF CSF levels could, therefore, be considered as a beneficial marker in Sativex®-treated patients. However, we did not find significant differences between the placebo and the Sativex® groups (Table 2; Fig. 2).

*Sensitivity to toxins in skin fibroblasts* We investigated the influence of Sativex® and placebo on skin fibroblasts and its potential for protection against toxic effects of several agents. Skin fibroblasts, obtained from the patients and grown in high serum (20 %) as described [36] were transferred to DMEM supplemented with low serum (0.2 %). Saline vehicle, 150 µM rotenone, 50 µM $H_2O$ [2, 30] 0 µM malonate, 300 µM quinolinic acid or 1.5 mM kainic acid was subsequently added and cell viability was measured after 48 h by the MTT method. There were no significant differences in the sensitivity to the toxins between cells from Sativex®-treated patients and cells from placebo-treated patients (Table 3).

*Gene expression of endocannabinoid system elements in peripheral lymphocytes* Endocannabinoid gene expression on lymphocytes is known to mirror metabolic changes in the brain and has been considered as a marker of disease and a potential tool to gage the action of chemical agents on the effects of the mutant huntingtin protein [37]. Gene expression for three key endocannabinoid elements was measured in lymphocytes of HD patients during the active (Sativex®) and placebo phases. Our data revealed no differences in the expression of FAAH and MAGL, the two major endocannabinoid-degrading enzymes, but an interesting elevation of CB2 receptor gene expression due to the Sativex treatment (Table 2). In addition to endocannabinoid elements, we also analyzed lymphocyte gene expression for two genes encoding proteins involved in metabolism and oxidative stress, aconitase-2 (ACO2) and uncoupling protein-2 (UCP2), which have been previously to be downregulated in HD patients compared to control subjects [42]. However, we did not find that such expected downregulation may be counteracted by the treatment with Sativex in agreement with the lack of effects on other biomarkers and in the progression of neurological decline in patients (Fig. 3).

## Safety and tolerability

Two SAEs were recorded, both occurring in the same patient during the placebo phase, and thus, were considered unrelated to the active treatment. The first SAE consisted on self-limited microcytic anemia, considered related to the use of non-steroidal antiinflamatory drugs (NSAIDs) and resolved after its discontinuation. The second SAE consisted on pregnancy, confirmed with a pregnancy test, taking place between visits 6 and 7 of the placebo phase. Both complications were reported as the protocol demanded, and followed until complete resolution.

No SAEs consisting on psychiatric alterations were detected neither during active Sativex® treatment or its withdrawal. Dizziness or disturbance in attention were the two most frequent adverse events (AA), with significant differences between drug and placebo periods, in agreement with other publications. Dizziness and disturbance in attention were both present in 16 % of patients during drug treatment period, while no case was reported during placebo period. The rest of AA showed a similar frequency in active, placebo, and no-treatment periods (Table 4).

## Scores in clinical UHDR scales

Period and sequence effects were discarded, and thus data from the two periods could be used for the analysis of the treatment effect. The results showing the differences between scores recorded in the basal and final visit of each period, are presented in Table 5. No differences on motor ($p = 0.286$), cognitive ($p = 0.824$), behavioral ($p = 1.0$) and functional ($p = 0.581$) scores were detected during

**Table 3** Sensitivity to toxins of skin fibroblasts from Sativex-treated and placebo-treated patients

| | $H_2O_2$ (50 µM) | Mal (300 µM) | QA (300 µM) | KA (1500 µM) | Rot (15 µM) |
|---|---|---|---|---|---|
| Sativex | 89 ± 2* | 96 ± 2 | 98 ± 2 | 97 ± 3 | 53 ± 6* |
| Placebo | 89 ± 3* | 101 ± 4 | 101 ± 4 | 98 ± 52 | 58 ± 7* |

Data are expressed as mean ± SEM

*Mal* malonate, *QA* quinolinic acid, *KA* kainic acid, *Rot* rotenone

* $P < 0.01$ from vehicle incubations

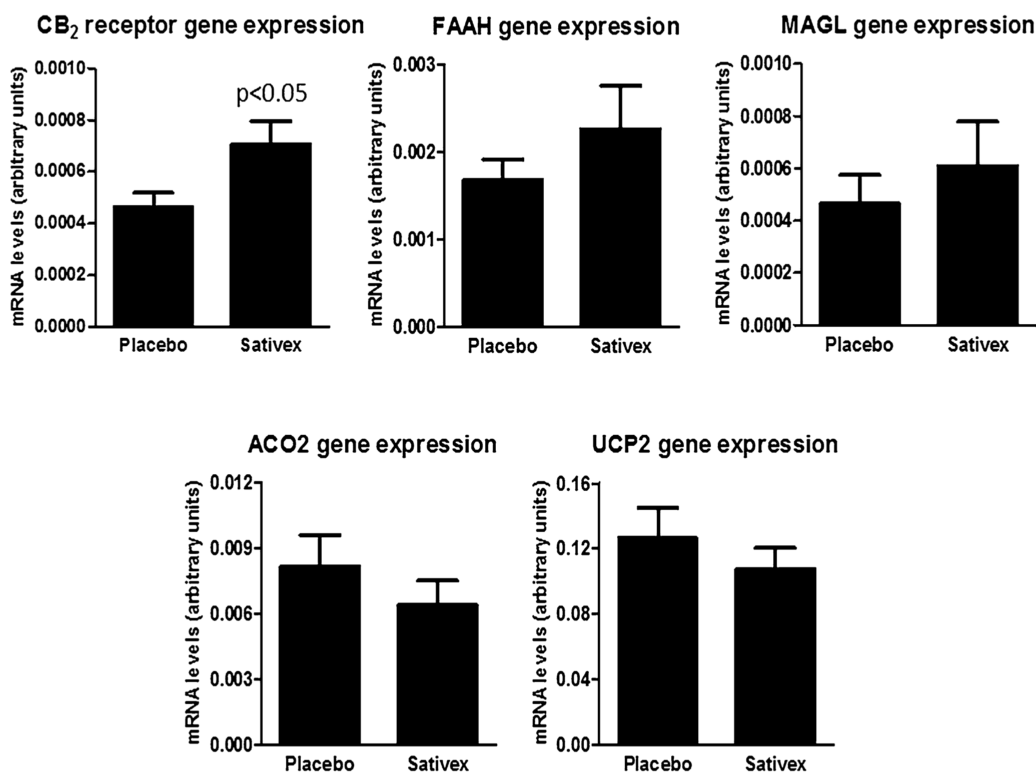


**Fig. 3** Gene expression

**Table 4** Reported adverse events

| | Sativex, $N = 25$ | Placebo, $N = 24$ | No treatment $N = 25$ | $p$ value |
|---|---|---|---|---|
| Anxiety | 6 (24) | 3 (12.5) | 5 (20) | 0.479 |
| Sleepiness | 5 (20) | 1 (4.17) | 3 (12) | 0.102 |
| Dizziness | 4 (16) | 0 | 2 (8) | 0.045* |
| Disturbance in attention | 4 (16) | 0 | 1 (0) | 0.045* |
| Insomnia | 2 (8) | 1 (4.17) | 3 (12) | 0.564 |
| Behavioral changes | 1 (4) | 3 (12.5) | 3 (12) | 0.317 |
| Fever | 1 (4) | 2 (8.33) | 2 (8) | 0.564 |
| Local infection | 4 (16) | 1 (4.17) | 1 (4) | 0.317 |
| Upper respiratory infection | 7 (28) | 8 (33) | 9 (36) | 0.317 |
| Diarrhea | 6 (24) | 3 (12.5) | 1 (4) | 0.414 |
| Vomits | 3 (12) | 0 | 3 (12) | 0.083 |
| Headache | 4 (16) | 4 (16.67) | 9 (36) | 1 |
| Muscular pain | 2 (8) | 2 (18.33) | 5 (20) | 1 |

Values are expressed as *n* (%), during the Sativex®, placebo and no-treatment periods

* $p < 0.05$

treatment with Sativex® as compared to placebo. Results of motor scale analysis showed no changes between active drug and placebo treatments (Table 5; Fig. 4). Individual evaluation of each of these clinical scales, indicates a non-significant trend towards improvement with placebo as compared to Sativex®.

## Discussion

Sativex® was safe and well tolerated in patients with HD. Neither clinical improvement nor worsening was detected for any motor, cognitive or psychiatric measures or the functional situation assessment. A remarkable finding was

**Table 5** Changes in outcomes between treatment allocations

| | Changes during Sativex® | Changes during Placebo | *p* value |
|---|---|---|---|
| Motor scores | | | |
| Eye movements | 0 (1.9) | 0.4 (1.3) | 0.238 |
| Maximal dystonia | −0.4 (1.6) | −0.4 (1.2) | 1.000 |
| Maximal chorea | 0 (2.2) | 0.6 (2.3) | 0.405 |
| Remaining items | −0.2 (3.4) | 1.4 (2.6) | 0.678 |
| TMS | −0.6 (6.4) | 2.0 (5.0) | 0.286 |
| Cognitive scores | | | |
| Verbal fluency | 0.6 (5.6) | −0.7 (6.6) | 0.405 |
| Categorical fluency | 11.7 (4.8) | 11.6 (5.1) | 0.824 |
| Stroop interference | 1.6 (6.2) | 0.7 (5.5) | 0.824 |
| Behavior scores | | | |
| bUHDRS | −2.6 (5.6) | −0.1 (8.0) | 1.000 |
| HADS | −2 (3.5) | −0.2 (5.1) | 0.405 |
| NPI | −2.6 (5.6) | −0.1 (7.9) | 0.134 |
| Function scores | | | |
| fUHDRS | 2.8 (11.6) | 0.3 (1.2) | 0.581 |

Data are presented as mean (SD)

*TMS* total motor score, *bUHDRS* behavioral Unified Huntington disease rating scale, *HADS* Hospital Anxiety and Depression Scale, *NPI* neuropsychiatric inventory, *fUHDRS* functional Unified Huntington Disease Rating Scale

* *P* value for the difference between changes in treatment allocations: Sativex® treatment vs. placebo treatment

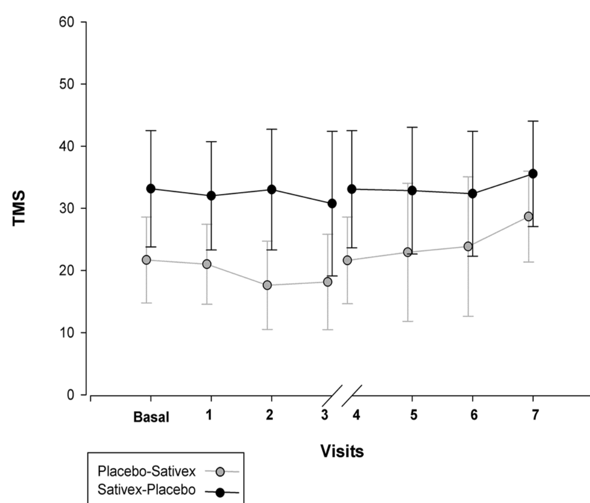


**Fig. 4** TMS

the absence of neuropsychiatric symptoms, a major concern due to their high prevalence in HD. There were no significant clinical variations, thus suggesting a relevant therapeutic response. Likewise, the lack of significant clinical effects of Sativex® was associated with a lack of changes in essentially all the biomarkers examined. The only exception was an elevation of CB2 receptor gene expression in peripheral lymphocytes due to the Sativex treatment. We interpret this elevation as a positive response elicited by the treatment and possibly aimed at enhancing the cytoprotective and anti-inflammatory role played by this cannabinoid receptor type in damaging conditions existing in HD, as described previously in patients and experimental models [20, 43]. However, it is possible that a longer period of treatment may be necessary to allow that the benefits derived from the activation of the CB2 receptor may be visible in the progression of the disease in patients. On the other hand, THC has been previously reported to increase BDNF levels in the brain of rodents [44] as well as in the plasma of human volunteers [45], while THC and CBD have been shown to modulate endocannabinoid degradation and, therefore, to change endocannabinoid levels [46, 47]. However, these short-term cannabinoid actions were not evident in the long-term cannabinoid treatments that we administered to the patients of our study.

Our results are in apparent contradiction with those reported in animal models [48], in which pharmacological administration of THC to mice expressing human mutant huntingtin exon 1 (R6/2 mice) exerted a therapeutic effect. It is likely that the therapeutic potential of cannabinoids in HD patients should be explored in the earlier phases of the disease, since progressive loss of CB1 receptors may attenuate its mechanisms of action [49]. It is possible that Sativex® high tolerability and the absence of clinical changes in HD symptomatology were related with the use

of the drug in too late disease stages, when the number of CB1 receptors is highly decreased. In this sense, the use of cannabinoids in earlier stages could maximize both, adverse events and clinical response.

In addition, it is likely that our experimental design was adequate to show safety but not well suited to prove efficacy. Additional studies of longer duration are likely to be more effective than crossed studies. Also, the use of other cannabinoids including CB1 and CB2 agonists with different ratios could exert different effects, and so might do TRPV1 receptor agonists, since the latter do not seem to disappear with HD progression [12]. The adequate safety showed in this clinical trial should encourage other studies with higher cannabinoid doses and longer administration periods.

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**Compliance with ethical standards**

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# CHAPTER 3

## Study of some of the molecular mechanisms involved in the effect of cannabinoid compounds in experimental models of HD.

In this Chapter we wanted to study more deeply some of the possible mechanisms involved in the positive effect of phytocannabinoids. In particular, we focused in the antioxidant effect and the possible involvement of NRF2 activation and promotion of antioxidant phase II response. To this aim, we tested the Sativex-like combination of $\Delta^9$-THC and CBD botanical extracts compared with each phytocannabinoid used individually in a HD cellular model of conditionally immortalized murine striatal neuroblasts (STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells). Firstly, we exposed these cells to agents that produced different cytotoxic events that occur in HD brains: glutamate (excitotoxicity), malonate (mitochondrial damage) and hydrogen peroxide (oxidative damage). In all cases, we confirmed an important extent of cell death, but our results showed that protection with phytocannabinoids was only visible against hydrogen peroxide insult. We found that CBD, but mainly Sativex, protected both cell lines against $H_2O_2$, although this effect was much more modest in STHdh$^{Q111/Q111}$ than in STHdh$^{Q7/Q7}$ cells. This effect was accompanied by an increase of HO1 gene expression, so next we tested the possible involvement of NRF2/ARE pathway in this phytocannabinoid-mediated neuroprotection. Our results did not confirm a role of NRF2/ARE in this process, so further research will be needed to dissect the potential mechanisms involved in the CBD and Sativex –mediated neuroprotection against oxidative damage in striatal cells.

A second specific objective in this chapter consisted in exploring whether endocannabinoids, in particular 2-AG, provide some type of tonic protection in HD, using the malonate model. Unexpectedly, we observed that inhibiting MAGL, rather than being neuroprotective, aggravated striatal damage, whereas the opposite happened with DAGL inhibition. This unexpected result was explained through a malonate-induced COX-2 up-regulation which contributes to convert 2-AG in prostaglandin-glycerol-esters. Such derivatives were highly neurotoxic. Using *in vitro* tools, M-213 cells, we proved that the induction of COX-2 and the generation of the 2-AG oxygenated derivatives, as well as that inhibition of COX-2 or the blockade of the targets for these derivatives reduced the neurotoxicity.

**Papers included in this Chapter:**

Valdeolivas, S., Rodriguez-Cueto, C. Sagredo, O. and Fernández-Ruiz, J. **Study of the involvement of NRF2-KEAP1/ARE signaling in the antioxidant effect of phytocannabinoids against hydrogen peroxide in a cellular model of Huntington's disease.** (Preliminary data not published).

Valdeolivas, S., Pazos, M.R., Bisogno, T., Piscitelli, F., Iannotti, F.A., Allara, M., Sagredo, O., Di Marzo, V. & Fernandez-Ruiz, J. (2013) **The inhibition of 2-arachidonoyl-glycerol (2-AG) biosynthesis, rather than enhancing striatal damage, protects striatal neurons from malonate-induced death: a potential role of cyclooxygenase-2-dependent metabolism of 2-AG.** Cell Death Dis, 4, e862.

# Study of the involvement of NRF2-KEAP1/ARE signaling in the antioxidant effect of phytocannabinoids against hydrogen peroxide in $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells, a cellular model of Huntington's disease

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## Abstract

Different phytocannabinoids have provided neuroprotection in experimental models of Huntington's disease (HD), even the issue has extended to the clinical scenario with the recent development of a trial using the phytocannabinoid-based medicine Sativex in HD patients. In the present study, we wanted to further investigate some of the molecular mechanisms underlying the positive effects of phytocannabinoids, using an in vitro strategy with striatal derived cell lines generated from a knock-in mouse containing homozygous huntingtin loci with a humanized exon 1 with either 7 ($STHdh^{Q7/Q7}$, WT cells) or 111 ($STHdh^{Q111/Q111}$, HD cells) polyglutamine repeats. We first investigated whether botanical extracts enriched in $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC-BDS) or cannabidiol (CBD-BDS), as well as their 1:1 combination as in Sativex®, were effective against different cytotoxic insults in $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells. We used excitotoxins, mitochondrial toxins, or hydrogen peroxide ($H_2O_2$), which resemble those cytotoxic events occurring in HD brains, although our data were positive only in the case of cells treated with $H_2O_2$. Thus, in the case of $STHdh^{Q7/Q7}$ cells, we found that CBD-BDS, alone or combined with $\Delta^9$-THC-BDS as in Sativex, but not $\Delta^9$-THC-BDS, prevented cell death after $H_2O_2$-induced oxidative damage. This protection was accompanied by an increase in heme oxygenase-1 (HO1) an enzyme induced by the activation of the transcription factor NRF2. Then we tried to confirm that the treatment of $STHdh^{Q7/Q7}$ cells with phytocannabinoids enhanced $H_2O_2$-induced NRF2 translocation to the nucleus, a response expected at the light of phytocannabinod effects of HO-1 gene expression. However, we were not able to confirm such response, even the nuclear translocation of NRF2 appeared to be a little bit lower after the treatment with the phytocannabinoids. In general, the responses in gene and protein expression in $STHdh^{Q111/Q111}$ cells were relatively similar compared to $STHdh^{Q7/Q7}$ cells, although much more moderate. Given that these differences did not influence the capability of phytocannabinoids to prevent cell death after $H_2O_2$, which resulted to be relatively similar in both types of cells, we assumed that other mechanisms of protection should work in the case of $STHdh^{Q111/Q111}$ cells. In summary, we predict, although additional evidence needs to be provided, a possible activation of NRF2-mediated mechanism of neuroprotection by CBD and Sativex, but not $\Delta^9$-THC, against oxidative damage in striatal neurons. The involvement of other signaling mechanisms cannot be ruled out, and further research is needed to elucidate the relevance of this mechanism for a cannabinoid-based treatment for HD.

**Key words:** Phytocannabinoids, Sativex, STHdhQ7/Q7 and STHdhQ111/Q111 cells, NRF2, oxidative stress.

## Introduction

Huntington's disease (HD) is a fatal inherited neurodegenerative disorder characterized by motor impairment, cognitive dysfunction and psychiatric illness (Huntington, 2003). The primary cause of the disease is a mutation in the huntingtin gene consisting of a variable expansion of the CAG triplet repeat translated into an abnormal polyglutamine tract in the amino-terminal portion of this protein, which becomes toxic for specific striatal and cortical neuronal subpopulations (THDCRG, 1993).

Nowadays, there is no specific pharmacotherapy to alleviate motor and cognitive symptoms and/or to delay disease progression in HD. Thus, even though a few compounds have produced encouraging effects in preclinical studies (e.g. minocycline, coenzyme Q10, unsaturated fatty acids, inhibitors of histone deacetylases), none of them have resulted yet in the

development of an effective treatment (Ross *et al.*, 2014).

A potential therapy that is being deeply evaluated in preclinical models of HD is the use of cannabinoid compounds. Preclinical studies with cannabinoids demonstrated preservation of striatal neurons against different cytotoxic stimuli that operate in HD pathogenesis (Pazos *et al.*, 2008), effects that were exerted through mechanisms of action that could involve or not the activation of $CB_1$ and/or $CB_2$ receptors. For instance, the positive effects exerted by the non-selective phytocannabinoid agonist $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) in the transgenic mouse model of HD R6/2 are likely produced through the activation of $CB_1$ receptors (Blázquez *et al.*, 2011). $\Delta^9$-THC can also be active at the $CB_2$ receptors, as selective agonists of this receptor preserved striatal neurons in this genetic model (Palazuelos *et al.*, 2009) and also in malonate-lesioned rats (Sagredo *et al.*, 2009), an acute model of HD in which striatal damage is produced primarily by apoptosis and glial activation/inflammatory events. Selective agonists of $CB_1$ (Pintor *et al.*, 2006) and $CB_2$ receptors (Palazuelos *et al.*, 2009) also preserved striatal neurons in *in vitro* or *in vivo* excitotoxic models. On the other hand, cannabinoids with antioxidant profile, e.g. $\Delta^9$-THC, cannabidiol (CBD) or cannabigerol (CBG) protected striatal neurons against toxicity caused by the mitochondrial complex II inhibitor 3-nitropropionic acid (3NP) that primes calpain activation and oxidative injury (Lastres-Becker *et al.*, 2004; Sagredo *et al.*, 2007; Valdeolivas *et al.*, 2015).

These results point to the hypotheses that the evaluation of cannabinoids as disease-modifying agents in HD patients should be necessarily conducted with a broad-spectrum cannabinoid or with combinations of various cannabinoids with different and complementary pharmacological profiles. Sativex is a cannabis-based medicine already approved for the treatment of spasticity and neuropathic pain in patients with multiple sclerosis in many countries (Sastre-Garriga *et al.*, 2011) and ,in Canada under the Notice of Compliance with Conditions (NOC/c) policy as adjunctive analgesic treatment in patients with advanced cancer (Johnson *et al.*, 2010). It has an appropriate profile for HD; it is antioxidant due to the structure of both of its components, can activate $CB_1$ and $CB_2$ receptors because of the presence of $\Delta^9$-THC, and can also exert cannabinoid receptor-independent properties due to $\Delta^9$-THC and, in particular, to CBD (Fernandez-Ruiz *et al.*, 2013). This non-psychoactive cannabinoid has proved effectiveness against cell death in multiple models of brain damage, acting through non-cannabinoid receptor based signaling pathways, such as the activation of 5-$HT_{1A}$ receptors (Pazos *et al.*, 2013), PPARγ nuclear receptors (Scuderi *et al.*, 2014) and NRF2 transcription factor (Juknat *et al.*, 2013). Moreover, CBD may counteract the possible psychoactive-like side effects related to the chronic use of $\Delta^9$-THC (Russo & Guy, 2006).

In the last years, we have performed experiments with the combination of $\Delta^9$-THC and CBD botanical extracts present in Sativex in those animal models of HD in which individual cannabinoid agonists have proved to be effective, with the objective of determining whether this mixture is also beneficial in these models. We obtained the first data in rats subjected to 3NP intoxication, a model in which the Sativex-like combination of phytocannabinoids preserved striatal neurons from death as the individual compounds had done previously (Lastres-Becker et al., 2004; Sagredo et al., 2007), and this effect was, as expected, independent of $CB_1$ and $CB_2$ receptors (Sagredo *et al.*, 2011). Next, we conducted similar studies in rats lesioned with malonate, an acute model in which selective $CB_2$ receptor agonists had yielded positive results (Sagredo *et al.*, 2009), while the blockade of $CB_1$ receptors aggravated the striatal damage (Lastres-Becker *et al.*, 2003). The Sativex®-like combination of phytocannabinoids also preserved striatal neurons from death caused by malonate and this effect was dependent on both $CB_1$ and $CB_2$ receptors (Valdeolivas *et al.*, 2012). These positive effects prompted us to extend our research to transgenic mouse models of HD, in particular the R6/2 mice which are frequently used for the evaluation of potential neuroprotective compounds that deserve to be studied at the clinical level. We found that a daily treatment with Sativex®-like combination of phytocannabinoids in R6/2 mice improved some motor deficits (Valdeolivas *et al.*, 2017), whereas it reversed the changes found in local metabolic activity in the basal ganglia using PET, and in a series of detectable biomarkers measured by ex-vivo proton magnetic resonance spectroscopy (H+-MRS), which reflect oxidative damage, energy failure, excitotoxicity, and other events typically found in HD, all of them being prognostic for brain integrity/damage (Tsang *et al.*, 2006; Pazos *et al.*, 2013).

In the present study, we aimed to further explore

the molecular mechanisms by which Sativex is neuroprotective in different models of HD using an *in vitro* strategy with striatal derived cell lines generated from a knock-in mouse containing homozygous huntingtin loci with a humanized exon 1 with either 7 ($STHdh^{Q7/Q7}$, WT cells) or 111 ($STHdh^{Q111/Q111}$ HD cells) polyglutamine repeats. Our first objective was to quantify possible differences between both types of cells in the expression of specific genes related to the endocannabinoid signaling and other markers of interest for the purpose of our study (e.g. inflammatory state, oxidative response, neurotrophins, and others). The second objective was evaluate the neuroprotective profile of $\Delta^9$-THC-BDS, CBD-BDS and Sativex against three different cytotoxic events that occur in the brains of the patients: excitotoxicity, mitochondrial damage and oxidative stress, all of them reproduced by using appropriate neurotoxins. Given the positive effects found with CBD and Sativex against oxidative stress-mediated cell death, our last objective was to explore the potential mechanisms involved in this action. Thus, we aimed to assess the possible involvement of the NRF2-KEAP1/ARE signaling pathway in the observed antioxidant effect by non-psychoactive cannabinoids.

## Materials and methods

### *Cell culture*

Conditionally immortalized striatal neuroblasts from wild-type mice ($STHdh^{Q7/Q7}$ cells), or knock-in mice expressing two copies of a mutant huntingtin allele ($STHdh^{Q111/Q111}$ cells), thus expressing endogenous levels of full-length huntingtin with 7 or 111 glutamine repeats respectively, were used. These cells are infected with a defective retrovirus transducing the temperature-sensitive A58/U19 large T antigen and geneticin-resistant colonies are selected at the permissive temperature of 33ºC, as described previously (Trettel *et al.*, 2000). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2mM ultra-glutamine, 1 mM sodium pyruvate, 1% penicillin/streptomycin and 400 µg/ml geneticin, under a humidified 5% $CO_2$ atmosphere at 33ºC.

### *Cell viability*

Cells were seeded at 38000 cells/$cm^2$ in 24-well plates in supplemented-DMEM medium previously described (complete medium). After 24h, complete medium was replaced by serum-free medium and cells were further incubated overnight. Afterwards, different conditions were applied depending on the cytotoxic agent used. For the mitochondrial damage experiment, cells were exposed to 100 mM malonate prepared in serum-free medium. After 5 hours of incubation, cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test. For the excitotoxicity experiment, serum-free medium was replaced by hand-made Locke's solution (Earle's balanced Salt Solution -EBSS-, 2.3 mM CaCl2, 5mM Hepes, 10 µM glycine) and cells were exposed to 10 mM glutamate for 6h. Then media from each well were used to measure lactate deshydrogenase (LDH) activity as an index of cell death, using the Cytotox 96 Non-Radioactive Cytotoxicity Assay Kit (G1780, Promega Biotech Ibérica, Madrid, Spain). For the oxidative stress experiment, complete medium was replaced by DMEM and cells were exposed to 2 mM hydrogen peroxide. After 2h, LDH activity was measured as in the excitotoxicity experiment. In all cases, 30 minutes before exposure to the cytotoxic agent, cells were treated with botanical extracts enriched with either $\Delta^9$ -THC, kindly provided by GW Pharmaceuticals Ltd., Cambridgeshire, U.K. ($\Delta^9$-THC botanical extract contains 66.7% $\Delta^9$-THC, 0.2% CBD, 0.8% cannabigerol, 1.1% cannabichromene, and 2.3% other phytocannabinoids) or CBD, also provided by GW Pharmaceuticals Ltd., Cambridgeshire, U.K. (CBD botanical extract contains 62.7% CBD, 3.6% $\Delta^9$-THC, 1.4% cannabigerol, 5.7% cannabichromene, and 1.8% other phytocannabinoids) or the Sativex-like 1:1 combination of both. Cannabinoid extracts were prepared in Tween-80 – culture medium solution (1:10) and always applied at a final concentration of 0.5 µM. In all experiments, negative control cells (not exposed to toxins and treatments, only cannabinoid extracts' vehicle added) and positive control cells (exposed to 1% triton X-100, representing 100% of cell death) were used. Additional experiments with cells only exposed to cannabinoid extracts were done (not shown), and cytotoxicity by these compounds at the indicated dose was ruled out.

### *Real-Time qTR-PCR*

Total RNA was isolated using SurePrep™ RNA/Protein Purification Kit (Fisher Bioreagents, Fair Lawn, NJ, USA). The total amount of RNA was quantified by spectrometry at 260 nm, its purity evaluated by the 260/280 nm absorbance ratio, and its integrity confirmed in agarose gels. cDNA was obtained using Quantitec Reverse Transcription Kit (Qiagen, Izasa,

Madrid, Spain) and samples were kept at -20ºC until amplification. Quantitative real-time PCR assays were performed using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA), in a 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). References of the probes used are shown as follows: KEAP1 (Mm00497268_m1), NQO1 (Mm01253561_m1), HO1 (Mm00516005_m1), iNOS (Mm01309902_m1), TNFα (Mm99999068_m1), GLAST (Mm00600697_m1), GLT-1 (Mm00441457_m1) IGF-1 (Mm00439560_m1), BDNF (Mm01334042_m1), DARPP32 (Mm00454892_m1), PPARγ (Mm01184322_m1), MAGL (Mm00449274_m1), FAAH (Mm00515684_m1), CB1 (Mm00432621_s1), GCLC (Mm00802655_m1), PRDX1 (Mm01621996_s1) and GAPDH (Mm99999915_g1). Relative gene expression data were determined by the $2^{-\Delta\Delta Ct}$ method. Each value was adjusted to GAPDH levels as reference.

***Preparation of nuclear and cytosolic extracts***

For subcellular fractionation experiments, cells were seeded in p100 plates (55000 cells/cm$^2$). After 24h, complete medium was replaced by serum-free medium and, after incubation overnight, cells were pre-treated with phytocannabinoids and exposed to 0.5 mM hydrogen peroxide for 2.5h. After that, cytosolic and nuclear fractions were prepared as described elsewhere (Lastres-Becker *et al.*, 2014). Briefly, cells were washed with cold PBS, harvested in 400 µl of cold Buffer A (20 mM HEPES, pH 7.0, 0.15 mM EDTA, 0.015 mM EGTA, 10 mM KCl, 1% NonidetTM P- 40, 1 mM phenylmethylsulphonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mg/ml leupeptin) and incubated on ice for 30'. Then samples were centrifuged 5' at 500g and the supernatants were stored at -80ºC as the cytosolic fraction. The nuclear pellet was resuspended carefully in 500 µl of cold Buffer B (10 mM HEPES, pH 8.0, 0.1 mM EDTA, 0.1 mM NaCl, 25% glycerol, 1 mM phenylmethylsulphonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mg/ml leupeptin) and centrifuged again 5' at 500g. The supernatant was then removed and the nuclear pellets were resuspended in Buffer A containing Laemmli SDS loading buffer. After sonication, these samples were stored at -80ºC as the nuclear fraction. Nuclear and cytosolic protein samples were boiled and resolved in 10% acrylamide gels (TGX Stain-Free™ FastCast™ Acrylamide Kit, Bio-Rad Laboratories, Hercules, CA, USA), and then transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) using mini Trans-Blot Electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% non-fat milk and incubated overnight at 4°C with the anti- NRF2 (C-20) primary antibody (sc-722, Santa Cruz Biotechnology). Incubation with anti-α-tubulin (TU-02) (sc-8035, Santa Cruz Biotechnology) and anti-laminB (C-20) (sc-6216, Santa Cruz Biotechnology) primary antibodies was used as a loading control for the cytosolic and nuclear fraction, respectively. This was followed by a second incubation during 1 hour at room temperature with an ECL™ Horseradish Peroxidase-linked whole secondary antibody (GE Healthcare UK Limited, Buckinghamshire, UK) at a 1:5000 dilution. Reactive bands were detected by chemiluminescence with the Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare UK Limited, Buckinghamshire, UK). Images were analyzed on a ChemiDoc station with Quantity one software (Bio-Rad Laboratories, Madrid, Spain). Data were calculated as the ratio between the optical densities of the specific protein band and the housekeeping protein band (α-tubulin or laminB), and they were normalized as fold changes over the control group for presentation.

***Statistical analyses***

Data are presented as mean ± SEM. All data were analysed by one-way ANOVA followed by the Student-Newman-Keuls test, or by unpaired Student's t-test, as required, using Graphpad Prism (version 6.0).

## Results

**Dysregulation in the expression of genes of interest in STHdh$^{Q111/Q111}$ compared to STHdh$^{Q7/Q7}$ cells.** As a first approach to these cell lines, we wanted to determine the differences in gene expression in STHdhQ111/Q111 cells versus STHdhQ7/Q7 cells in endocannabinoid genes, as well as others related to cell survival (e.g. neurotrophins) or to the response to cytotoxic events such as inflammation, oxidative stress, mitochondrial damage and excitotoxicity. The results are shown in *Figure 1*. As expected, we observed a decrease in the expression of neurotrophins (BDNF and IGF1), which has been already described in other HD models and in patients (Ferrer *et al.*, 2000; Zuccato & Cattaneo, 2007). Regarding the cannabinoid system, we found an increase in FAAH enzyme, which is also elevated in other models of early HD, but decreased at early stages and also in human lymphocytes (Battista

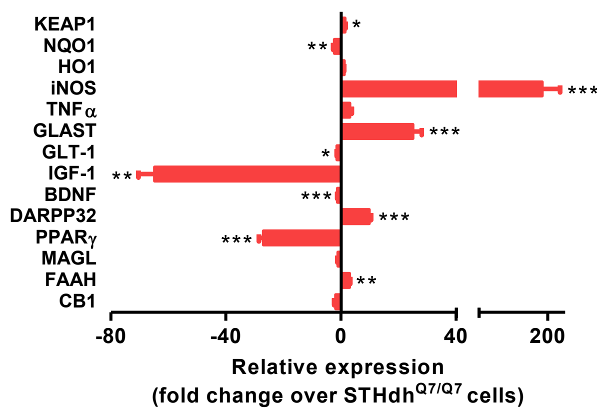


**Figure 1.** Changes in gene expression in STHdh$^{Q111/Q111}$ cells compared to STHdh$^{Q7/Q7}$ cells. Values are expressed as means ± SEM of 3 cases *per* group. Data were subjected to unpaired t-test. *p<0.05, **p<0.01, ***p<0.005 compared to compared to STHdh$^{Q7/Q7}$ cells.

*et al.*, 2007; Bari *et al.*, 2013). Besides, we found a decrease, although not significant, in $CB_1$ receptor expression that has been widely reported as a pathogenic hallmark of the disease (Blazquez *et al.*, 2011). We also observed significant differences in genes related to the cellular response to oxidative damage, such as NQO1 and Keap-1 and to inflammation, such as PPARγ. Of particular interest is the huge increase in iNOS found in STHdh$^{Q111/Q111}$ cells, nearly 200 times higher than in WT cells, which had not been described before. The changes observed in the expression of glutamate transporters GLT-1 and GLAST are also surprising given that these two glutamate transporters are characteristic of glial cells (Perego *et al.*, 2000; Plachez *et al.*, 2004). *A priori* all these data made us hypothesize that STHdh$^{Q111/Q111}$ cells might be more sensitive to the cytotoxic insults that will be described in the following results, and likewise could be differently affected by the treatment with phytocannabinoids. Unexpectedly, our results showed the opposite (see below).

**Effects of phytocannabinoid combinations on cell death induced by different insults.** Next, we aimed to determine the potential effects of phytocannabinoids on cell death produced by different damaging insults that resemble neuropathological events that occur in the brains of HD patients. First, we exposed both cell lines to malonate, a mitochondrial complex-II inhibitor that has been previously used in models of neuroinflammation and mitochondrial damage in HD. As expected, malonate induced a decrease in cell viability in both cell lines (STHdh$^{Q7/Q7}$ $F(4,40)=26.87$, $p<0.005$; STHdh$^{Q111/Q111}$ $F(4,39)=15.80$, $p<0.005$) with these last cells being apparently more vulnerable to

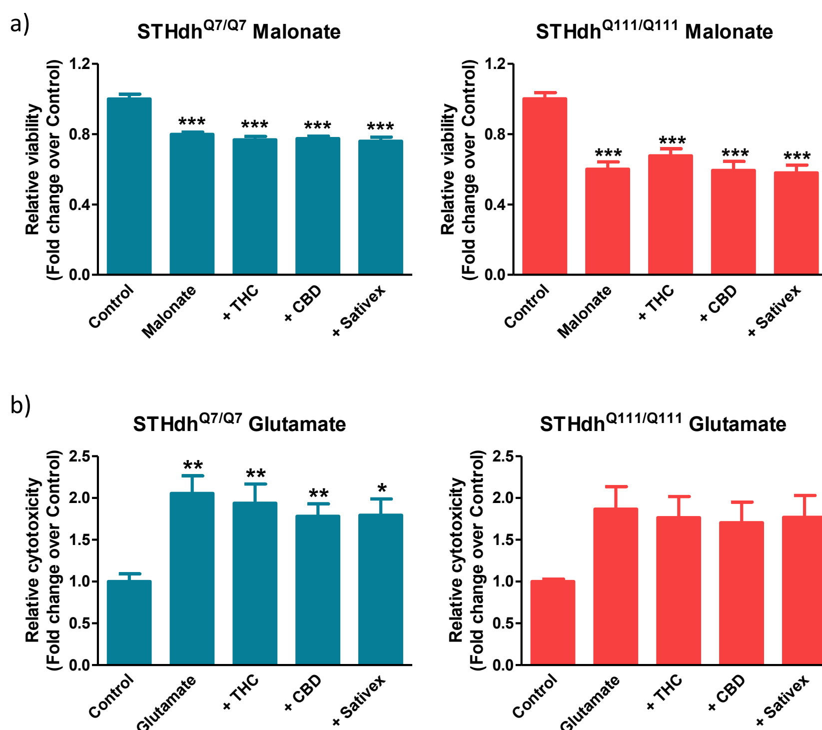


**Figure 2.** Viability and cytotoxicity assays of STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells pretreated with phytocannabinoids and exposed to malonate and glutamate. Values are expressed as means ± SEM of 8-9 cases *per* group. Data were subjected to one-way analysis of variance, followed by the Student-Newman-Keuls test. a) Relative viability, measured by MTT assay, of STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells pretreated with either 0.5 µM $\Delta^9$-THC, CBD or Sativex for 30' and exposed to 100 mM malonate for 5h. ***p<0.005 compared to control group. b) Relative cytotoxicity, measured by LDH-release assay, of STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells pretreated with either 0.5 µM $\Delta^9$-THC, CBD or Sativex for 30' and exposed to 100 mM glutamate for 6h. *p<0.05, **p<0.01 compared to control group.

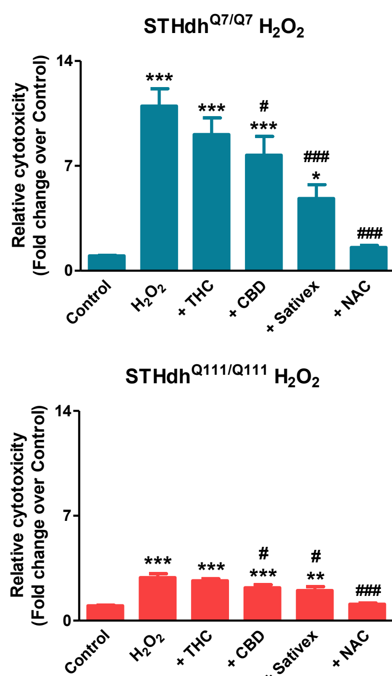


**Figure 3.** Relative cytotoxicity, measured by LDH-release assay, of of $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells pretreated with either 0.5 µM $\Delta^9$-THC, CBD or Sativex for 30' and exposed to 2mM $H_2O_2$ for 2h. 1 mM NAC was used as antioxidant control. Values are expressed as means ± SEM of 6-12 cases *per* group. Data were subjected to one-way analysis of variance, followed by the Student-Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.005 compared to control group. #p<0.5, ###p<0.005 compared to $H_2O_2$ group.

this kind of damage (*Fig. 2a*). Anyway, the treatment with none of the phytocannabinoids ($\Delta^9$-THC, CBD and Sativex) could prevent cell loss. Next, we investigated the response of these cells to glutamate exposure (*Fig. 2b*). Glutamate caused cytotoxicity in a similar way in both cell lines, although, in $STHdh^{Q111/Q111}$ cells the elevation in cell death was not statistically significant ($STHdh^{Q7/Q7}$ $F(4,40)=5.17$, $p<0.01$; $STHdh^{Q111/Q111}$ $F(4,39)=2.35$, $p>0.05$), and the pre-treatment with phytocannabinoids did not cause any effect in cell death in none of these cell lines. The third approach consisted on the exposure of the cells to hydrogen peroxide ($H_2O_2$) to produce cell death through oxidative stress. In this experiment, the antioxidant N-acetylcysteine (NAC) was added at the same time than phytocannabinoids, as a positive control. Although $STHdh^{Q111/Q111}$ cells showed a higher LDH release in basal conditions (data not shown), surprisingly, they were much less vulnerable to $H_2O_2$ than $STHdh^{Q7/Q7}$. However, the treatment with CBD-BDS and Sativex, but not $\Delta^9$-THC-BDS, reduced significantly the cytotoxicity ($STHdh^{Q7/Q7}$ $F(4,40)=26.87$, $p<0.05$ CBD vs $H_2O_2$, $p<0.005$ Sativex vs $H_2O_2$; $STHdh^{Q111/Q111}$ $F(4,39)=15.80$, $p<0.05$), although the reversion was not complete as occurred in those cells treated with NAC (*Fig. 3*).

**Effect of phytocannabinoids on the expression of genes related to NRF2 and antioxidant response.** After the robust effect exerted by CBD-BDS and Sativex, we wanted to further investigate the possible mechanisms responsible of this neuroprotective action. Although this strong antioxidant profile was not unexpected, as CBD has intrinsically more antioxidant capacity than $\Delta^9$-THC because of its chemical structure, the fact that the combination of both in the Sativex-like treatment has a higher neuroprotective effect suggested that specific intracellular signaling mechanisms (e.g. NFR2 signaling) might be involved. Thus, we repeated the same experiment but using a reduced dose and a prolonged exposure of $H_2O_2$, with the objective of avoiding early cell necrosis. Therefore, cells were pre-treated with phytocannabinoids and exposed to 0.5 mM hydrogen peroxide for 3.5h, and then total RNA was extracted to analyze gene expression of different enzymes induced by oxidative stress. As can be seen in *Fig. 4.*, the expression of antioxidant enzymes induced by the activation of NRF2, as well as kelch like ECH associated protein 1 (KEAP1), was evaluated. Surprisingly, there were no changes in gene expression of neither glutamate-cysteine ligase catalytic subunit (GCLC) nor peroxiredoxin-1 (PRDX1) in none of the cell lines, although the activity of these enzymes is directly related to the insult with $H_2O_2$. However, the expression of heme oxygenase-1 (HO1) was significantly increased after the exposure to $H_2O_2$ in $STHdh^{Q7/Q7}$ cells and not affected by the treatment with $\Delta^9$-THC-BDS ($F(4,10)=19.38$, $p<0.01$). Moreover, the treatment with CBD-BDS and Sativex duplicated the increase in the expression of this enzyme, suggesting that an induction of antioxidant response may be elicited by the treatment with these compounds. In $STHdh^{Q111/Q111}$ the effect was much more moderate and did not reach statistical significance, although a similar trend was appreciated. Regarding the expression of the enzyme NAD(P)H quinone dehydrogenase 1 (NQO1), and the repressor of NRF2-dependent transcription KEAP1, no changes by the treatment with phytocannabinoids were observed. The increase in HO-1 expression made us hypothesize that CBD and Sativex could exert a neuroprotective effect by inducing the NRF2 pathway against an oxidative insult.

**Effects of phytocannabinoids on NRF2 nuclear translocation.** In order to confirm the potential action

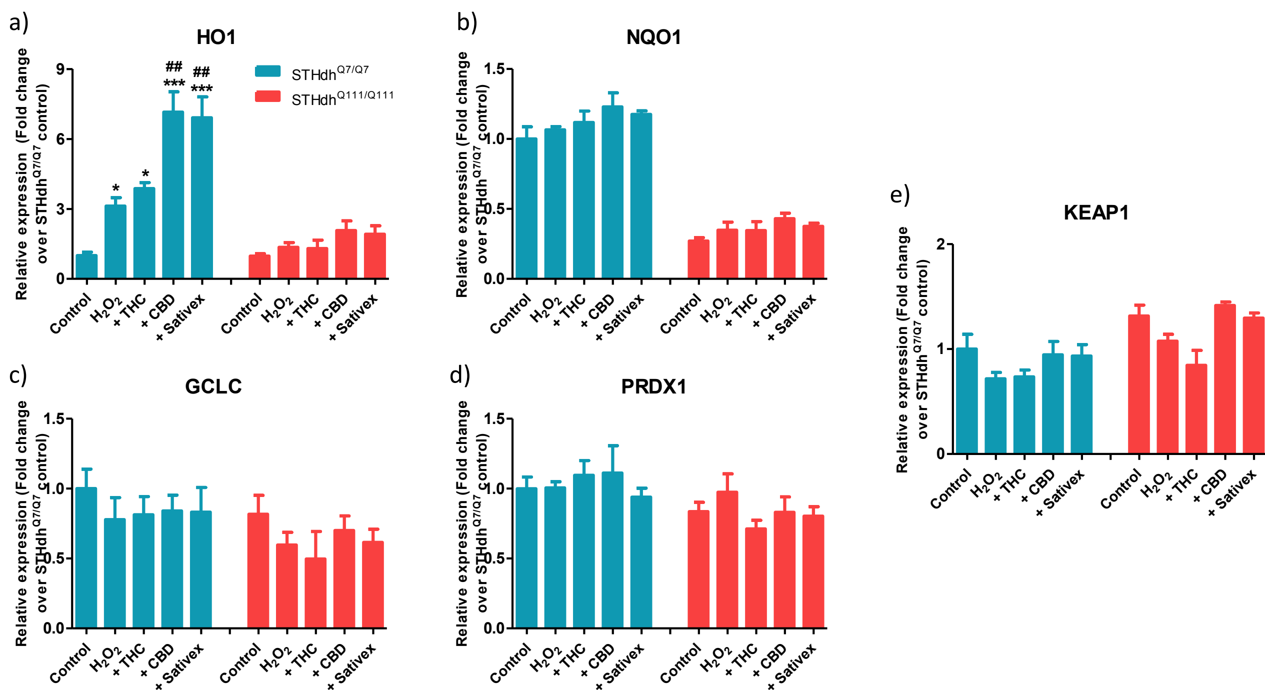


**Figure 4.** Changes in gene expression in STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells pretreated with either 0.5 µM Δ$^{9}$-THC, CBD or Sativex for 30' and exposed to 0.5 mM $H_2O_2$ for 3.5h. Values are expressed as means ± SEM of 3 cases *per* group. Data were subjected to one-way analysis of variance, followed by the Student-Newman-Keuls test. *p<0.05, ***p<0.005 compared to control group. ##p<0.01 compared to $H_2O_2$ group. a) HO1 relative expression. b) NQO1 relative expression. c) GCLC relative expression. d) PRDX1 relative expression. e) KEAP1 relative expression.

of phytocannabinoids on the activation of the NRF2 pathway, we performed a subcellular fractionation and measured the levels of nuclear and cytosolic NRF2 protein in both cell lines after the cannabinoid pre-treatments and the insult with $H_2O_2$. As shown in *Fig. 5*, hydrogen peroxide clearly induced the translocation of NRF2 to the nucleus in STHdh$^{Q7/Q7}$ cells ($F(1,8)=14.32$, $p<0.05$), but the treatment with cannabinoid compounds did not produce any additional effect.

## Discussion

Despite the extensive preclinical research performed in the last years to find potential treatments for Huntington's disease, only a few of them have shown promising effects to prompt their evaluation in clinical trials. Among them, the drugs approved for HD patients up to now are focused mainly on ameliorating the clinical symptoms (Bates *et al.*, 2015). In this context, cannabinoid compounds have proved to be a very interesting therapeutic option both for the management of symptoms and to delay the progression of the disease. For example, $CB_1$ agonists have shown anti-hyperkinetic and thus symptom-relieving effects in acute models of excitotoxicity (Lastres-Becker *et al.*, 2002)). Besides, phytocannabinoids have been extensively evaluated in the last few years to prove their neuroprotective potential in different models of the disease. Thus, Δ$^{9}$-THC showed positive results in a mouse model of excitotoxicity and in R6/2 transgenic mice (Palazuelos *et al.*, 2009) and CBD proved to be highly neuroprotective in an acute model of oxidative damage (Sagredo *et al.*, 2011). Interestingly, the 1:1 combination of these two cannabinoids, so-called Sativex, has been evaluated in different models of the disease with very promising results (Sagredo *et al.*, 2011; Valdeolivas *et al.*, 2012; Valdeolivas *et al.*, 2017). This drug, that has already obtained approval for the treatment of spasticity and pain in multiple sclerosis in 17 countries, was indeed evaluated in a pilot clinical trial with HD patients very recently (Lopez-Sendon Moreno *et al.*, 2016). Unfortunately, and similarly to other clinical trials with cannabinoids such CBD (Consroe et al., 1991), it was safe and well-tolerated, but did not show any clinical improvement.

Because of its broad-spectrum profile and the very good results obtained in different animal models of HD, we consider Sativex a very promising therapeutic agent for this disease and have provided preclinical evidence that this is the case using neurotoxin-based models (Sagredo et al., 2011; Valdeolivas et al., 2012) and R6/2 mice (Valdeolivas et al., 2017). In the present study, we wanted to further explore

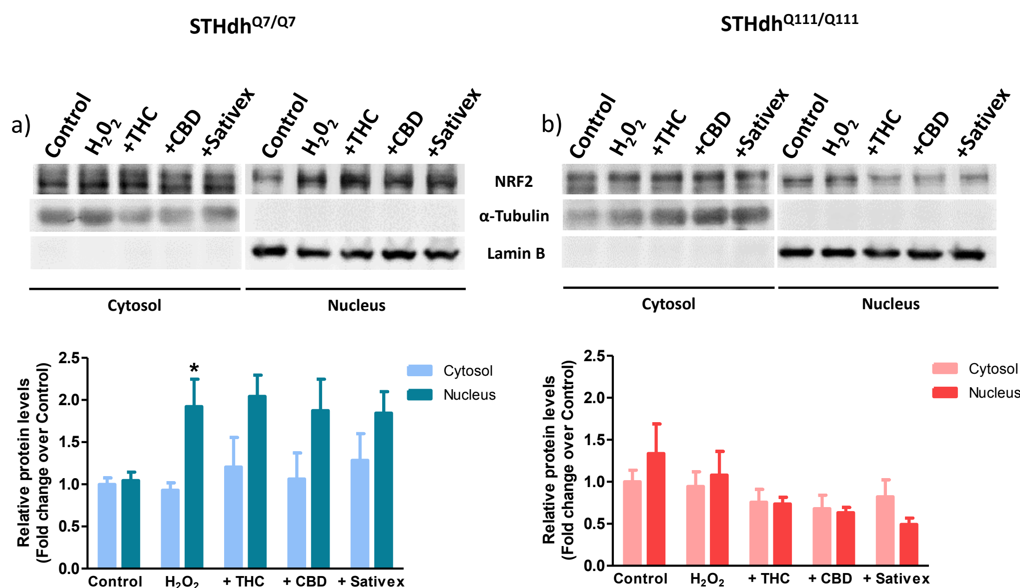


**Figure 5.** NRF2 protein levels in the cytosol and nucleus of a) STHdh$^{Q7/Q7}$ and b) STHdh$^{Q111/Q111}$ cells, pretreated with either 0.5 µM $\Delta^9$-THC, CBD or Sativex for 30' and exposed to 0.5 mM $H_2O_2$ for 2.5h. Values are expressed as means ± SEM of 5 cases *per* group. NRF2 band volumes were normalized to α-tubulin and LaminB as cytosolic and nuclear loading controls, respectively. Data were subjected to unpaired t-test (control-cytosol *vs* control-nucleus, $H_2O_2$-cytosol *vs* $H_2O_2$-nucleus and so on). *p<0.05 compared to cytosolic protein levels.

the potential molecular mechanisms underlying these beneficial effects using an in vitro approach and replication of the different cytotoxic stimuli that operate in the brains of HD patients. For this purpose, we used STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells, striatal derived cell lines from knock in transgenic HD mice, a model that has been widely used for basic research and preclinical drug testing (Blazquez *et al.*, 2011; Ruiz *et al.*, 2012). Moreover, we used three different approaches that could resemble, as in animal models of the disease, different cytotoxic events: that is, a model of mitochondrial damage and apoptosis (exposure to malonate), a model of excitotoxicity (exposure to glutamate) and a model of oxidative stress (exposure to $H_2O_2$). We brought the cells under the action of these toxic agents, and pretreated them with either $\Delta^9$-THC-BDS or CBD-BDS alone or the Sativex-like combination of them. We repeated both manipulations in both cell lines, with the purpose of testing if the response to damage by the cells or the possible effect of phytocannabinoids could be affected by the expression of mutant huntingtin. In the first set of experiments, in which cells were exposed to the mitochondrial complex II inhibitor malonate, we could prove that STHdh$^{Q111/Q111}$ cells are more sensitive to mitochondrial damage than WT cells, something that has been already reported elsewhere (Milakovic & Johnson, 2005). Next, we investigated the response to excitotoxicity, but, in our hands, we could not observe the higher vulnerability of STHdh$^{Q111/Q111}$ that has been shown in previous publications (Blázquez *et al.*, 2011). This could be due to the use in our case of a different agent (glutamate instead of NMDA) and also a different viability assay (that measures mainly necrotic cell death instead of mitochondrial integrity). Anyhow, the treatment with phytocannabinoids was not able to prevent the cell death caused by neither of these agents. In the case of the exposure to oxidative damage, we found that STHdh$^{Q111/Q111}$ cells were much less sensitive to this insult than STHdh$^{Q7/Q7}$. This has been reported before (Ribeiro *et al.*, 2013) but not at such a high extent, and this unequal response could be due to a different dose and time of exposure to $H_2O_2$ in our experiment. Another explanation for this response would be a reduced expression of the $Ca^{2+}$-binding protein downstream regulatory element antagonist modulator (DREAM) by STHdh$^{Q111/Q111}$ cells, which would make them less sensitive to this kind of damage, as it was published during the time we were conducting our experiments (Naranjo *et al.*, 2016). In this case, we did observe an protective effect of phytocannabinoids in this model: not $\Delta^9$-THC-BDS but CBD-BDS and specially Sativex were able to prevent cell death, which suggests that the combination of these two compounds is more effective against this kind of damage than in a separate way. Interestingly, this was observed both in STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cell lines, although in the last ones the effect was more modest, presumably because the level of damage was lower as well by the reasons explained before. The fact that

CBD and Sativex were more antioxidant than $\Delta^9$-THC was not surprising for us, being that CBD, because of its structure containing two hydroxyl groups, has intrinsically more ROS-scavenger activity. However, as CBD has been proven recently to induce NRF2-mediated antioxidant response (Juknat *et al.*, 2013) and the activation of this pathway has been proposed as a potential therapeutic target for HD (Rotblat *et al.*, 2014), we wanted investigate whether this could be the mechanism of the neuroprotective effect of Sativex in this model.

Therefore, the next step was to evaluate the levels of gene expression of some antioxidant enzymes induced by NRF2, in both cell lines and in all experimental groups. Surprisingly, the expression of two antioxidant enzymes typically related to the response against $H_2O_2$ damage, GCLC and PRDX1, did not change in any cell type, in any experimental group in this model. However, we observed a marked increase of the enzyme HO1 in STHdh$^{Q7/Q7}$ cells, that was even higher in the cells treated with CBD and Sativex. This trend was also observed in the relative mRNA levels of NQO1, an enzyme which expression is specifically activated by NRF2. Moreover, although no significant, we observed a decrease in the expression of KEAP-1 in cells exposed to $H_2O_2$, that was reverted by the treatment with, again, CBD and Sativex but not $\Delta^9$-THC. The changes in KEAP-1 must be anyway taken with certain caution, as the mechanism by which this protein regulates NFR2 does not imply changes in KEAP-1 gene expression, but to interact directly in the cytoplasm with this transcription factor, so changes in KEAP-1 gene expression may be a consequence rather than a cause of the changes elicited by $H_2O_2$ exposure and/or phytocannabinoid treatments. These results suggested that the activation of NRF2 signaling pathway could indeed be playing a role in the phytocannabinoid-mediated protection against ROS. Regarding STHdh$^{Q111/Q111}$, no changes were found in the gene expression of GCLC and PRDX1 either, and the mRNA levels of HO-1 and NQO1 after oxidative damage were very low compared to STHdh$^{Q7/Q7}$ cells. It has been reported indeed that mHTT expression leads to an impairment of NRF2 signaling in these cells (Jin *et al.*, 2013), so we assumed that CBD and Sativex-mediated neuroprotection in these cells might be due to the activation of additional signaling mechanisms.

Finally, we aimed to confirm the activation of NRF2 antioxidant response by assessing the protein levels of this transcription factor in the nucleus against cytosolic protein levels, in order to show its nuclear translocation after an oxidative insult. In STHdh$^{Q7/Q7}$ cells, we only observed a significant increase of nuclear protein levels in the experimental group exposed to hydrogen peroxide, but this change was not clearly appreciated in any of the treatment groups, even the significance of the differences between the nuclear and cytosolic levels of NRF2 disappeared, which may suggest that additional mechanisms may be involved in the effects of phytocannabinoids and, in particular, in the induction of HO-1 expression. In addition, it is also important to remark that previous work has demonstrated certain non-specific effects for the antibodies developed against NRF2, so it is possible that this has affected our experimental design.

In conclusion, and considering these preliminary data, we cannot confirm that the activation of NRF2-mediated antioxidant pathway is the main mechanism for the phytocannabinoid-mediated neuroprotection against oxidative stress in STHdh$^{Q7/Q7}$ cells. It should be noted that the levels of NQO1, whose expression is known to be specifically induced by the NRF2-KEAP1/ARE pathway, did not change significantly in this experiment, despite an increase in the expression of HO-1 was absolutely patent. However, the expression of this last enzyme, unlike NQO1, is reported to be activated by other signaling pathways, such as JNK (Lin *et al.*, 2013), PI3K/AKT (Li *et al.*, 2014) and p38 (Ning *et al.*, 2002). Therefore, the involvement of other mechanisms cannot be ruled out, and further experiments are needed to elucidate the mechanism of Sativex-mediated neuroprotection in this experimental approach.

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# The inhibition of 2-arachidonoyl-glycerol (2-AG) biosynthesis, rather than enhancing striatal damage, protects striatal neurons from malonate-induced death: a potential role of cyclooxygenase-2-dependent metabolism of 2-AG

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**The cannabinoid $CB_2$ receptor, which is activated by the endocannabinoid 2-arachidonoyl-glycerol (2-AG), protects striatal neurons from apoptotic death caused by the local administration of malonate, a rat model of Huntington's disease (HD). In the present study, we investigated whether endocannabinoids provide tonic neuroprotection in this HD model, by examining the effect of O-3841, an inhibitor of diacylglycerol lipases, the enzymes that catalyse 2-AG biosynthesis, and JZL184 or OMDM169, two inhibitors of 2-AG inactivation by monoacylglycerol lipase (MAGL). The inhibitors were injected in rats with the striatum lesioned with malonate, and several biochemical and morphological parameters were measured in this brain area. Similar experiments were also conducted *in vitro* in cultured M-213 cells, which have the phenotypic characteristics of striatal neurons. O-3841 produced a significant reduction in the striatal levels of 2-AG in animals lesioned with malonate. However, surprisingly, the inhibitor attenuated malonate-induced GABA and BDNF deficiencies and the reduction in Nissl staining, as well as the increase in GFAP immunostaining. In contrast, JZL184 exacerbated malonate-induced striatal damage. Cyclooxygenase-2 (COX-2) was induced in the striatum 24 h after the lesion simultaneously with other pro-inflammatory responses. The COX-2-derived 2-AG metabolite, prostaglandin $E_2$ glyceryl ester ($PGE_2$-G), exacerbated neurotoxicity, and this effect was antagonized by the blockade of $PGE_2$-G action with AGN220675. In M-213 cells exposed to malonate, in which COX-2 was also upregulated, JZL184 worsened neurotoxicity, and this effect was attenuated by the COX-2 inhibitor celecoxib or AGN220675. OMDM169 also worsened neurotoxicity and produced measurable levels of $PGE_2$-G. In conclusion, the inhibition of 2-AG biosynthesis is neuroprotective in rats lesioned with malonate, possibly through the counteraction of the formation of pro-neuroinflammatory $PGE_2$-G, formed from COX-2-mediated oxygenation of 2-AG. Accordingly, MAGL inhibition or the administration of $PGE_2$-G aggravates the malonate toxicity.**

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Cannabinoids have been recently proposed as neuroprotecting agents in Huntington's disease (HD).[1–3] HD is an adult-onset and autosomal-dominant neurodegenerative disorder characterized by the progressive death of specific neuronal subpopulations within the striatum and the cerebral cortex.[4] HD is caused by a CAG triplet expansion in the gene coding the protein huntingtin,[5] which is toxic for these neuronal subpopulations, although the mechanism(s) responsible for this particular preference in neuronal degeneration in HD remains to be fully identified. An important challenge in HD is

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**Keywords:** endocannabinoids; CB1 and CB2 receptors; diacyl-glycerol lipase; monoacyl-glycerol lipase; cyclooxygenase-2; Huntington's disease; malonate; neuroprotection

**Abbreviations:** 2-AG, 2-arachidonoyl-glycerol; BDNF, brain-derived neurotrophic factor; COX-2, cyclooxygenase-2; DAGL, diacylglycerol lipase; DMSO, dimethylsulfoxide; GABA, $\gamma$-aminobutiric acid; GFAP, glial fibrillary acidic protein; HD, huntington's disease; HPLC, high-performance liquid chromatography; iNOS, inducible nitric oxide synthase; LC-APCI-MS, liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry; LC-ESI-IT-ToF, liquid chromatography-electrospray-ion trap-time of flight; OEA, oleylethanolamide; OPA, o-phthaldehide; PBS, phosphate-buffered saline; PEA, palmitoylethanolamide; $PGE_2$-G, prostaglandin $E_2$-glycerol ester

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the development of efficient therapies to halt or slow down disease progression,[6,7] because the therapies investigated to date have been poorly effective. Compounds like minocycline, unsaturated fatty acids, coenzyme Q-10 and inhibitors of histone deacetylases are presently under clinical investigation,[8] whereas not only certain cannabinoid compounds, including both $CB_1$ and $CB_2$ receptor agonists, but also antioxidant phytocannabinoids have been successfully assayed in preclinical models[9–16] and have recently been applied in the clinical testing.[17]

The endocannabinoid system, which encompasses not only $CB_1$ and $CB_2$ receptors but also their endogenous ligands, the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG), and the enzymes for endocannabinoid biosynthesis and degradation, is subject to dysregulation in animal models of HD.[1,2,18] In particular, a strong correlation exists between reduced striatal levels of anandamide and 2-AG and locomotor signs of HD in R6/2 mice, genetic model of HD,[19] as well as in 3-nitropropionate-lesioned rats,[20] thus suggesting that impaired biosynthesis or exaggerated degradation of endocannabinoids might underlie in part the hyperkinesias typical of this disorder. Although this hypothesis was supported in part by the beneficial effect of inhibitors of endocannabinoid degradation,[21] it is not known whether the inhibition of endocannabinoid biosynthesis can worsen HD signs.

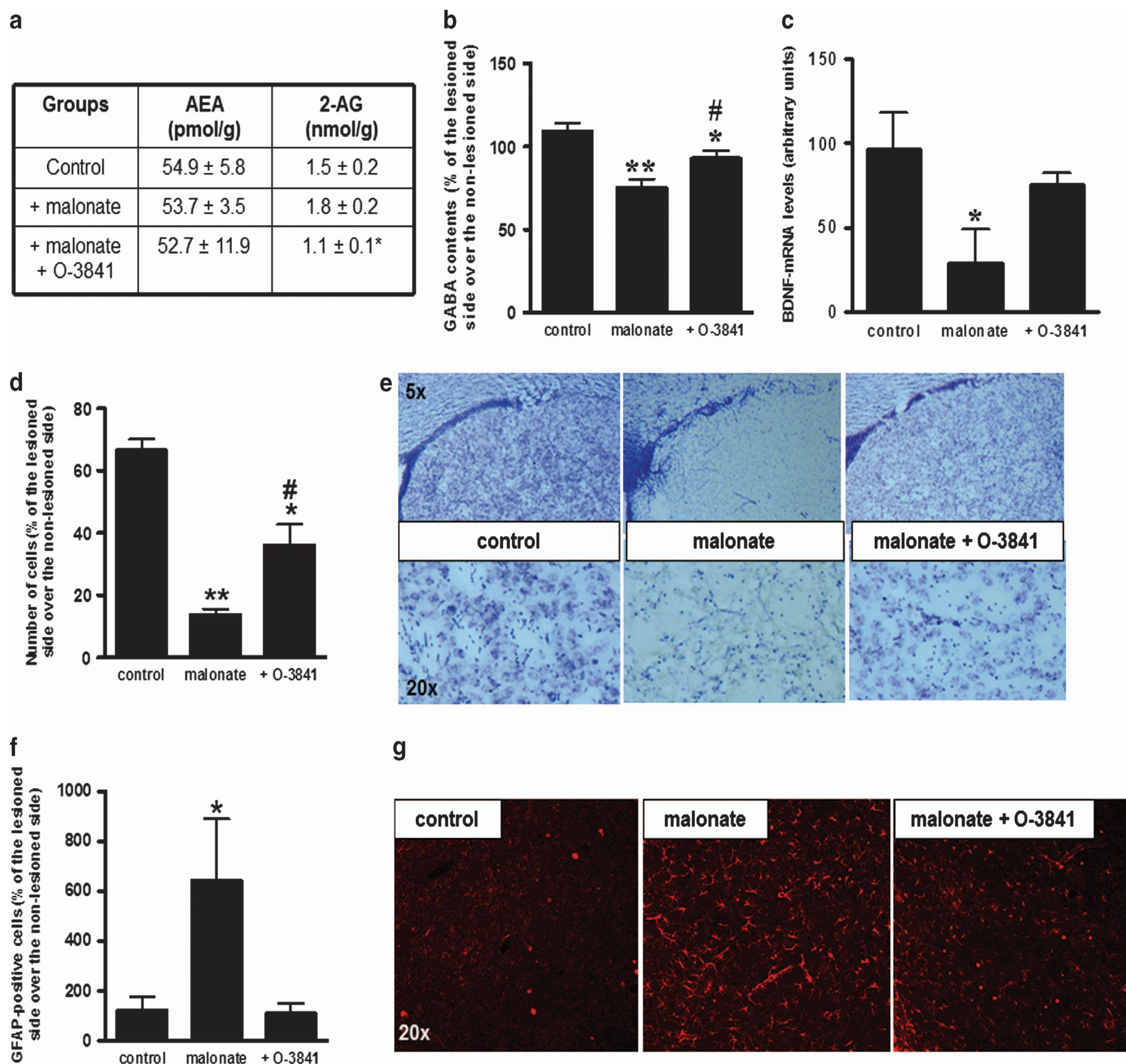
In the present study, we focused on striatal degeneration generated by the complex II inhibitor malonate, which is known to result in the death of striatal neurons through mechanisms that involve mainly the apoptotic machinery.[22] In this model, $CB_2$ receptor agonists protected striatal neurons from apoptotic death,[12] whereas the blockade of $CB_1$ receptors enhanced the magnitude of malonate-induced lesion.[23] Now, we wanted to determine the effects that the lesion with malonate produces on the striatal endocannabinoid levels. In particular, we expected that the inhibition of diacylglycerol lipases (DAGLs), the enzymes that catalyse the biosynthesis of 2-AG, with the compound O-3841,[24] would aggravate the effect of this lesion. However, on the basis of the initial unexpected finding of a neuroprotective effect of this inhibitor, our study progressed to additional objectives: (i) to elucidate what type of effect would be produced on the lesion by the opposite pharmacological manipulation, that is the blockade of the monoacylglycerol lipase (MAGL) and (ii) to investigate whether the biotransformation of 2-AG in prostaglandin glyceryl esters (PG-Gs) by cyclooxygenase-2 (COX-2) might explain the paradoxical effects found with O-3841, given that this type of oxygenated derivatives of 2-AG has been previously related to neurotoxic effects.[25] For these studies, we also used cultured M-213 cells, which share many phenotypic characteristics with striatal neurons.[26]

## Results

**Effects of DAGL inhibition on the striatal degeneration caused by malonate.** The original objective of this study was to examine the effects of the DAGL inhibitor O-3841[27] on the striatal damage caused by malonate. As expected, the local administration of O-3841 produced a significant reduction in the striatal levels of 2-AG in animals lesioned with malonate ($F(2,17) = 4.111$, $P < 0.05$), without affecting the levels of anandamide (Figure 1a). Importantly, unlike other models of HD,[19,20] neither anandamide nor 2-AG was altered by the lesion of the striatum with malonate. Given the neuroprotective properties described for 2-AG,[28] a reduction in the levels of this endocannabinoid by O-3841 was expected to enhance the magnitude of malonate-induced lesion. However, our data indicate that the inhibitor enhanced the survival of striatal neurons as could be demonstrated by its capability to partially attenuate the malonate-induced deficit in GABA ($F(2,15) = 12.56$, $P < 0.01$; Figure 1b) and BDNF ($F(2,12) = 4.04$, $P < 0.05$; Figure 1c). It also enhanced the number of Nissl-stained neurons that had been markedly reduced by malonate ($F(2,18) = 26.46$, $P < 0.0001$; Figures 1d and e) and attenuated the glial activation caused by this neurotoxin, as indicated by the reduction in the intensity of immunostaining for the marker of astrocytes GFAP ($F(2,13) = 4.623$, $P < 0.05$; Figures 1f and g). Looking at the representative microphotographs of the three groups under investigation, it was evident that astrocytes underwent morphological alterations after the lesion, similar to those previously reported.[29] Such alterations were notably reduced by the treatment with O-3841 (Figure 1g).

**Effects of malonate lesion on COX-2 and other pro-inflammatory responses and $PGE_2$-G formation.** The above data contrast with the notion that 2-AG is neuroprotective, although a few studies demonstrated that, under certain circumstances, 2-AG may be also neurotoxic, in part through the generation of COX-2-derived metabolites.[25] We examined this possibility by analysing the effects of malonate on the expression of COX-2 as well as on the levels of one of the most representative COX-2 derivative of 2-AG, the $PGE_2$-G. We have also quantitated the expression of other mediators (e.g., iNOS, PPAR-$\alpha$, PPAR-$\gamma$) that have been reported to have an instrumental role in proinflammatory events in neurodegenerative disorders.[30] Our experiments revealed that malonate lesion upregulated COX-2 in the striatum, although this effect was evident only 24-h after the lesion ($F(2,10) = 10.17$, $P < 0.01$; Figure 2a), and the same pattern was seen for iNOS ($F(2,11) = 12.46$, $P < 0.005$; Figure 2b). In agreement with these responses, we found a reduction in the expression of PPARs (only significant in the case of PPAR-$\alpha$: $F(2,8) = 15.21$, $P < 0.005$, but showing a trend towards a decrease in the case of PPAR-$\gamma$: $F(2,9) = 1,503$, ns; see Figures 2c and d), the activation of which is known to inhibit the expression of these pro-inflammatory enzymes—an effect that has been related to a reduction in NF$\kappa$B signalling.[31]

The increase in COX-2 expression by malonate might be in favour of an increased generation of COX-2-derived 2-AG metabolites. We tried to demonstrate such an increase by analysing the levels of $PGE_2$-G in the malonate-lesioned striatum, and, particularly, after the inhibition of MAGL, which should facilitate the formation of $PGE_2$-G (see below). Although our method, described here for the first time, was extremely sensitive (by allowing the quantification of as little as 50 fmols of $PGE_2$-G), we did not detect any $PGE_2$-G-like peak after LC-ESI-IT-ToF analysis. This, considering the average amount of striatal tissue that we analysed, indicates



| Groups | AEA (pmol/g) | 2-AG (nmol/g) |
|---|---|---|
| Control | 54.9 ± 5.8 | 1.5 ± 0.2 |
| + malonate | 53.7 ± 3.5 | 1.8 ± 0.2 |
| + malonate + O-3841 | 52.7 ± 11.9 | 1.1 ± 0.1* |



**Figure 1** Levels of endocannabinoids (panel **a**), GABA (panel **b**), BDNF-mRNA (panel **c**), number of Nissl-stained cells (panel **d** and representative microphotographs in panel **e**) and GFAP immunostaining (panel **f** and representative microphotographs in panel **g**), measured in the striatum of malonate-lesioned rats after DAGL inhibition with O-3841 (250 ng administered locally) and of their sham-operated controls. See details in the text. Values are means ± S.E.M. of 5–7 animals *per* group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (*$P<0.05$, **$P<0.005$ *versus* controls; $^{\#}P<0.05$ *versus* the group treated with malonate)

that less than 1.9 pmol/g wet tissue weight of $PGE_2$-G are present in the striatum of malonate-treated rats. However, this finding does not necessarily imply that $PGE_2$-G was not formed under our experimental conditions, as the generation of this compound might be restricted only to those striatal areas where the lesion is more intense, and hence impossible to detect when the whole striatum is analysed.

**Effects of MAGL inhibition on striatal degeneration caused by malonate.** Next, we investigated whether the increase in 2-AG levels after MAGL inhibition would produce the opposite effect to the protection found with DAGL inhibition. We first worked with the non-covalent MAGL inhibitor OMDM169,[32] but the local administration of this compound did not produce any significant change in the levels of 2-AG and anandamide (Figure 3a), and it did not aggravate the effects of malonate on the striatal parenchyma measured by Nissl staining (Figure 3b). Therefore, we used the more potent MAGL inhibitor JZL184, which, compared with OMDM169, is covalent and also more selective and elicits an eightfold increase in 2-AG levels.[33] The Nissl staining in the striatal parenchyma revealed some apparent reduction in the number of stained cells that did not reach statistical significance (see Figure 3d), but the differences were evident and statistically significant in the case of GFAP immunostaining, which was higher in the malonate-lesioned animals treated with JZL184 ($F(2,10)=41.22$, $P<0.0001$; Figure 3c), thus indicating stronger astrogliosis in the striatal parenchyma of these animals. We assumed that this aggravating effect was caused by the biotransformation of 2-AG in PG-Gs despite the fact that, also in this case, we were unable to detect *in vivo* generation of $PGE_2$-G. To further explore this possibility, we decided to inject this 2-AG derivative directly into the striatum of malonate-lesioned rats

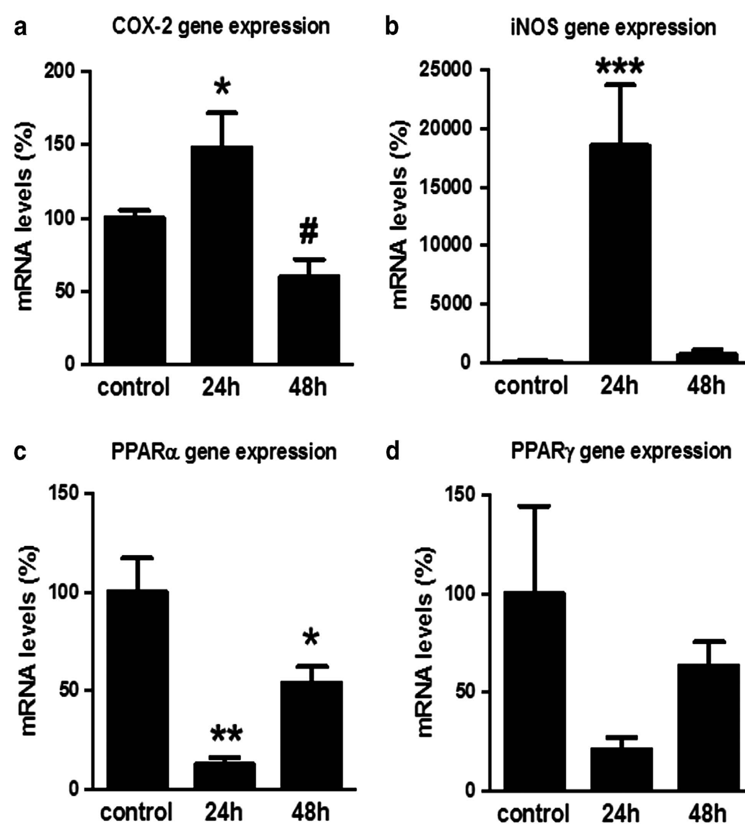
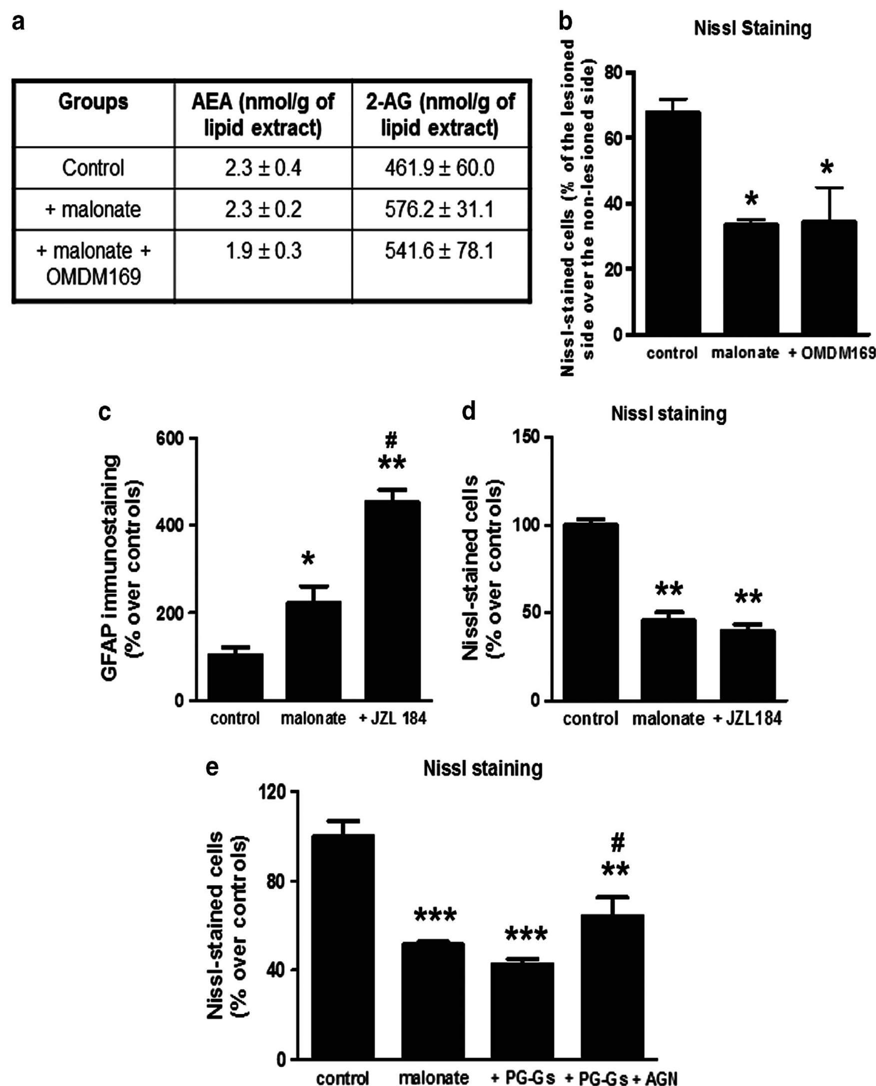



**Figure 2** mRNA levels for COX-2 (**a**), iNOS (**b**), PPAR-$\alpha$ (**c**) and PPAR-$\gamma$ (**d**) measured in the striatum of malonate-lesioned rats (at 24 or 48 hour after the injection) and of their sham-operated controls. See details in the text. Values are presented as means ± S.E.M. of 4–6 animals per group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (*$P<0.05$, **$P<0.005$, ***$P<0.0005$ *versus* the other groups; $^{\#}P<0.05$ *versus* the group treated with malonate)

and found a further reduction in the number of Nissl-stained cells in the striatal parenchyma that were partially attenuated by the co-administration of the PGE2-G antagonist AGN220675 ($F(3,15)=28.48$, $0<0.0001$; Figure 3e).

**Induction of COX-2 in response to malonate and effects of MAGL inhibition in cultured M-213 cells.** Given the difficulties to detect *in vivo* generation of PG-Gs in malonate-lesioned rats, we moved to an *in vitro* strategy using a cell line, M-213, which recapitulates some phenotypic characteristics of striatal neurons.[26] These cells were sensitive to malonate, which reduced the number of surviving cells by more than 50% at 6 h after the addition of the neurotoxin (Figure 4a). This was paralleled by a marked upregulatory response (>6 fold) in the expression of COX-2 (Figure 4b), reproducing the same response found *in vivo*, whereas the exposure to malonate downregulated the expression of MAGL and FAAH (Figures 4d and f). Accordingly, the activities of 2-AG and anandamide-hydrolysing enzymes, which in neurons are mostly accounted for MAGL and FAAH, respectively, were reduced (Figure 4c), and the same happened with the MAGL and FAAH levels (Figures 4e and g). Malonate did not affect the levels of anandamide and related *N*-acylethanolamines in these cells, whereas there was a trend towards an increase in the levels of 2-AG, although we were still unable to detect any significant change in the levels of $PGE_2$-G (Figure 4c). However, the addition of the MAGL inhibitor OMDM169 to the culture media tended to reduce the levels of 2-AG (Figure 5a) and allowed to detect for the first time $PGE_2$-G levels ($F(2,8)=17.22$, $P<0.005$; Figure 5b), precisely in the experimental condition in which the generation of this 2-AG derivative should be maximal. In agreement with this, we found an increase in malonate-induced cell death when 2-AG levels were elevated with OMDM169 ($F(2,14)=243.5$, $P<0.0001$; Figure 5c) and a trend towards an increase when we used JZL184 (Figure 5d). We also found that the addition of 2-AG *per se* enhanced malonate-induced cell death ($F(2,15)=130.6$, $P<0.0001$; Figure 5e). We conducted a pilot concentration–response study (0.1–50 $\mu$M), which showed that doses <25 $\mu$M did not enhance malonate-induced cell death, whereas a significant potentiating effect was found with 25 and 50 $\mu$M (data not shown).

We next investigated whether the enhancing effect of MAGL inhibitors on malonate-induced cell death may be attenuated by the blockade with the $PGE_2$-G antagonist AGN220675 or by inhibiting COX-2 with celecoxib, which would provide further evidence of the role of 2-AG biotransformation in the effects investigated in this study. We used for these experiments the MAGL inhibitor JZL184, which produced a slight increase in malonate-induced cell death, and we found that both AGN220675 ($F(3,30)=8.605$, $P<0.005$; Figure 6a) and celecoxib ($F(3,21)=30.04$, $P<0.0001$; Figure 6b; we used two concentrations, 12.5 and 25 $\mu$M, both

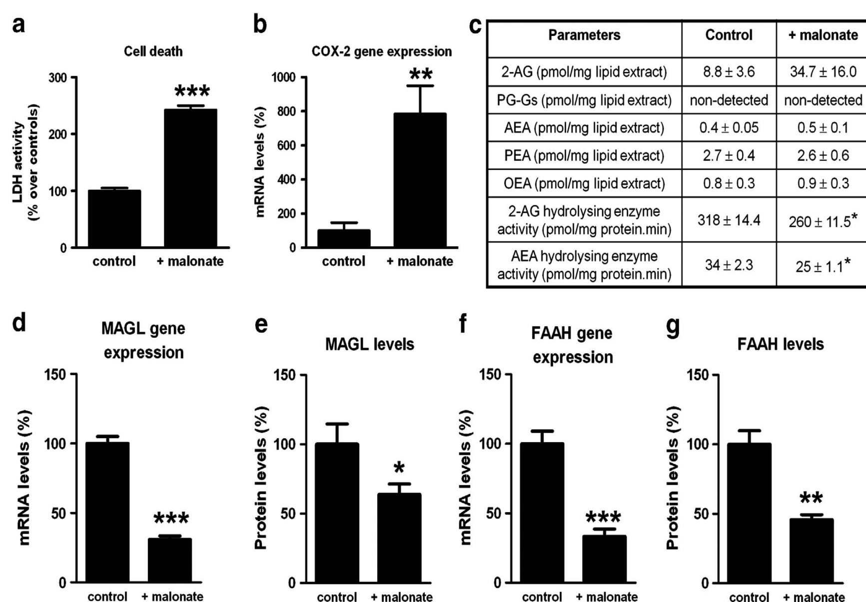


| Groups | AEA (nmol/g of lipid extract) | 2-AG (nmol/g of lipid extract) |
|---|---|---|
| Control | 2.3 ± 0.4 | 461.9 ± 60.0 |
| + malonate | 2.3 ± 0.2 | 576.2 ± 31.1 |
| + malonate + OMDM169 | 1.9 ± 0.3 | 541.6 ± 78.1 |



**Figure 3** Amounts of endocannabinoids (panel **a**), number of Nissl-stained cells (panels **b**, **d** and **e**) and GFAP immunostaining (panel **c**), measured in the striatum of malonate-lesioned rats after MAGL inhibition with OMDM169 (500 ng administered locally) or JZL184 (4 mg/kg weight administered i.p.), or after the administration of $PGE_2$-G (10 $\mu$g administered locally) and/or AGN220675 (300 ng administered locally), and of their sham-operated controls. See details in the text. Values are means ± S.E.M. of 4–6 animals *per* group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (*$P<0.05$, **$P<0.001$, ***$P<0.005$ *versus* controls; #$P<0.05$ *versus* the group treated with malonate and $PGE_2$-G)

equally effective) reduced cell death compared with the effect found with the combination of malonate and JZL184. We next tried to obtain a similar result with the direct addition of 2-AG. We again found that 2-AG was able to enhance malonate-induced cell death ($F(4,29)=36.42$, $P<0.0001$; Figure 6c), but this response was not altered by the celecoxib-induced inhibition of COX-2 or by the $PGE_2$-G antagonist AGN220675 (Figure 6c), used at the same concentrations as in the experiments with JZL184. In addition, higher concentrations of AGN220675 (1 and 50 mM) were ineffective in this case (data not shown). This latter finding may be related to the rapid degradation of 2-AG to arachidonic acid and glycerol in absence of MAGL inhibition, and the subsequent generation of prostaglandins, rather than PG-Gs, by the action of COX-1 and, to a smaller extent, COX-2, which would explain the highest cell death found after the combination of 2-AG and malonate compared with the cells exposed to malonate alone (Figure 6c). In fact, this is supported by the observation that the incubation of these cells with 2-AG at the same concentration (25 $\mu$M) as in the above experiment, and also at a higher concentration (50 $\mu$M), but in absence of malonate, increased cell death (more than twofold for 25 $\mu$M 2-AG and almost fourfold for 50 $\mu$M 2-AG), and both responses were significantly enhanced by celecoxib ($F(4,31)=26.6$, $P<0.0001$; Figure 6d). This inhibitor might have blocked the formation of $PGE_2$-Gs by COX-2 acting on exogenous 2-AG and enhanced the amount of 2-AG available for arachidonic acid generation and the action of COX-1, with a subsequent greater generation of prostaglandins, which would cause cell death. Indeed, 2-AG hydrolysis is an important source of arachidonic acid and neuroinflammatory prostaglandins,[34] such that genetic or pharmacological inactivation of 2-AG hydrolysis provides beneficial effects in experimental Parkinsonism[34] and Alzheimer's disease.[35]




| Parameters | Control | + malonate |
|---|---|---|
| 2-AG (pmol/mg lipid extract) | 8.8 ± 3.6 | 34.7 ± 16.0 |
| PG-Gs (pmol/mg lipid extract) | non-detected | non-detected |
| AEA (pmol/mg lipid extract) | 0.4 ± 0.05 | 0.5 ± 0.1 |
| PEA (pmol/mg lipid extract) | 2.7 ± 0.4 | 2.6 ± 0.6 |
| OEA (pmol/mg lipid extract) | 0.8 ± 0.3 | 0.9 ± 0.3 |
| 2-AG hydrolysing enzyme activity (pmol/mg protein.min) | 318 ± 14.4 | 260 ± 11.5* |
| AEA hydrolysing enzyme activity (pmol/mg protein.min) | 34 ± 2.3 | 25 ± 1.1* |

**Figure 4** LDH activity (panel **a**), mRNA levels for COX-2 (panel **b**), MAGL (panel **d**) and FAAH (panel **f**), protein levels of MAGL (panel **e**) and FAAH (panel **g**) and levels of 2-AG, PGE2-G, anandamide and related *N*-acylethanolamines and activities of 2-AG- and anandamide-hydrolysing enzymes (panel **c**), measured in cultured M-213 cells treated for 6 h with 40 mM malonate. See details in the text. Values are presented as means ± S.E.M. of 5–7 cases per group (except in the measures of enzyme activities and protein levels that were three cases per group). Data were assessed by the unpaired Student's *t*-test (*$P<0.05$, **$P<0.01$, ***$P<0.005$ *versus* controls)

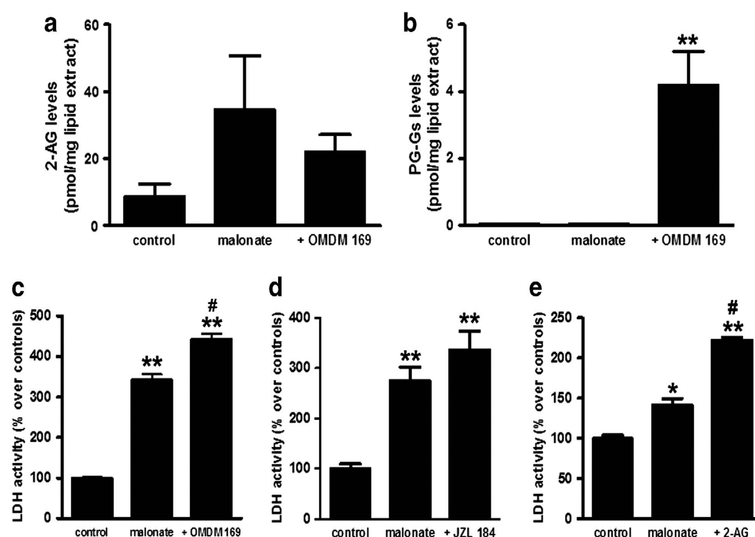



**Figure 5** Amounts of 2-AG (panel **a**) and $PGE_2$-G (panel **b**), and LDH activity, measured in cultured M-213 cells treated for 6 h with 40 mM malonate and/or the inhibitors of MAGL, OMDM169, at 1 $\mu$M (panel **c**) or JZL184 at 10 nM (panel **d**), or with 40 nM malonate and/or 2-AG at 25 $\mu$M (panel **e**). See details in the text. Values are presented as means ± S.E.M. of 4–6 cases *per* group. Data were assessed by the one-way analysis of variance followed by the Student–Newman–Keuls test (*$P<0.05$, **$P<0.005$ *versus* controls; $^{\#}P<0.05$ *versus* malonate)

## Discussion

Previous studies have shown that $CB_2$ receptor-deficient mice exhibit a greater sensitivity to malonate,[12] whereas the blockade of $CB_1$ receptors with rimonabant also enhanced the magnitude of striatal degeneration caused by this neurotoxin in rats.[23] In this context, we predicted that the blockade of 2-AG biosynthesis with the recently developed DAGL inhibitor O-3841[27] should also result in a greater sensitivity to malonate, given the previously reported neuroprotective effects of 2-AG through its capability to activate both cannabinoid receptors with similar efficacy.[28] Surprisingly, however, the administration of O-3841 to malonate-lesioned rats, rather than aggravating the striatal lesion, was accompanied by greater survival of striatal neurons and by the attenuation of glial activation typically associated with the malonate lesion.[12]

These unexpected observations found some support in recent studies that have demonstrated that 2-AG may also behave as a neurotoxic factor under special circumstances,

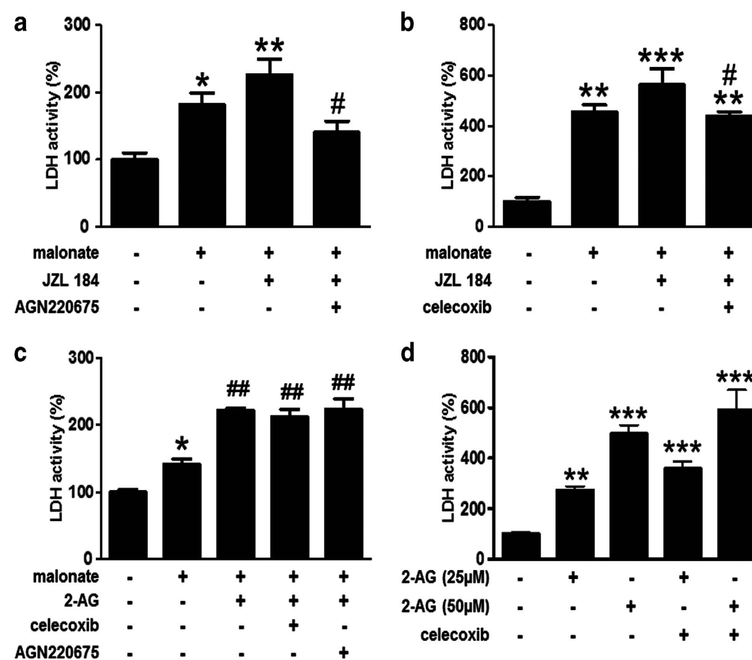



**Figure 6** LDH activity measured in cultured M-213 cells treated for 6 h with 40 mM malonate and/or the inhibitor of MAGL JZL184 at 10 nM in the presence or absence of $PGE_2$-G antagonist AGN220675 at 25 $\mu$M (panel **a**) or the COX-2 inhibitor celecoxib at 12.5 $\mu$M (panel **b**), or with 40 nM malonate and/or 2-AG at 25 $\mu$M combined with celecoxib at 12.5 $\mu$M or AGN220675 at 25 $\mu$M (panel **c**), or treated directly with 2-AG at 25 or 50 $\mu$M and/or celecoxib at 12.5 $\mu$M (panel **d**). See details in the text. Values are presented as means ± S.E.M. of 6-8 cases *per* group. Data were assessed by the one-way analysis of variance followed by the Student–Newman–Keuls test (*$P<0.05$, **$P<0.005$, ***$P<0.0005$ *versus* controls; #$P<0.05$ *versus* malonate + JZL184; ##$P<0.005$ *versus* malonate)

namely, during the overexpression of COX-2, a key response in neuroinflammatory events accompany neurodegeneration.[36–38] Thus, Sang *et al.*[25] reported that 2-AG may be a natural substrate of inducible COX-2 enzyme and generate a series of metabolites, known as PG-Gs,[39] the most abundant of which, $PGE_2$-G, is known to induce excitotoxic damage[25] and to exert pro-inflammatory and hyperalgesic effects.[40] In fact, 2-AG is oxygenated by COX-2 as effectively as arachidonic acid,[25] whereas the oxygenation of anandamide by this enzyme is slower.[41] Therefore, it appears that the key condition for the activation of this metabolic pathway would be that COX-2 enzyme is abnormally upregulated in response to a brain insult. In such situation, there would be an imbalance between the previously reported neuroprotective effects of 2-AG (mediated by the activation of either $CB_1$, $CB_2$ or other cannabinoid-related receptors;[28,42–44]) and its transformation by COX-2, which would generate potential neurotoxic metabolites like $PGE_2$-G. Our data support the existence of this imbalance in our experimental conditions, because we found an elevated COX-2 gene expression in the malonate-lesioned striatum compared with controls. We also found a similar early upregulatory response in other pro-inflammatory enzyme such as iNOS, which frequently accompanies COX-2 elevation.[30] In addition, we observed the expected down-regulation of PPAR-$\alpha$ and PPAR-$\gamma$, for which certain cannabinoids, including 2-AG (see below), have been recently found to act as agonists.[43,45] This may explain the observed increases in COX-2 and iNOS with the reduction of the PPAR-dependent inhibitory effect on NF$\kappa$B signalling, which in turn is involved in the stimulation of the expression of these pro-inflammatory enzymes.[31] It is important to remark that the elevation of COX-2 has been already found in neurodegenerative disorders including HD,[36,46–48] having an instrumental role in the pathogenesis,[38] so that its reduction through different strategies (e.g., pharmacological inhibition) may reduce brain damage.[49] Interestingly, 2-AG may represent an important source of arachidonic acid and neuroinflammatory prostanoids in some of these disorders,[34,35] and, therefore, the neuroprotective and anti-inflammatory actions of O-3841 effect could be also explained by the interference with this mechanism. However, although in the studies in which 2-AG was shown to act as a biosynthetic precursor of neuroinflammatory prostanoids, MAGL inhibitors produced neuroprotective and anti-inflammatory effects,[34,35] here we found that MAGL inhibition results in a greater striatal damage both *in vivo* and *in vitro*. Therefore, our findings with O-3841 and JZL184/OMDM169 can only be interpreted by the interference with, or enhancement of, the generation of oxygenated derivatives of 2-AG by elevated activity of COX-2 (see Figure 7 panels A and B, respectively). In other words, the inhibition of DAGL might re-establish the balance between the neuroprotective and neurotoxic mechanisms of 2-AG, whereas the inhibition of MAGL would enhance this imbalance, which is, in fact, likely to be facilitated under conditions of reduced expression of this enzyme, as in the present case (see below).

On these premises, the next objective of our study became the demonstration that PG-Gs, in particular $PGE_2$-G, are generated in the striatum in response to malonate, and that they are reduced by DAGL inhibition and increased after MAGL inhibition. However, we were unable to find detectable levels of $PGE_2$-G after these manipulations *in vivo*, in

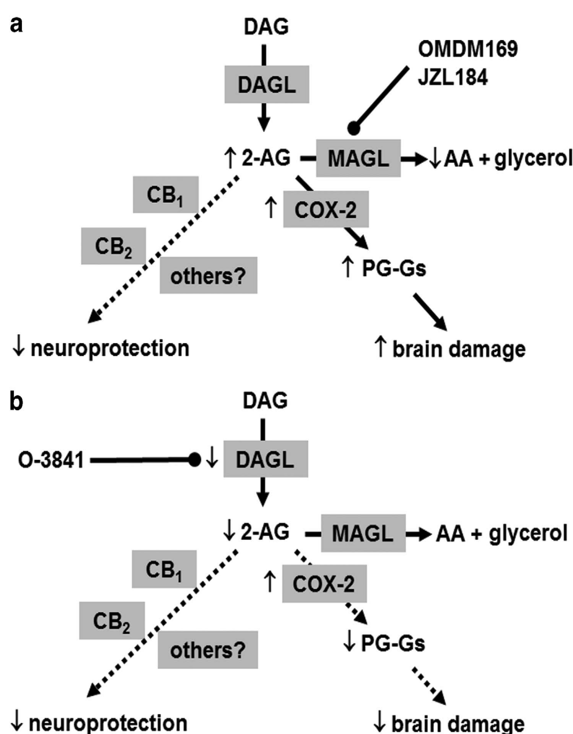



**Figure 7** Schematic overview of possible mechanisms explaining the effect of 2-AG following malonate lesion and after MAGL (panel **a**) or DAGL (panel **b**) inhibition

particular after the lesion with malonate combined with MAGL inhibition, that is, the experimental conditions in which the generation of these derivatives would be maximal. On the basis of the detection limit of our analytical method, we estimated that an amount of less than 1.9 pmol/g wet tissue weight of this compound is present in the whole striatum from both unlesioned and lesioned rats, in agreement with previous findings indicating that the tissue concentrations of this compound, for example, in the rat paw, are in the order of the fmol/g wet tissue weight.[40] It is possible that the generation of this metabolite is restricted to the lesioned cells within the striatum, then rendering impossible its detection when the whole striatum needs to be used for biochemical determinations (see Results section).

Given the difficulties to detect the generation of PG-Gs from 2-AG in our *in vivo* model, we considered the possibility that pathways other than COX-2 metabolism of 2-AG may be involved in the generation of putative pro-inflammatory 2-AG-derived compounds, or that the neuroprotection shown by O-3841 was due to its interaction with targets other than DAGL. An attractive possibility would be the expected increase in the DAG levels derived from the inhibition of DAGL, as DAG signalling has been associated with cell proliferation, differentiation and survival/death decision.[50] However, our next experiments provided further evidence in support of a mechanism involving the generation of PG-Gs from 2-AG by the action of COX-2. First, we administered synthetic $PGE_2$-G to malonate-lesioned rats and found an aggravation of striatal damage that was partially reversed by the $PGE_2$-G antagonist AGN220675. Secondly, in experiments conducted *in vitro* with cultured M-213 cells, which also experience a marked upregulation of COX-2 in response to malonate, we showed that the increase in endogenous 2-AG levels derived from MAGL inhibition results in a greater cell death. This effect appeared to be related to the generation of PG-Gs as it was reversed by pharmacological manipulations that either inhibit the formation (e.g., inhibition of COX-2 with celecoxib) or block the action at its target(s) (e.g., AGN220675) of $PGE_2$-G. Indeed, the only experimental condition in which we were able to detect $PGE_2$-G was in these cells after the incubation with malonate combined with the MAGL inhibitor OMDM169, that is, the same experimental conditions in which cell death was maximal. Notably, malonate treatment of M-213 cells was accompanied by the downregulation of MAGL mRNA, protein and enzymatic activity, that is, another condition that, together with COX-2 upregulation, is expected to favour the formation of PG-Gs.

Another issue arising from our data and deserving some comments is the role that 2-AG may have in the upregulation of COX-2 observed here. A recent study[51] showed that COX-2 upregulation may be elicited by a loss in the endogenous inhibition of this enzyme exerted by 2-AG via $CB_1$ receptors. This was found in the hippocampus under pro-inflammatory and excitotoxic stimuli both *in vivo* and *in vitro*,[51] thus supporting the idea that neuroprotection exerted by 2-AG through the activation of $CB_1$ receptors might be used also to prevent the COX-2 elevation typical of several neuroinflammatory/neurodegenerative conditions. *A priori*, this possibility would be in contrast with our present hypothesis of 2-AG exerting a neurotoxic, rather than neuroprotective, effect. It is possible that neuroprotection with 2-AG is the predominant response when the levels of 2-AG are strongly elevated by the neurotoxic stimuli, as in the case of traumatic brain injury,[44] as this elevation would allow to prevent, among others, the upregulation of COX-2. In contrast, in our present case, in which 2-AG levels remained unaffected by the malonate-induced lesion, this might have allowed the upregulation of COX-2 and the subsequent potential transformation of basal 2-AG levels into PG-Gs by the action of this enzyme, thus supporting the above-mentioned idea of an imbalance between the neuroprotective and neurotoxic effects of 2-AG. Interestingly, a follow-up study by the same group[52] revealed that the inhibitory effect by 2-AG of COX-2 expression also involves the activation of PPAR-$\gamma$, for which we have found, under our experimental conditions, a trend towards downregulation. This downregulation effect might counteract the inhibition by 2-AG of COX-2, participate in COX-2 upregulation and facilitate the conversion by this enzyme of 2-AG in PG-Gs. It is also important to remark that the downregulation of PPARs observed in the malonate-lesioned rats would be another factor, in addition to elevated levels of COX-2, aggravating the imbalance between the neuroprotective and neurotoxic effects of 2-AG, as these nuclear receptors have been involved in part of the neuroprotective actions of 2-AG,[43,45] which would then be lost in malonate-lesioned rats.

In summary, the inhibition of 2-AG biosynthesis is accompanied by neuroprotection in rats lesioned with malonate. This effect seems to be related to a potential inhibition of certain pro-neuroinflammatory actions of 2-AG, particularly those involving its transformation into oxygenated metabolites by COX-2, an enzyme that was found here to be elevated in

the striatum after the lesion with malonate. In contrast, MAGL inhibition or the administration of $PGE_2$-G *in vivo* aggravated the malonate toxicity, and these effects were reproduced in cultured M-213 cells in which upregulation of COX-2 was also elicited by malonate.

## Materials and methods

**Animals, treatments and sampling.** Male Sprague–Dawley rats were housed in a room with controlled photoperiod (08 : 00–20 : 00 light) and temperature ($22 \pm 1\,^{\circ}C$). They had free access to standard food and water and were used at an adult age (3-month old; 300–350 *g* weight) for experimental purposes, all conducted in an attempt to minimize animal pain and discomfort, and following local and European rules (directive 86/609/EEC). Rats were injected stereotaxically (coordinates: +0.8 mm anterior, +2.9 mm lateral from the bregma, −4.5 mm ventral from the dura mater) into the left striatum with 2 M malonate (dissolved in PBS 0.1 M, pH = 7.4) in a volume of 1 $\mu$l or were sham operated in the same left striatum but with no injection of the toxin. The contralateral striatum of each animal always remained unaffected, allowing analyses performed in this animal model lead to data frequently expressed as percentage of the lesioned side over the corresponding non-lesioned side. Animals were used for these experimental analyses 48 h later, except in the case of the time–course analysis of gene expression for COX-2, inducible nitric oxide synthase (iNOS) and peroxisome-proliferator activated receptors (PPAR) for which animals were sacrificed at 24 and 48 h after the lesion.

O-3841 (octadec-9-enoic acid 1-methoxymethyl-2-(fluoro-methyl-phosphinoyloxy)-ethyl ester), a kind gift by Dr. A Mahadevan, Organix, Woburn, Massachussets, was synthesized as previously described.[27] The compound was dissolved in dimethylsulfoxide (DMSO) and diluted in 0.1 M PBS (pH = 7.4) for administration (final concentration of DMSO < 1%). As this compound is not expected to cross the blood–brain barrier, it was locally administered 30 min before the administration of malonate. Each animal received a unique administration of 250 ng of O-3841 in a volume of 1 $\mu$l. In additional experiments, 500 ng of OMDM169, synthesized as previously described,[32] 10 $\mu$g of prostaglandin $E_2$-glycerol ester ($PGE_2$-G; purchased from Cayman Chemical, Ann Arbor, Michigan, USA) and 300 ng of AGN220675 (generously provided by Dr. Woodward, Allergan, Irvine, CA, USA), prepared in 5% DMSO-saline, were also administered following the same procedure as in the case of O-3841. In another experiment, JZL184 (purchased from Tocris Bioscience, Bristol, UK, and dissolved in DMSO-Tween 80-saline, 1 : 1 : 18) was administered to malonate-lesioned rats at the dose of 4 mg/kg weight in two injections, 30 min before and 2 h after the intrastriatal injection of malonate, following our previously published procedure.[12] Malonate-lesioned animals, administered with the corresponding vehicle for all treatments (malonate group), as well as sham-operated animals (control group), were always included for each pharmacological experiment.

Depending on the experiment, animals were killed 24–48 h after the administration of malonate, and their brains were rapidly removed and frozen in 2-methylbutane, cooled in dry ice and stored until evaluation of neurochemical parameters indicating the degree of malonate-induced striatal injury. Some animals from each experimental group were perfused with 10% formalin, and their brains were post fixed during 3 h, cryoprotected and stored at −20 °C before being sliced in a vibratome. This material was used for histological analyses. In a separate group of animals (only in the experiments with the DAGL inhibitor O-3841 or the MAGL inhibitor OMDM169), brains were dissected before freezing and their striata were weighed, frozen on dry ice and stored in eppendorf tubes at −80 °C until lipid extraction and analysis of endocannabinoids and related compounds ($PGE_2$-G or *N*-acylethanolamines). The entire procedure took less than 5 min, which is significantly less than the amount of time required for postmortem generation of 2-AG, arachidonoylethanolamide (anandamide) or related compounds. Finally, in another separate experiment, malonate-injected and sham-operated rats were decapitated twice after the formation of the lesion (24 and 48 h). Their brains were quickly and carefully removed, the striata dissected and frozen for qRT-PCR analysis.

**Cell culture.** M-213-2O cells were a generous gift from Dr. WJ Freed (National Institute on Drug Abuse, Bethesda, MD, USA; see 26). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM ultra-glutamine, 1% non-essential amino acids and 1% penicillin/streptomycin (LONZA, Verviers, Belgium) and under a humidified 5% $CO_2$ atmosphere at 37 °C. For cytotoxicity experiments, cells were seeded at 50000 cells/$cm^2$ in 24-well plates and maintained under a humidified atmosphere (5% CO2) at 37 °C overnight. Afterwards, normal medium was replaced by serum-free medium and cells were exposed to either OMDM169 (1 $\mu$M), JZL184 (10 nM), 2-AG (25 and 50 $\mu$M; purchased from Tocris Bioscience, Bristol, UK), celecoxib (12.5 or 25 $\mu$M; purchased from Sigma-Aldrich, Madrid, Spain) or AGN220675 (25 $\mu$M) added from more-concentrated solutions (prepared in serum-free medium and containing low amounts of DMSO) to obtain these final concentrations (final DMSO was always < 0.1%). Thirty min later, cells were exposed to malonate for 6 h, added from a solution prepared in a serum-free medium to obtain a final concentration of 40 mM in the wells. Control cells were maintained in the serum-free medium, but were not exposed to malonate or the different treatments. After malonate exposure, media from each well were used to measure lactate deshydrogenase (LDH) activity as an index of cell death, using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (G1780, Promega Biotech Ibérica, Madrid, Spain). In all experiments, the data of LDH activity were compared with the values obtained in a positive control with 1% Triton X-100 (Sigma-Aldrich, Madrid, Spain) that was taken as 100% of cell death. Compared with the Triton X-100-exposed cells, control cells (not exposed to malonate) accounted for a 10–20% of cell death, whereas malonate accounted for a 60–80%. For graphical presentation, the data in malonate-exposed cells (with or without the different additional treatments) were always expressed as the percentage over the data in the control cells (not exposed to malonate).

**Analysis of endocannabinoid levels.** Tissues or cells were homogenized in 5 volume of chloroform/methanol/ Tris–HCl 50 mM (2 : 1 : 1) containing 10 pmol of $d^8$-anandamide, $d^4$-palmitoylethanolamide (PEA), $d^4$-oleylethanolamide (OEA) and $d^5$-2-AG. Deuterated standards were synthesized from $d^8$ arachidonic acid and ethanolamine or glycerol, or from $d^4$-ethanolamine and palmitic or oleic acid, as described ([53,54] respectively). Homogenates were centrifuged at 13 000 *g* for 16 min (4 °C), and the aqueous phase plus debris were collected and extracted again twice with 1 volume of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized extracts were resuspended in chloroform/methanol 99 : 1 by volume. The solutions were then purified by open-bed chromatography on silica as described.[54] Fractions eluted with chloroform/ methanol 9 : 1 by volume. (containing anandamide, 2-AG, OEA and PEA) were collected and the excess solvent evaporated with a rotating evaporator, and aliquots were analysed by isotope dilution liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC-APCI–MS) carried out under conditions described previously,[55] and allowing the separations of 2-AG, anandamide, OEA and PEA. MS detection was carried out in the selected ion-monitoring mode using *m/z* values of 356 and 348 (molecular ion +1 for deuterated and undeuterated anandamide), 384.35 and 379.35 (molecular ion +1 for deuterated and undeuterated 2-AG), 304 and 300 (molecular ion +1 for deuterated and undeuterated PEA) and 330 and 326 (molecular ion +1 for deuterated and undeuterated OEA). The amounts of endocannabinoids and related *N*-acylethanolamines were expressed as pmols/mg of lipids extracted. An amount of 1 mg of extracted lipids corresponds to ~0.010–0.015 g of wet tissue weight.

**Method for the extraction, purification and quantification of PGE2-G.** Tissues and cells were homogenized in 5 volume of acetone containing 25 pmol of d4-$PGE_2$-G (a kind gift by Dr. DF Woodward, Allergan, Irvine, CA, USA). Homogenates were centrifuged at 13 000 *g* for 5 min (4 °C), and the debris was extracted again four times with 1 volume of acetone. The organic phases from the five extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized extracts were resuspended in chloroform/methanol 99 : 1 by volume. The solutions were then purified by open-bed chromatography on silica using a gradient elution of chloroform/methanol to separate different class of lipids. Fractions eluted with chloroform/methanol 7 : 3 by volume. (containing $PGE_2$-G) were collected and the excess solvent evaporated with a rotating evaporator, and aliquots were analyzed by Liquid Chromatography-Electrospray-Ion Trap-Time of Flight (LC-ESI-IT-ToF) mass spectrometry. An IT-ToF mass spectrometer (Shimadzu) was used in conjunction with an LC-20AB (Shimadzu). LC analysis was performed in the isocratic mode using a DiscoveryC18 column (15 cm × 2.1 mm, 5 $\mu$m) and methanol/water/acetic acid (53 : 47 : 0.05 by volume.) as mobile phase with a flow rate of 0.15 ml/min. Identification of $PGE_2$-G (retention time 22.5 min) was carried out using ESI ionization in the positive mode with nebulizing gas flow of 1.5 ml/min and a curved desolvation line temperature of 250 °C. $PGE_2$-G quantification was performed by isotope dilution by using *m/z* values of 453.2766 and 449.2515, corresponding to the sodium adduct of the molecular ion $(M+23)^+$ for deuterated and undeuterated $PGE_2$-G, respectively. The full recovery of $PGE_2$-G from tissue due to the analytical procedure reported above was 44.9 ± 9.1. The LC-ESI-IT-ToF method described here for the first time is specific and sensitive with a limit of detection (defined as the concentration at which the signal/noise ratio is greater than 3 : 1) of 25 fmol. Moreover, the ratio between the $(M+23)^+$ peak areas of undeuterated (0.025–20 pmol) *versus.* deuterated (1 pmol) $PGE_2$-G varied linearly with the amount of undeuterated $PGE_2$-G. The quantification limit of $PGE_2$-G was 50 fmol.[56]

**HPLC determination.** Brain coronal slices (around 500 $\mu$m thick) were made at the level of the striatum.[57] Subsequently, this structure was dissected and homogenized in 20–40 volumes of cold 150 mM potassium phosphate buffer, pH 6.8, and distributed into two aliquots, one to be used for the analysis of protein concentration.[58] The other aliquot was used for the measurement of GABA contents by HPLC coupled to electrochemical detection.[11] It was diluted (½) with 0.4 N perchloric acid containing 0.4 mM sodium disulphite, 0.90 mM EDTA and $\beta$-aminobutirate as internal standard. Afterwards, samples were centrifuged for 3 min (15000 g) and the supernatants subjected to a previous derivatization process with *o*-phthaldehide (OPA)-sulphite solution (14.9 mM OPA, 45.4 mM sodium sulphite and 4.5% ethanol in 327 mM borate buffer, pH 10.4; see details in 11), previous to their injection into the HPLC system. This consisted of the following elements. The pump was an isocratic Spectra-Physics 8810. The column was an RP-18 (Spherisorb ODS-2; 150 mm, 4.6 mm, 5 $\mu$m particle size; Waters, Massachusetts, USA). The mobile phase, previously filtered and degassed, consisted of 0.06 M sodium dihydrogen phosphate, 0.06 mM EDTA and 20-30% methanol (pH 4.4). The flow rate was 0.8 ml/min. The effluent was monitored with a Metrohm bioanalytical system amperometric detector using a glassy carbon electrode. The potential was 0.85V 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. The results were obtained from the peaks and calculated by comparison with the area under the corresponding internal standard peak. Values were expressed as $\mu$g/mg of protein.

**Histological analyses.** Fixed brains were sliced (35 $\mu$m-thick sections) in a vibratome. Free-floating sections obtained from each brain were incubated with a monoclonal anti-GFAP-Cy3 conjugate antibody (1 : 500, Sigma-Aldrich, Madrid, Spain) 24 h at room temperature. Following extensive washing, the brain sections were mounted on glass slides using the Vectashield Mounting Medium (Vector Laboratories, Burlingame, USA). In addition, the Hoescht staining was used for nuclear counterstaining. Adjacent sections to those used in the immunohistochemical analysis were used for Nissl staining, which allowed for the

determination of the number of cells after the different treatments done. A Nikon Eclipse 90i confocal microscope and a Nikon DXM 1200F camera were used for observation and photography, and all image processing techniques were performed using ImageJ, the software developed and freely distributed by the US National Institutes of Health (Bethesda. MD, USA).

**Real time qRT-PCR analysis.** Total RNA was isolated from lesioned or non-lesioned rat striata, or from cultured cells, using the RNATidy reagent (AppliChem., Cheshire, CT, USA). 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. After the removal of genomic DNA (to eliminate DNA contamination), single-stranded complementary DNA was synthesized from 1 $\mu$g of total RNA using a commerical kit (Rneasy Mini Quantitect Reverse Transcription, Qiagen, Izasa, Madrid, Spain). The reaction mixture was kept frozen at $-20\,^{\circ}$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 COX-2 (reference: Rn01483828_m1), BDNF (reference: Rn01484928_m1), iNOS (reference: Rn00561646_m1), PPAR-$\alpha$ (reference: Rn00566193_m1), PPAR-$\gamma$ (reference: Rn00440945_m1), MAGL (reference: Rn00593297_m1) and FAAH (reference: Rn00577086_m1), using $\beta$-actin expression (reference: Rn00667869_m1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA, USA).

**Enzyme assays.** 2-AG and anandamide hydrolysing activities were measured in cells after malonate treatment as described above for $PGE_2$-G measurement. In particular, 2-AG hydrolysis, which is mostly accounted for at least 80% by MAGL in neurons,[59] was measured by incubating the 10 000 *g* cytosolic fraction (70–100 $\mu$g/sample) of cells in Tris-HCl 50 mM, at pH 7.0 at 37 °C for 20 min, with synthetic 2-arachidonoyl-($^3$H)-glycerol (40 Ci/mmol, ARC St. Louis, MO, USA) properly diluted with 2-AG (Cayman Chemicals, Ann Arbor, MI, USA) to the final concentration of 50 $\mu$M. After incubation, the amount of [$^3$H]-glycerol produced was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH$ 1 : 1 (by volume). Anandamide hydrolysis, which is almost uniquely accounted for FAAH in neurons, was measured by incubating the 10 000 *g* membrane fraction (70–100 $\mu$g/sample) of tissues in Tris-HCl 50 mM, at pH 9.0–10.00 at 37 °C for 30 min, with synthetic *N*-arachidonoyl-[$^{14}$C]-ethanolamine (55 mCi/mmol, ARC St. Louis, MO, USA) properly diluted with anandamide (Tocris Bioscience, Avonmouth, Bristol, UK) to the final concentration of 10 $\mu$M. After incubation, the amount of [$^{14}$C]-ethanolamine produced was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH$ 1 : 1 (by volume). In both cases, values were expressed as pmol of product formed/mg of protein in 1 minute of incubation.

**Western blot analysis.** Cultured cells (approximately $10^6$/sample), exposed or not to 40 mM malonate for 6 h, were collected and washed twice in cold PBS and lysed on ice using the RIPA lysis solution. Lysates were maintained in ice for 20 min before a centrifugation of 15 min at 15 000 *g*. Supernatants were recovered and quantified by Bio-Rad DC Protein Assay Kit (Bio-Rad, Segrate, MI, Italy). Subsequently, 40–50 $\mu$g of lysates were boiled for 5 min in the Laemmli SDS loading buffer and loaded on precast polyacrylamide gels (4–12% gradient; Bolt Bis-Tris Plus gels; Life Technologies, Milan, Italy) and then transferred to a PVDF membrane. Filters were incubated overnight at 4 °C with the following antibodies: mouse anti-FAAH antibody (1 $\mu$g/ml; from Sigma-Aldrich Milan, Italy; batch number: 11011-4H8) or rabbit anti-MAGL (1 : 200; Cayman Chemical, Ann Abor MI, USA). An anti $\alpha$-tubulin antibody (1 : 5000; Sigma-Aldrich Milan, Italy) was used to check for equal protein loading. Reactive bands were detected by chemiluminescence (ECL or ECL-plus; Biorad, Segrate, MI, Italy). Images were analysed on a ChemiDoc station with Quantityone software (Bio-Rad, Segrate, MI, Italy). Data were calculated as the ratio between the optical densities of the protein and $\alpha$-tubulin, but they were normalized as a percentage over the control group for presentation.

**Statistical analyses.** All data were assessed by one-way ANOVA followed by the Student–Newman–Keuls test, or by the unpaired Student's *t*-test, as required, using the GraphPad software (version 4.0).

## Conflict of Interest

All authors declare that they have no conflicts of interest.

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# DISCUSSION

Neurodegenerative diseases can certainly be considered as emerging diseases in the 21st century. The progressive increase in human life span occurring in the last decades, in particular in developed countries, have led to the emergence of disorders that involve the progressive and unstoppable degeneration of certain parts of the nervous system resulting in important physical and cognitive inabilities. In parallel with the increase in the prevalence of neurodegenerative disorders in the world population, it is necessary to progress in the knowledge of these disorders and, in particular, in the development of new therapies that can delay the progression of these devastating illnesses.

Huntington's disease, despite having a known origin (a single mutation in the HTT gene that leads to a protein with an abnormally long poly-glutamine tract), is not different from other neurodegenerative disorders in terms of aging influence and brain atrophy. The degeneration of basal ganglia and cerebral cortex results not only in motor symptoms, but cognitive decline and psychiatric problems as well. The prognosis of these patients is really poor, as to date there is no cure and the treatments available are merely palliative (e.g. antidepressants and benzodiacepines for the control of psychiatric issues). For the management of choreic movements, tetrabenazine (a vesicular monoamine transporter type 2 inhibitor) is the only treatment available (Kenney *et al.*, 2007). Its derivative deutetrabenazine, which has a longer half-life and reduced metabolic variability, is under clinical evaluation (Huntington Study *et al.*, 2016). However, beyond the management of symptoms, there is an urgent need to find a therapy that can delay the progression of the disease. In this context, only pridopidine (a dopamine stabilizer) has provided a weak hope, as it showed certain ability to improve Total Functional Capacity, which may be an indirect indicator of disease progression, in patients compared to placebo, in a phase II clinical trial (Shannon, 2016), although it did not show improvement in any other parameter evaluated.

Pharmacological manipulation of the ECS may have an impact in both motor and cognitive symptoms as well as in disease progression in HD. As explained in the Introduction, HD patients show alterations in various components of this system during the development of the disease (Glass *et al*., 200; Palazuelos *et al*., 2009). Moreover, several preclinical studies conducted during the last years have shown that certain cannabinoid compounds are capable of attenuating cytotoxic processes involved in the neuropathology of the disease, such as oxidative, inflammatory and excitotoxic injury, thus improving neuronal survival (reviewed in Fernández-Ruiz *et al.*, 2015). All these data has generated a hope on the potential of cannabinoids as disease modifiers in HD.

As explained in previous sections, not only $CB_1$ (Pintor *et al*., 2006; Blázquez *et al.*, 2011) and $CB_2$ agonists (Sagredo *et al*., 2009, Palazuelos *et al.*, 2009) have shown efficacy in preclinical models of the disease. The use of antioxidant cannabinoids (Sagredo *et al*., 2011) and inhibitors of endocannabinoid degrading enzymes (Lastres-Becker *et al*., 2003) have also been tested with positive results in different HD experimental models. Given the multiple dysregulation of the endocannabinoid system in HD subjects, the multi-target capability of cannabinoids (acting by cannabinoid receptor dependent and independent mechanisms), and the action on different pathways separately by cannabinoids in different experimental approaches, the best pharmacological strategy would be the use of a broad-spectrum treatment, with only one cannabinoid having multiple sites of action or combining various cannabinoid compounds active at different neuroprotective mechanisms of action. The purpose of this multi-target strategy would be to act at the greatest number of pathological events that occur in the disease.

With this purpose, the pharmacological evaluations performed in this Doctoral Thesis have been designed to point towards three directions: first, the use of different experimental models that can resemble most of the cytotoxic events of the pathology; second, the validation of a cannabinoid medicine that has already been approved for other prescriptions (Sativex for the treatment of spasticity in multiple sclerosis), and that owns the desired broad-spectrum profile; third, the study of cannabinoid compounds that have barely been investigated, such as CBG, and whose pharmacological profile, as happens with CBD, is different from classic cannabinoids,

being devoid of psychoactive effects, and having interest for medical development.

As reported in Chapter 1, we have demonstrated that the broad-spectrum strategy has a good neuroprotective potential in this disease. The Sativex-like combination of phytocannabinoids attenuated neuronal loss and glial activation in the malonate model of HD, characterized by inflammatory and apoptotic damage (Valdeolivas *et al.*, 2012), and improved some motor deficits and brain metabolic alterations in R6/2 transgenic mice (Valdeolivas *et al.*, 2017). These results can be added to the ones obtained in the previously published study with this phytocannabinoid formulation in the 3NP model, which is characterized by oxidative stress and calpain activation (Sagredo *et al.*, 2011). Moreover, we could also demonstrate that the neuroprotective effect of Sativex was exerted by the activation of $CB_1$ and $CB_2$ receptors in the case of the malonate model, whereas Sativex apparently worked by cannabinoid receptor-independent mechanisms (although the possibility of a role of PPARs in this model cannot be ruled out) in the case of the 3NP-lesioned animals, which supports our idea of a multi-target strategy. The mechanism(s) involved in the beneficial effects of Sativex in R6/2 mice was(were) not investigated in this work, but previous work in which our group has participated, demonstrated that both $CB_1$ and $CB_2$ receptor appear to be involved. It is important to remark that the same formulation provided beneficial effects in three different models in which striatal damage was predominantly caused by one specific cytotoxic insult, then stressing the pleiotrophic properties of cannabinoids and their suitability to be used as neuroprotective agents. We wanted to maintain the same approach in the evaluation of CBG, so we investigated this non-psychoactive phytocannabinoid in the model of mice lesioned with 3NP and in the R6/2 model, and in both cases the treatment with this phytocannabinoid attenuated some of their characteristic neurological deficits and histopathological signs (Valdeolivas *et al.*, 2015). CBG has a low affinity for cannabinoid receptors, but we showed its capability to activate PPARγ receptors instead, so we suggest that this could be one of the mechanisms involved in the neuroprotective effects of this compound in these two models, and, by extension, this may be also one of the active mechanisms, as mentioned before, in the effects of Sativex, which also contains a cannabinoid receptor-independent phytocannabinoid as CBD, in 3NP-lesioned rats (Sagredo et al., 2011). Therefore, we can conclude that the phytocannabinoids evaluated are able to provide neuroprotection against oxidative stress, inflammation, apoptotic processes and mitochondrial damage, and that different molecular targets implicated in these effects, including $CB_1$ and $CB_2$ receptors, antioxidant responses and may be PPAR receptors. This represents an important advantage for the development of novel neuroprotective therapies with cannabinoids in comparison with current strategies which are based on very selective compounds active exclusively on only one of these cytotoxic insults. Considering these results, we suggest that focusing not only in $CB_1$ receptors, which are lost very early in the disease, but also in other endocannabinoid-related targets active on pathogenic events such as oxidative damage, inflammation and gliosis, would be a good strategy for developing cannabinoid compounds as a novel therapy useful to alleviate symptoms and delay disease progression in HD. For the future, we assume that a further evaluation of CBD and CBG is highly justified, since these non-classic phytocannabinoids, besides being potent antioxidants, can act on different, even complementary targets; some of them would be NRF2, NFκB, PPARγ and $5HT_{1A}$ activation and the inhibition of FAAH in the case of CBD (Bisogno *et al.*, 2001; Esposito *et al.*, 2006; O'Sullivan & Kendall, 2010; Fernández-Ruiz *et al.*, 2013), and TRPV1, TRPA1 and PPARγ activation and $5HT_{1A}$ inhibition in the case of CBG (Borrelli *et al.*, 2014; Granja *et al.*, 2012; Cascio *et al.*, 2010). This completely fits with the idea of developing broad-spectrum therapies against the multiple cytotoxic mechanisms operating in HD.

After the different preclinical evidences provided, the clinical evaluation of Sativex as a neuroprotective therapy in HD patients was highly justified. Sativex has a promising broad-spectrum profile, has been already approved for other indications (as explained in the Introduction) and appears to be safe with no relevant psychoactive side-effects despite the

presence of $\Delta^9$-THC. In Chapter 2, we present the results of a double-blind, randomized, cross-over, placebo controlled, pilot trial with Sativex in a small cohort of HD patients (Chapter II, Lopez-Sendon Moreno *et al.*, 2016). The primary endpoint of this study, which was the safety and tolerability of the compound, was fully achieved (only 2 SAEs were reported, both drug-unrelated), and no psychoactive effects were detected. However, there was no clinical response in the arms treated with Sativex, in parallel with the absence of changes in the biomarkers analyzed, except an increase of $CB_2$ in the lymphocytes of Sativex-treated subjects.

Although the primary endpoint of the study was reached, the treatment with Sativex did not offer the clinical benefits expected according to the data obtained in the preclinical models. We believe that this lack of efficacy is directly related to an excessively short time for the active treatment, which could be prevented in future studies with some changes in the study design. As stated in the paper (López-Sendón *et al*., 2016), a new study with presymptomatic subjects should be considered, as in this stage the loss of $CB_1$ receptors is still not relevant and Sativex could thus be more effective delaying the onset of motor symptoms. Moreover, although cross over is a well design for a pilot study, it renders a high placebo effect and the wash out period might not be long enough to make a clear deference between arms. We would also need to extend the period of treatment to attenuate this placebo effect and to enhance the possibilities for Sativex to be active.An analogous situation was reported before in a clinical trial with creatine (Tabrizi *et al.*, 2003), also failed and in which the treatment duration was one year. Authors suggested that this short treatment period could be the main factor for its lack of efficacy. Therefore, we would suggest performing a parallel study with a longer treatment duration. In addition to these changes, an increase in the dose should be considered, as the highest dose applied did not render any psychoactive effect, and the establishment of a fixed instead of a variable dosing scheme might offer more robust results. It is important also to highlight that the increase in the expression of $CB_2$ receptors, although yet unexplained, might be attributed to a protective response, as found in postmortem samples from HD patients and from experimental models, a fact that also occurs in other neurodegenerative disorders (Ashton & Glass, 2007; Aso & Ferrer, 2016). In addition, the activation of $CB_2$ receptors by selective and non-selective cannabinoids has proven to be neuroprotective in inflammatory and excitotoxic models of the disease (Palazuelos *et al.*, 2009; Sagredo *et al.*, 2009), and Sativex is active at the $CB_2$ receptors Therefore, a prolonged treatment scheme with Sativex could facilitate the appearance of positive effects related to $CB_2$ activation. Lastly, besides a higher dose of Sativex, we propose the use of other cannabinoid combinations that are not only cannabinoid receptor –mediated. The positive results offered by CBG in preclinical studies and the preclinical evidences of a protective effect of cannabinoids able to act at TRPV1 and PPARγ receptors, among other additional targets, in HD models, support the evaluation of new combinations of cannabinoids.

In the last Chapter of this Thesis, we wanted to further study the mechanisms that could underlie the positive effects of cannabinoids as neuroprotectant observed in animal models of HD. We used first $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells, in order to study the effect of mHTT on the cellular response against excitotoxic, mitochondrial and oxidative damage, although we focused on the last one. First, we were surprised to observe that $STHdh^{Q111/Q111}$ cells were less vulnerable to $H_2O_2$. A possible explanation to this result could be a reduced expression of the $Ca^{2+}$-binding protein DREAM by $STHdh^{Q111/Q111}$ cells triggered by the presence of mHTT, which would make them less sensitive to this kind of damage, as shown by other authors during the time we were conducting our experiment (Naranjo *et al.*, 2016). Dysregulation in $Ca^{2+}$ homeostasis is a key event for neurodegeneration in HD, so the reduced expression of this factor in mutant cells could be interpreted as an endogenous protective mechanism against oxidation. In this model, CBD, but particularly in the form of Sativex-like combination of phytocannabinoids, afforded neuroprotection against $H_2O_2$ exposure. This effect was equally significant in both cell lines, but it was much more moderate in $STHdh^{Q111/Q111}$ than in $STHdh^{Q7/Q7}$ cells. This positive effect in

$STHdh^{Q7/Q7}$ cells, which are wild type neuroblasts, means that these results can be applied not only to HD, but also to other neurodegenerative diseases in which mHTT is not involved. Therefore, we could prove Sativex and CBD are highly effective protecting neurons against oxidative insults, a fact that has been frequently claimed (Borges et al., 2013; Rajan et al., 2016; Hosseinzadeh et al., 2016). This neuroprotection correlated with a clearly enhanced expression of the phase II (inducible) antioxidant enzyme HO-1, so we tried to prove if the activation of the NRF2/ARE pathway, which is currently widely investigated in neurodegeneration (Calkins *et al.*, 2009; Johnson & Johnson, 2015) was the main responsible of this neuroprotective effect. Unfortunately, we could not completely prove this involvement, but it is important to highlight that these data are preliminary and that further experiments will be needed to confirm if Sativex activates NRF2-mediated gene transcription and/or if other pathways are involved, such as the NFkB as has been reported with CBD (Esposito *et al.*, 2006) or others in which the activity of HO-1 is also increased, such as JNK (Lin *et al.*, 2013), PI3K/AKT (Li *et al.*, 2014) and p38 (Ning *et al.*, 2002).

Lastly, although the pharmacological manipulation of the endocannabinoid system has provided promising results as a novel neuroprotective therapy in preclinical studies of HD and has demonstrated to be safe in HD patients, there exist some circumstances that may alter such beneficial effects. In the last publication of this Thesis, presented in Chapter 3, we have addressed this “dark face” of the endocannabinoid system that sometimes has been found to have a dual activity at different processes (Fowler *et al.*, 2010). Examples of this duality can be found in the anticonvulsant effects of different cannabinoids, in particular those active at the $CB_1$ receptor, which may behave as anticonvulsant or proconvulsant depending on the neuronal site in which the receptor is located, e.g. anticonvulsant when acting at $CB_1$ receptors located in glutamatergic neurons, proconvulsant if these receptor are located in GABAergic neurons. A similar situation happens with the protective/proapoptotic effects of cannabinoids which depend on the cellular context, so they can protect/preserve healthy neurons, as it has been investigated in this Doctoral Thesis, but they can also activate the apoptosis of tumoral cells. Moving to the work conducted in this thesis, we have obtained experimental evidence showing that the alteration of the endocannabinoid tone may have positive or deleterious effects depending on the context. . It is important to remark that, speaking on the pharmacological manipulation of this system, acting on $CB_1$ receptors in this pathology may not exert a very potent effect, since these receptors are downregulated from initial stages of the disease and the striatal neurons in which these receptors are more highly expressed are the ones more vulnerable to neurodegeneration. Therefore, maybe the treatment with $CB_1$ agonists would only provide a significant effect during pre-symptomatic, early stages of the disease. However, a recent study suggests that a small pool of $CB_1$ receptors, located in corticostriatal projections of glutamatergic neurons and not in MSNs as expected, are the target of the neuroprotective effect of cannabinoids (Chiarlone *et al.*, 2014), which represents a good news. On the contrary, targeting the elevation of $CB_2$ has shown very positive effects in different models, both of inflammation, glial activation and excitotoxicity, as explained previously (Sagredo *et al*., 2009, Palazuelos *et al*., 2009). Moreover, the enhancement of 2-AG and AEA levels has proven anti-inflammatory effects in various models of neurodegeneration (see Turcotte *et al.*, 2015 for review). These data, together with the changes in FAAH levels observed in HD *postmortem* brain tissue (Battista *et al*., 2007) and in animal models (Blázquez *et al.*, 2011) which ran in opposite directions, prompted us to evaluate consequences of the variation in 2-AG levels elicited by the inhibition of DAGL and MAGL enzymes in the malonate model of HD, an aspect that has not been significantly investigated in this disease. Despite what we expected, inhibition of 2-AG biosynthesis with a DAGL inhibitor prevented the damage by malonate, and on the contrary, the inhibition of its degradation with a MAGL inhibitor enhanced striatal damage in this model. These effects were completely independent on the activation of $CB_1$/$CB_2$ receptors (the protective targets of the endocannabinoid system), and they seemed to be related to a proinflammatory action of 2-AG that involves its transformation into oxygenated

metabolites by the enzyme COX-2 (which is also elevated in this model), named prostaglandin glyceryl esters (PG-Gs), which have been related to aggravation of inflammatory and excitotoxic processes in other experimental models (Sang *et al.*, 2007; Hu *et al.*, 2008). In this context, recent reports suggest the use of "substrate-selective" COX-2 inhibitors (SSCIs), which inhibit endocannabinoid inactivation without affecting prostaglandin generation from arachidonic acid, to avoid the neurotoxic effects exerted by the increase of endocannabinoid levels and their conversion to oxygenated metabolites observed in certain circumstances (Hermanson *et al.*, 2014). Therefore, certain caution must be taken when considering the modulation of the endocannabinoid system in neurodegenerative disorders, since elements of this system may experience dysregulation and trying to target many of them at the same time could facilitate the occurrence of unexpected harmful effects.

As an overall conclusion, the results shown in this Thesis allow us to confirm that the treatment with phytocannabinoids is neuroprotective in different preclinical models of HD. This has led to the evaluation of Sativex in a small group of HD patients that proved to be safe and well-tolerated, but did not offer any clinical improvement. Hence, more research must be done to unveil the multiple mechanisms involved in the neuroprotective effects of cannabinoid compounds in HD. We also propose the evaluation of new phytocannabinoid combinations in preclinical models and in the clinical field, in order to develop multitarget therapies that can limit the multiple neuropathological processes involved in the disease. Moreover, we also suggest a cautious approach when manipulating the endocannabinoid system in this disease, since under certain circumstances the enhancement of the endocannabinoid tone might have deleterious effects, and this might be one of the causes for the lack of positive effects with cannabinoid formulations in preclinical and clinical testing.

# CONCLUSIONS

The results obtained in this Doctoral Thesis allow us to conclude that:

1. CBG protects striatal neurons and improves neurological decline in the 3NP model of acute striatal oxidative damage, by acting specifically on improving antioxidant defenses and reducing glial reactivity, and these effects are exerted by cannabinoid receptor-independent mechanisms, presumably by acting at the PPARs.

2. CBG also exerts neuroprotection in the transgenic R6/2 model, in this case, by reducing the accumulation of huntingtin aggregates and acting positively on specific neurotrophins and specific genes related to HD transcriptional dysregulation. Again the effects are cannabinoid receptor-independent.

3. The Sativex-like combination of phytocannabinoid botanical extracts protects striatal cells in the malonate model of acute inflammatory and apoptotic damage, acting specifically on limiting glial reactivity and improving neurotrophic support. This effect involves the activation of both $CB_1$ and $CB_2$ receptors.

4. The Sativex-like combination of phytocannabinoid botanical extracts also improves some motor deficits and corrects the metabolic deficiencies and prognostic markers related to energy deficit and mitochondrial failure in the R6/2 transgenic model of HD.

5. Sativex is safe and well-tolerated in HD patients as shown in a pilot Phase II clinical trial conducted in a cohort of early symptomatic HD patients.

6. However, Sativex did not exert any clinical benefit in motor and cognitive scales and also in different biomarkers, other than an elevation in the levels of $CB_2$ receptors that could be an endogenous protective response. The short time used for the active treatment in this clinical trial suggests the need to consider longer periods of treatment in future clinical studies.

7. Using in vitro strategies ($STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells), we could demonstrate that the protective effects of CBD against oxidative damage in the form of botanical extract, alone or combined with $\Delta^9$-THC as in Sativex, may involve a certain activation of the NRF2 signaling, although the contribution of additional cellular mechanisms appears to be necessary.

8. Contrarily to the expected result, $STHdh^{Q111/Q111}$ cells are not more vulnerable to the oxidative insult, and the contribution of NRF2 signaling in these cells is rather limited.

9. The inhibition of 2-AG biosynthesis in malonate-lesioned rats, contrarily to the expected result, preserves striatal neurons from death, whereas the inhibition of 2-AG degradation aggravates striatal damage.

10. Using this in vivo model and also an in vitro strategy (M-213 cells), we could demonstrate that such unexpected result was facilitated by the up-regulation of COX-2 enzyme, the increase in 2-AG availability, and its transformation by this enzyme into prostaglandin-glyceryl-esters, which are highly neurotoxic.

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