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FACULTAD DE CIENCIAS BIOLÓGICAS

DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR I

TESIS DOCTORAL

PAPEL NEUROPROTECTOR DEL RECEPTOR CB_2 CANNABINOIDE EN LA ENFERMEDAD DE HUNTINGTON

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

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Madrid, Junio de 2014

RESUMEN

Los derivados de la planta *Cannabis Sativa L*. (cannabis o cáñamo) se han utilizados desde hace siglos, tanto con fines lúdico-espirituales como industriales y medicinales. Las primeras evidencias del empleo del cannabis con fines medicinales se remontan a la cultura China hacia el año 5000 a.c., un uso que fue extendido a la región de Oriente Medio, Norte de África, Europa, el Caribe y los Estados Unidos alrededor del año 500 a.c.. Así, el empleo del cannabis a lo largo de los siglos ha destacado por sus propiedades analgésicas de uso tópico, antisépticas y sedantes, y posteriormente en la medicina occidental europea del siglo XIX por sus efectos antiespasmódicos y estimuladores del apetito. A pesar de este largo recorrido histórico, no fue hasta la segunda mitad del siglo XX que se describió la estructura química de sus componentes. En 1964 se describió la estructura química del principal componente psicoactivo de la planta, el Δ^9-tetrahydrocannabinol (THC). Posteriormente se describieron otros compuestos activos presentes en proporciones menores en los extractos de la inflorescencia y hojas de la planta, en total alrededor de 100, que se agruparon bajo la denominación genérica de cannabinoides.

En un principio se pensó que, debido a su naturaleza altamente lipofílica, los compuestos cannabinoides actuaban directamente alterando las propiedades de las membranas celulares, pero estudios farmacológicos con THC indicaron la posible existencia de un receptor de cannabinoides a través del cual éste ejercería sus efectos. El primer receptor cannabinoide, CB_1, se clonó en 1990 y poco después se identificaron un segundo receptor, CB_2, y los dos principales ligandos endógenos de estos, la *N*–araquidonoiletalonamina, también llamada anandamida (AEA), y el 2-araquidonoilglicerol (2-AG). En los años sucesivos a estos primeros hallazgos, se describió un sistema de enzimas de transporte, síntesis y degradación de endocannabinoides, que junto con los ligandos cannabinoides endógenos (endocannabinoides) y sus receptores, constituye el sistema endocannabinoide.

El receptor cannabinoide CB_1 es el receptor acoplado a proteínas G más abundante en el cerebro y se expresa en muy altos niveles en áreas del sistema nervioso central implicadas en el control de la actividad motora (ganglios basales, cerebelo), memoria y aprendizaje (corteza, hipocampo), emociones (amigdala) y diversas funciones autónomas y endocrinas (hipotálamo, médula). El segundo receptor cannabinoide, CB_2, se encuentra expresado

principalmente en la periferia, en células del sistema inmune y hematopoyético, en el páncreas endócrino, hueso y tejido adipso, etc. Además, estudios recientes han demostrado la presencia de CB_2 en el sistema nervioso central, principalmente en microglía y probablemente en astrocitos y enalguna población neuronal. Además de los dos receptores clásicos de cannabinoides, se ha postulado la existencia de otros receptores activados por cannabinoides tanto endógenos como exógenos. Entre ellos se encuentran el receptor huérfano acoplado a proteínas G GPR55, el receptor de potencial transitorio V1 (TRPV1) y la familia de receptores activados por proliferadores peroxisomales (PPAR).

El sistema endocannabinoide está involucrado en una gran variedad de procesos fisiológicos, entre ellos la modulación de la transmisión neuronal y la neuroprotección. En este contexto, se observó que los ligandos endógenos de los receptores cannabinoides son generados en respuesta a la actividad neuronal, y el sistema de señalización que desencadena su producción es utilizado en el sistema nervioso central para disminuir el exceso de actividad presináptica, modulando la funcionalidad y plasticidad de distintos tipos de sinapsis, en particular las sinapsis glutamatérgicas y GABAérgicas. Estudios en diferentes modelos animales confirman la hipótesis de que el receptor CB_1 juega un papel importante en promover la supervivencia neuronal en situaciones patofisiológicas y que esta acción neuroprotectora depende en parte de la inhibición de la neurotransmisión glutamatérgica excitadora. Además, el papel neuroprotector del sistema endocannabinoide se ve reforzado por el aumento en la producción de endocannabinoides en respuesta a distintos tipos de daño neuronal.

Asímismo, se han observado variaciones en los niveles de diversos componentes del sistema endocannabinoide ante diversas situaciones neuropatológicas. Por ejemplo, se ha descrito que la expresión del receptor CB_1 disminuye en los ganglios basales de pacientes y modelos animales de la enfermedad de Huntington (EH), una patología neurodegenerativa autosómica dominante, causada por la expansión anormal del trinucleótido CAG en el exón 1 del gen IT15, que codifica la proteína huntingtina. La proteína mutante porta una expansión de poliglutaminas en su extremo N-terminal. La EH se caracteriza por movimientos coreicos, alteración motora progresiva, demencia y alteraciones cognitivas.

Actualmente no se dispone de cura para la EH, existiendo tan solo tratamientos paliativos de la sintomatología, produciéndose la muerte de los pacientes en 10-20 años tras

la aparición de los primeros síntomas. El mecanismo patogénico que subyace la enfermedad es aún desconocido, y se atribuye globalmente a mecanismos de ganancia de función por la expresión de la proteína mutada: una de las principales consecuencias de la huntingtina mutada es una importante alteración de los sistemas de regulación de expresión génica, aunque también se sostiene la hipótesis de una pérdida de función de la proteína silvestre. A pesar de que la EH sea una enfermedad monogénica, los mecanismos que inducen la progresiva degeneración de las neuronas estriatales parecen extremadamente complejos y no se han elucidado completamente. El descenso temprano en los niveles del receptor CB_1 ocurre en estadios asintomáticos y anteriores a los cambios neuroquímicos, y podría reflejar la disminución en la actividad inhibitoria GABAérgica característica de la enfermedad.

Considerando estas evidencias, el **objetivo** global de esta Tesis Doctoral ha sido estudiar más profundamente el papel neuroprotector del receptor CB_1, su relevancia fisiológica y su potencial terapéutico en neurodegeneración, con particular énfasis en el estriado y en la EH.

Este objetivo general se puede dividir en 3 objetivos específicos:

1- Elucidar el papel del receptor CB_1 en los procesos neurodegenerativos que ocurren en la Enfermedad de Huntington.
2- Estudiar los mecanismos moleculares que subyacen la inhibición de la expression del receptor CB_1 mediada por la huntingtina mutada.
3- Evaluar la relevancia fisiológica y el potencial terapéutico de receptores CB_1 expresados en neuronas GABAérgicas (médium-sized spiny neurons) o glutamatérgicas (corticoestriatales) en modelos de neurodegeneración.

Los resultados obtenidos en esta Tesis Doctoral se han dividido en 2 capítulos.

En el **primer capítulo** se evaluó la relevancia fisiopatológica del receptor CB_1 en el contexto neurodegenerativo de la enfermedad de Huntington. En primer lugar estudiamos las consecuencias de la deleción genética del receptor en la sintomatología, la

neuropatología y la patología molecular de ratones R6/2, que constituyen un modelo animal bien establecido de la enfermedad. A continuación, en este mismo modelo murino, se analizaron los efectos de la activación farmacológica del receptor CB_1. También, se estudió el papel neuroprotector de dicho receptor en neuroblastos estriatales murinos condicionalmente inmortalizados (células $STHdh^{Q7/Q7}$, $STHdh^{Q7/Q111}$ y $STHdh^{Q111/Q111}$). Por último, se caracterizó el mecanismo molecular por el cual la huntingtina mutada inhibe la expresión del gen del receptor CB_1.

Los resultados obtenidos en este primer capítulo confirmaron que la pérdida temprana del receptor CB_1 está involucrada de manera crítica en la etiopatología de la Enfermedad de Huntington. Sin embargo, todavía no se conocen qué subpoblaciones precisas de receptores cannabinoides están implicadas en los efectos neuroprotectores. Por eso, en el **segundo capítulo** de esta Tesis Doctoral se estudió la relevancia fisiológica y el potencial terapéutico en modelos de neurodegeneración de las poblaciones de receptores CB_1 localizadas en neuronas GABAérgicas o glutamatérgicas. Con este fin, se usó en primer lugar un modelo de excitotoxicidad, la inyección intraestriatal de acido quinolínico, en ratones mutantes condicionales que carecen de receptores CB_1 en neuronas GABAérgicas o glutamatérgicas. A continuación, se empleaó un modelo farmacogenético (*designer receptor exclusively activated by designer drug*) para sobreactivar la trasmisión excitadora en neuronas corticoestriatales. Finalmente, se evaluaó si las consecuencias deletéreas de la deleción genética del receptor CB_1 en ratones R6/2 se deben a una de-inhibición de la transmisión GABAergica o glutamatergica. Este objetivo se abordó con dos aproximaciones experimentales: por un lado, en ratones R6/2 que carecen de receptor CB_1, se modulará farmacológicamente la activación de los receptores $GABA_A$ o NMDA; por otro lado, en ese mismo modelo animal, se delecionará específicamente el gen del receptor CB_1 en neuronas GABAérgicas o glutamatérgicas.

Conclusiones

En esta Tesis Doctoral se han estudiado la relevancia fisiológica y el potencial terapéutico del receptor CB_1 en procesos neurodegenerativos que se observan en el estriado y en la Enfermedad de Huntingon.

Los resultados obtenidos nos permiten concluir que:

1-La disminución en la expresión del receptor CB_1 que se observa en la Enfermedad de Huntington es consecuencia de una represión aberrante de la transcripción del receptor. El repressor element-1 silencing transcription factor (REST), que, en condiciones normales, se encuentra inactivo en el citoplasma, en presencia de la Huntingtina mutada trasloca al núcleo, donde inhibe la transcripción de sus genes diana, entre ellos CB_1.

2- La perdida de CB_1 está involucrada en la patofisiología de la Enfermedad de Huntington, ya que la ablación genética del receptor exacerba los síntomas, la neuropatología y la patología molecular de la enfermedad, mientras que su activación farmacológica es beneficiosa.

3-La actividad neuroprotectora del sistema endocannabinoide depende de una población restringida de receptores CB_1, que residen en las terminales glutamatérgicas corticoestriatales.

SUMMARY

Preparations from the hemp plant *Cannabis sativa* L. have been used for many centuries both for medicinal and recreational purposes. Even though the effects of the plant derivatives and their medicinal potential have been exploited during centuries, the chemical structure of their unique, active components – the cannabinoids – was not elucidated until the second half of the XXth century. It was in 1964 that the chemical structure of the main psychoactive constituent of the plant, Δ^9-tetrahydrocannabinol (THC), was described. This work represented a milestone in cannabinoid research: since then, up to 100 derivatives of the plant, referred to as phytocannabinoids, have been isolated. Moreover, it stimulated the generation of a whole range of synthetic analogs, and prompted a large number of studies, which contributed to elucidate the mechanism of action of these compounds.

Due to the lipophilic nature of THC, it was initially believed that this compound exerted its effects via a non-selective interaction with the plasma membrane. However, data from several groups suggested a specific interaction of cannabinoids with as yet undiscovered membrane receptors. Thus, the first cannabinoid receptor, named CB_1, was eventually cloned from rat cerebral cortex, and subsequently from human brain and testis, and from mouse brain. Few years later, a second cannabinoid receptor, named CB_2, was identified in immune cells and tissues.

Cannabinoid receptors are members of the G protein-coupled receptor (GPCR) superfamily, with 7 transmembrane domains, and are generally coupled to $G_{i/o}$ proteins. Currently, we know that CB_1 receptor is the most abundant GPCR in the mammalian brain. It is particularly abundant within certain regions of the brain that underlie with the observed effects of cannabinoids, including alteration of cognition and memory, food intake, anxiety, nociception, learning and motor coordination. Hence, CB_1 receptors are highly expressed, for example, in the hippocampus, basal ganglia, cerebral cortex, amygdala and cerebellum and can be found, to a lesser extent, in many peripheral tissues such as spleen, eye, testis, uterus, adipose tissue and skin. The second cannabinoid receptor, CB_2 is mainly expressed in the periphery, in cells of the immune system and hematopoietic systems, but also in the endocrine pancreas, bone and adipose tissue, among others. Additionally, several studies have shown the presence of CB_2 receptors in the brain, mainly in microglia and perhaps also in astrocytes, although more recently they have been found at low levels in some restricted neuronal populations.

Although CB_1 and CB_2 are well known and characterized, several pharmacological studies suggest the existence of additional cannabinoid receptors such as the orphan GPCR GPR55, the transient receptor potential cation channel subfamily V member I ($TRPV_1$) ion cannel, and some members of the peroxisome proliferator-activated (PPAR) nuclear receptor family.

The discovery of specific receptors in mammalian cells that recognize *Cannabis sativa*-derived compounds stimulated the hunt for the missing piece of the puzzle: the endogenous ligands of the cannabinoid receptors. The first endocannabinoid identified, the *N*-arachidonyletanolamine or anandamide (AEA), was purified from porcine brain. It is expressed in the brain areas in which CB_1 expression is also elevated, and to a lower extent in peripheral tissues (heart, spleen, testis and uterus), and is able to bind and stimulate cannabinoid receptors. As the search for endogenous receptor-binding compounds continued, the second endocannabinoid, 2-arachidonylglycerol (2-AG), was isolated. This monoacylglycerol is highly expressed in the brain, at about 200-fold higher levels than AEA and is also found in peripheral tissues, such as pancreas, spleen, liver, lungs and kidney.

Altogether, this fascinating experimental effort led the scientific community to describe a new signaling system, the so-called endocannabinoid system, composed of specific receptors, their endogenous ligands and the protein responsible for their production, release, uptake and degradation. Thus, along with the description of endogenous cannabinoids, specific pathways for their synthesis, transport and degradation have also been defined. A distinctive feature of endocannabinoids is their synthesis "on demand": they are produced and released only upon a particular physiological or pathological stimulus, rather than being synthesized and stored in vesicles, like many other water-soluble neuromodulators.

Endocannabinoids act in a broadly paracrine fashion, modulating the action of numerous processes in different neighboring cells. In the central nervous system, they act mostly as neuromodulators. Once synthesized, they are released to the synapse, and activate cannabinoid receptors, as well as some ionic channels. Even though, as lipophilic compounds, they can diffuse passively through the cell membrane, this process is accelerated by a rapid and selective transport system. Once inside the cell, endocannabinoids are deactivated and degraded by specific enzymes. FAAH catabolizes

AEA to arachidonic acid and ethanolamine, while monoacylglycerol lipase (MAGL) degrades 2-AG into arachidonic acid and glicerol, although other pathways of degradation have been described.

Cannabinoid receptors are primarily coupled to inhibitory $G_{i/o}$ proteins. Thus, activation of CB_1 and CB_2 receptors promotes the dissociation of αi and βγ subunits. The αi subunit inhibits adenylyl cyclase, decreasing the production of cAMP and thus the activity of the cAMP-dependent protein kinase (PKA), which is involved in many biological processes and gene expression control. On the other hand, βγ dimers can participate in the activation of the extracellular signal-regulated kinase (ERK), the mitogen-activated protein kinase (MAPK) p38 and the c-Jun N-terminal protein kinase (JNK). Cannabinoids also regulate other pathways involved in the control of cell proliferation and survival, including the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, ceramide production, sphingomyelin hydrolysis through the adaptor protein FAN and the regulation of various ion channels. For example, the CB_1 receptor induces the inhibition of N- and P/Q-type voltage sensitive Ca^{2+} channels and activation of G-protein-activated inwardly rectifying K^+ (GIRK) channels, which hyperpolarize and thus reduces the excitability of the plasma membrane.

The endocannabinoid system participates in the regulation of many different physiological processes. Hence, the pharmacological manipulation of the endocannabinoid system constitutes a powerful tool to get a better insight into these physiological processes and may represent a potential therapeutic target. Within the central nervous system, the major function of the endocannabinoid system accepted so far is the modulation of synaptic transmission by retrograde signaling. Unlike traditional synaptic transmission, in which neurotransmitter release from the presynaptic terminal leads to postsynaptic receptor activation, retrograde signaling involves postsynaptic release of a compound that then acts on presynaptic receptors.

CB_1 activation leads essentially to presynaptic inhibition of neurotransmitter reléase: depolarization of the postsynaptic neuron induces the release of endocannabinoids, which travel retrogradely and suppress presynaptic activity.

Besides its well-established neuromodulatory functions, an extensive amount of data has highlighted that neuronal damage activates endocannabinoid signaling as an intrinsic neuroprotective response; this, in turn, leads to the stimulation of molecular events downstream of the cannabinoid receptors that promote neuronal survival and function. The

neuroprotective action of endocannabinoid signaling relies mainly on the inhibition of excitotoxic glutamatergic neurotransmission. Functional CB_1 receptors located on glutamatergic terminals become activated by endocannabinoids upon excitatory synaptic transmission and prevent massive glutamate release. In addition, CB1 receptors can trigger cell-autonomous intracellular signal transduction events that promote cell survival.

This neuromodulatory and neuroprotective functions of the endocannabinoid system led to the hypothesis of its implication in the pathophysiology and/or treatment of many of the disorders associated with these circuits, including Huntington's disease, where a down-regulation of the receptor levels has been described as one of the most characteristic neurochemical alteration observed.

Huntington's disease is an autosomal dominant inherited neurodegenerative disorder, disease is caused by an abnormal CAG repeat expansion in the Huntingtin (Htt) gene; therefore, the protein encoded carries a polyglutamine (polyQ) region near its N-terminus. HD is characterized by several symptoms that comprehend progressive emotional and psychiatric disturbances, generalized motor dysfunctions and gradual cognitive decline. The neuropathological hallmark of HD is mainly brain specific, with extreme but selective neurodegeneration in the striatum (caudate nucleus and putamen). Nowadays, there is no effective treatment to cure Huntington's disease, and the pathogenic mechanisms underlying this disorders are still unclear. The early presymptomatic reduction of CB1 receptor might reflect a decrease in the inhibitory GABAergic activity characteristic of the disease.

In this context, the global **aim** of this **Doctoral Thesis** is to investigate in further detail the neuroprotective activity of the CB_1 receptor and unravel its physiological relevance and therapeutic potential in neurodegeneration, with especial emphasis on the striatum and Huntington's disease.

This main aim can be divided into 3 specific aims:

1. To elucidate the molecular mechanism by which mutant huntingtin downregulates CB_1 receptor gene expression.

2. To clarify the possible neuroprotective role of the CB_1 receptor in the neurodegenerative processes that occur in Huntington's disease.

3.To assess the precise relevance of two differently-located CB_1 receptor pools, namely those situated on GABAergic neurons (medium-sized spiny neurons) or glutamatergic neurons (corticostriatal projection neurons), in Huntington's disease-associated excitotoxicity.

The results obtained have been divided into two chapters:

In this **first chapter** we studied the pathophysiological relevance of the CB_1 receptor in the context of a neurodegenerative disease (Huntington's disease). Firstly, we focused on the consequences of CB_1 receptor genetic ablation on the Huntington's disease-like symptomatology, neuropathology and molecular pathology in a well-established model of the disease, namely the R6/2 mouse. Subsequently, we analyzed the effect of the pharmacological activation of CB_1 receptors in these mice, as well as the neuroprotective activity of the receptor in conditionally-immortalized murine striatal neuroblasts ($STHdh^{Q7/Q7}$, $STHdh^{Q7/Q111}$ and $STHdh^{Q111/Q111}$ cells). Lastly, we tried to elucidate the molecular mechanism by which mutant huntingtin downregulates CB_1 receptor gene expression.

To further dissect the neuroprotective activity of the CB_1 receptor, in the **second chapter** we analyzed the physiological relevance and therapeutic potential in neurodegeneration of the CB_1 receptor pools located on GABAergic or glutamatergic neurons. Firstly, we induced excitotoxic damage in the mouse brain by administering quinolinic acid to conditional mutant animals lacking CB_1 receptors in either GABAergic or glutamatergic neurons. Subsequently, we enhanced corticostriatal glutamatergic transmission with a *designer receptor exclusively activated by designer drug* pharmacogenetic approach. We next examined whether the detrimental consequences of knocking-out CB_1 receptors were due to the de-inhibition of glutamatergic and/or GABAergic transmission, (i) by the pharmacological modulation of $GABA_A$ receptors or NMDA receptors in R6/2 mice that do not express CB_1 receptors, and (ii) by the selective deletion in those mice of the CB_1 receptor gene in either corticostriatal glutamatergic or striatal GABAergic neurons.

Hence, the results obtained in this Thesis allow us to conclude:

1-The down-regulation of CB_1 receptors by mutant huntingtin is dependent on a loss of WT huntingtin function, involves the control of the CB_1 receptor gene promoter by repressor element-1 silencing transcription factor.

2- CB_1 receptor is strongly involucrated in the pathophysiology of Huntington's disease. The loss of the receptor aggravates symptoms, neuropathology and molecular pathology of the disease, while the pharmacological stimulation of the receptor evokes beneficial effects.

3-The neuroprotective effects of the cannabinoid stimulation rely on the activation of a specific pool of CB_1 receptors, namely those located on the glutamatergic neurons that project from the cerebral cortex to the striatum.

ABBREVIATIONS

2-AG: 2-Arachidonoylglycerol

ABHD6: α/β-hydrolase-6

AC: Adenylyl ciclase

AD: Alzheimer's disease

AEA: Anandamide or N-arachidonoylethanolamine

Akt: also called RAC-alpha serine/threonine-protein kinase; protein kinase B

BDNF: Brain-derived neurotrophic factor

bFGF: Basic fibroblast growth factor

cAMP: 3'-5'-cyclic adenosine monophosphate

CB_1: Cannabinoid receptor, type 1

CB_2: Cannabinoid receptor, type 2

CBD: Cannabidiol

CCK: Cholecystokinin

CNS: Central nervous system

COX-2: Cyclooxigenase 2

D2: Dopamine receptor, type 2

DAG: Diacylgycelol

DAGL: Diacylgycelol lipase

DG: dentate gyrus

DSE: Depolarization-induced suppression of excitation

DSI: Depolarization-induced suppression of inhibition

eCB: Endocannabinoids

EGF: Epidermal growth factor

ERK: Extracellular signal-regulated kinase

FAAH: Fatty acid amide hydrolase

FADD: Fas-Associated protein with Death Domain

FAN: Factor associated with neutral sphingomyelinase activation

FLAT: FAAH-like anandamide transporter

GABA: γ-Aminobutyric acid

GIRK: G-protein-activated inwardly rectifying K^+ channels

GLT-1: Glial Glutamate Transporter 1

GPCR: G-protein coupled receptor

GSK-3: Glycogen synthase 3

HD: Huntington's disease

HIP1: Huntingtin interacting protein 1

HIV: Human immunodeficiency virus

HTT: Huntingtin

IL-2: Interleukin 2

IP_3: Inositol 1,4,5-trisfosfato

JNK: c-Jun N-terminal protein kinase

KA: Kainic acid

KO: Knock-out

LTD: Long-term synaptic depression

MAGL: Monoacylglycerol lipase

MAPK: Mitogen-activated protein kinase

mHtt: Mutant Huntingtin

MSNs: Medium-sized spiny neurons

mTORC1: Mammalian Target of Rapamycin complex 1

NAE: N-acetylethanolamine

NAPE: N-acetylphosphatidylethanolamine

NMDA: N-methyl-D-aspartate

NMDAR: N-methyl-D-aspartate receptor

nNOS: Neural nitric oxide synthases

NO: nitric oxide

NP: Neural progenitors

NPY: Neuropeptide Y

PA: Phosphatidic acid

PEA15: Phosphoprotein enriched in astrocytes 15

PI: Phosphoinositides

PI3K: Phosphatidylinositol 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

PLD: Phospholipase D

polyQ: Polyglutamine

PPAR: *Peroxisome proliferator-activated receptors*

PSD95: post-synaptic density 95

QA: Quinolinic acid

REST: RE1-Silencing Transcription factor

SGZ: Subgranular zone

SSI: Slow self inhibition

STD: Short-term synaptic depression

SVZ: Subventricular zone

THC: Δ^9-tetrahydrocannabinol

TNFα: Tumor necrosis factor

TrkB: Neurotrophic tyrosine kinase, receptor, type 2

TRPV1: Transient receptor potential cation channel subfamily V member I

VGCCs: Voltage-gated Ca2+ channels

vGluT: Vesicular glutamate transporter

WT: wild type

INTRODUCTION

THE ENDOCANNABINOID SYSTEM

CANNABINOIDS AND THEIR RECEPTORS

Preparations from the hemp plant *Cannabis sativa* L. have been used for many centuries both for medicinal and recreational purposes. Even though the effects of the plant derivatives and their medicinal potential have been exploited during centuries, the chemical structure of their unique, active components – the cannabinoids – was not elucidated until the second half of the XXth century (*Figure 1*). It was in 1964 that Gaoni and Mechoulam (Gaoni Y., 1964) described the chemical structure of the main psychoactive constituent of the plant, Δ^9-tetrahydrocannabinol (THC). This work represented a milestone in cannabinoid research: since then, up to 100 derivatives of the plant, referred to as phytocannabinoids, have been isolated. Moreover, it stimulated the generation of a whole range of synthetic analogs, and prompted a large number of studies, which contributed to elucidate the mechanism of action of these compounds [(Pertwee et al., 2010), *Box 1*]

Figure 1: *Cannabis sativa* is a plant of the Cannabaceae family. Preparations from this plant have been used for centuries as a source of industrial fiber, seed oil, food, recreation, religious and spiritual enlightment, as well as for medicinal applications.

Due to the lipophilic nature of THC, it was initially believed that this compound exerted its effects via a non-selective interaction with the plasma membrane, stimulating or inhibiting membrane associated proteins, and altering membrane permeability and fluidity (Hillard et al., 1985). However, data from several groups suggested a specific interaction of cannabinoids with as yet undiscovered membrane receptors: the incubation of neuroblastoma cells with THC induced a functional inhibition of adenylyl cyclase (AC), with the consequent reduction in 3'-5'-cyclic adenosine monophosphate (cAMP) levels (Howlett and Fleming, 1984). This inhibition was counteracted by pertussis toxin incubation, thus suggesting the involvement of a $G_{i/o}$ protein (Howlett et al., 1986). This finding, as well as studies demonstrating the stereoselectivity of the (-)-enantiomers of THC (Dewey, 1986), and the specific binding of radiolabeled agonists in rat brain membranes (Devane et al., 1988), indicated that the majority of the central effects of cannabinoids was mediated by specific membrane receptors. Thus, the first cannabinoid receptor, termed CB_1, was eventually cloned from rat cerebral cortex (Matsuda et al., 1990), and subsequently from human brain and testis (Gerard et al., 1991), and from mouse brain (Chakrabarti et al., 1995). Few years later, a second cannabinoid receptor, named CB_2, was identified in immune cells and tissues (Munro et al., 1993).

BOX 1: Phytocannabinoids and synthetic cannabinoids

Cannabinoids are usually classified into three main families depending on their origin: endocannabinoids (see Introduction for an extensive description), phytocannabinoids and synthetic cannabinoids (sometimes referred to as "synthocannabinoids").

Phytocannabinoids are the plant-derived natural products. Δ^9-Tetrahydrocannabinol (THC) is the main psychoactive constituent of the plant and acts as a partial agonist at CB_1 and CB_2 receptors. Cannabidiol (CBD) may represent up to 40% in some extracts of the plant resin, is not psychoactive and has antioxidant properties. It shows low affinity for classical cannabinoids receptors and could act as an antagonist on GPR55. Other relevant *C. sativa*–derived cannabinoids include cannabinol, cannabigerol, Δ9-tetrahydrocannabivarin and Δ^8-tetrahydrocannabinol. Synthetic cannabinoids encompass a large variety of chemical entities and can be classified according to their structure. Classical synthetic cannabinoids share structural similarity with phytocannabinoids; relevant examples of them are the highly potent CB_1/CB_2-mixed agonist HU-210 and two CB_2-selective agonists, JWH-133 and HU-308. Among the non-classical cannabinoids we find CP-55,940, whose tritiated form was important to identify the CB_1 receptor. Aminoalkylindols include the CB_1/CB_2-mixed agonist WIN-55,212-2 and the CB_2-selective antagonist AM630. Additionally, the diacylpirazols comprise the CB_1-selective antagonist SR141716, also known as rimonabant, as well as AM251 and AM281, two structural analogues of that compound with similar CB_1-antagonistic characteristics, and the CB_2-selective antagonist SR144528.

Cannabinoid receptors are members of the G protein-coupled receptor (GPCR) superfamily, with 7 transmembrane domains, and are generally coupled to $G_{i/o}$ proteins. They share 44% sequence homology (Munro et al., 1993) and are differentially distributed in the body. Their low homology, compared to other families of the GPCR superfamily, and their distinct location may indicate that they diverged early in the phylogenetic tree, even though they shared a common ancestor gene. These receptors are widely expressed in a large number of organisms, showing a 97-99% of aminoacidic sequence identity among vertebrates (Elphick, 2012).

Currently, we know that CB_1 receptor is the most abundant GPCR in the mammalian brain (Herkenham et al., 1990; Kano et al., 2009). It is particularly abundant within certain regions of the brain that underlie with the observed effects of cannabinoids, including alteration of cognition and memory, food intake, anxiety, nociception, learning and motor coordination (Katona and Freund, 2008; Piomelli, 2003). Hence, CB_1 receptors are highly expressed, for example, in the hippocampus, basal ganglia, cerebral cortex, amygdala and cerebellum (Glass et al., 1997; Herkenham et al., 1990; Tsou et al., 1998) (*Figure 2*), and can be found, to a lesser extent, in many peripheral tissues such as spleen, eye, testis, uterus, adipose tissue and skin (Mackie, 2005). Additionally, two splice variants of the CB_1 receptor have been identified, so-called CB_1a (Shire et al., 1995) and CB_1b (Ryberg et al., 2005). These alternative receptors are expressed in a variety of tissues, albeit at lower levels, and have a unique pharmacological profile (Ryberg et al., 2005).

The second cannabinoid receptor, CB_2 is mainly expressed in the periphery, in cells of the immune system and hematopoietic systems (Cabral and Staab, 2005), but also in the endocrine pancreas (Juan-Pico et al., 2006), bone (Idris et al., 2005) and adipose tissue (Pagano et al., 2008), among others. Additionally, several studies have shown the presence of CB_2 receptors in the brain, mainly in microglia and perhaps also in astrocytes (Maresz et

al., 2005; Stella, 2004), although more recently they have been found at low levels in some restricted neuronal populations (*Figure 2*) (Ashton et al., 2006; Lanciego et al., 2011; Onaivi et al., 2008; Van Sickle et al., 2005).

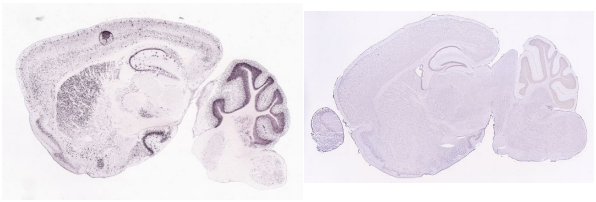

Figure 2 . CB_1 and CB_2 cannabinoid receptor distribution in the mouse brain (taken from Allen Brain Atlas).

Although CB_1 and CB_2 are well known and characterized, several pharmacological studies suggest the existence of additional cannabinoid receptors. One of them may be the orphan GPCR GPR55. This receptor shares only 14% sequence homology with CB_1 and CB_2 receptors and is mostly coupled to $G_{\alpha 13}$ and $G_{q/11}$. It is found in the CNS (Sylantyev et al., 2013), mainly in the basal ganglia, as well as in the gut and in large dorsal root ganglion neurons (Lauckner et al., 2008). GPR55 binds some cannabinoids; however, its pharmacology remains quite controversial (Pertwee et al., 2010). It has also been reported that the transient receptor potential cation channel subfamily V member I ($TRPV_1$) ion channel is able to bind some fatty acylethanolamides and *N*-arachidonoyldopamine -though not 2-arachidonyl glycerol (2-AG), so many authors consider it a cannabinoid receptor that can participate in the endocannabinoid-mediated control of sensory pain (Bisogno et al., 2001); (De Petrocellis et al., 2008; Pertwee et al., 2010). Lastly, several studies indicate that endocannabinoids can also activate some members of the peroxisome proliferator-activated (PPAR) nuclear receptor family (O'Sullivan, 2007) (Pertwee et al., 2010), even though the potencies of endocannabinoids and their metabolites as PPAR agonists or antagonists are relatively low compared with those as agonists of canonical cannabinoid CB_1/CB_2 receptors.

ENDOCANNABINOID STRUCTURE, SYNTHESIS AND DEGRADATION

The discovery of specific receptors in mammalian cells that recognize *Cannabis sativa*-derived compounds stimulated the hunt for the missing piece of the puzzle: the endogenous ligands of the cannabinoid receptors. The first endocannabinoid identified, the *N*-arachidonyletanolamine or anandamide (AEA), was purified from porcine brain (Devane et al., 1992). It is expressed in the brain areas in which CB_1 expression is also elevated, and to a lower extent in peripheral tissues (heart, spleen, testis and uterus) (Felder et al., 1996), and is able to bind and stimulate cannabinoid receptors. As the search for endogenous receptor-binding compounds continued, the second endocannabinoid, 2-arachidonylglycerol (2-AG), was initially isolated from intestine samples, where CB_2 is highly expressed (Mechoulam et al., 1995). For this reason, 2-AG was initially postulated as a CB_2 agonist. However, Sugiura et al (Sugiura et al., 1999; Sugiura et al., 1995) demonstrated that 2-AG has high affinity for both cannabinoid receptors, at which it binds as a full agonist (Pertwee et al., 2010). This monoacylglycerol is highly expressed in the brain, at about 200-fold higher levels than AEA (Beltramo et al., 1997; Sugiura et al., 1995), and is also found in peripheral tissues, such as pancreas,

spleen (Mechoulam et al., 1995), liver, lungs and kidney (Kondo et al., 1998).

Other putative endogenous ligands are noladin ether, virhodamine, *N*-arachidonoyldopamine and some AEA-like fatty acylethanolamides (Pacher et al., 2006). All of them share a similar chemical structure, have cannabimimetic activity, even though their levels are significantly lower than those of AEA and 2-AG, and their physiological relevance remains still unclear. In this context, it is also worth mentioning that recent experimental evidence has shown that at least other 3 endogenous molecules chemically unrelated to cannabinoids, namely hemopressin (Bauer et al., 2012), lipoxin A4 (Pamplona et al., 2012) and pregnenolone (Vallee et al., 2014), can act as allosteric modulators of the CB_1 receptor.

Altogether, this fascinating experimental effort led the scientific community to describe a new signaling system, the so-called endocannabinoid system, composed of specific receptors, their endogenous ligands and the protein responsible for their production, release, uptake and degradation (reviewed by (Piomelli, 2003)). Thus, along with the description of endogenous cannabinoids, specific pathways for their synthesis, transport and degradation have also been defined (Beltramo et al., 1997; Bisogno et al., 2003; Di Marzo et al., 1994; Liu et al., 2008; Stella et al., 1997). These are tightly regulated processes (*Figure 3*). A distinctive feature of endocannabinoids is their synthesis "on demand": they are produced and released only upon a particular physiological or pathological stimulus (Mechoulam et al., 1998; Piomelli, 2003), rather than being synthesized and stored in vesicles, like many other water-soluble neuromodulators.

In animal tissues, AEA is mainly generated from membrane phospholipid precursors by two successive enzymatic reactions (Di Marzo et al., 1994; Okamoto et al., 2004). The first step consists in the *N*-acylation of phosphatidylethanolamine to generate *N*-acylphosphatidylethanolamine (NAPE) by Ca^{2+}-dependent *N*-acyltransferase. Subsequently, *N*-acetylethanolamine (NAE) is released from NAPE by a phosphodiesterase of the phospholipase D type (NAPE-PLD). This produces phosphatidic acid, a metabolic intermediate that is used by cells in the synthesis of the other glycerol-derived phopspholipids, and a fatty acylethanolamyde as AEA. Recently, other mechanisms for the synthesis of AEA have been proposed (Pacher et al., 2006) (Liu et al., 2008). AEA synthesis can be inusually dependent on cytosolic Ca^{2+} elevation, but can also be Ca^{2+}-independent (Di Marzo et al., 1994) (Leung et al., 2006). Furthermore, the activation of some GPCRs, like dopamine D2 receptors, metabotropic glutamate receptors and muscarinic acetylcholine receptors can trigger AEA production and release (Piomelli, 2003).

The other main endocannabinoid, 2-AG, is produced from the hydrolysis of diacylglycerols (DAGs) containing an arachidonyl chain in the sn-2 position, a reaction that is catalyzed by a DAG lipase selective for the sn-1 position. There are two *sn*-1 DAG lipase isozymes, DAGLα and DAGLβ (Bisogno et al., 2003). DAGs, in turn, can be produced from the hydrolysis either of phosphoinositides (PI), catalyzed by a PI-selective phospholipase C (PI-PLC), or of phosphatidic acid

(PA), catalyzed by a PA phosphohydrolase (Di Marzo et al., 1996), (Stella and Piomelli, 2001), (Bisogno et al., 1999), (Carrier et al., 2004) (Figure 3). Neuronal 2-AG production can be initiated by an increase in the concentration of cytosolic Ca^{2+} (Stella et al., 1997) by the activation of metabotropic (Jung et al., 2010) and ionotropic receptors (Stella and Piomelli, 2001), as well as in microglial cells (Witting et al., 2004).

Endocannabinoids act in a broadly paracrine fashion, modulating the action of numerous processes in different neighboring cells. In the central nervous system, they act mostly as neuromodulators (Wilson and Nicoll, 2002). Once synthesized, they are released to the synapse (Liu et al., 2008), and activate cannabinoid receptors, as well as some ionic channels (reviewed in (Pertwee et al., 2010)). Even though, as lipophilic compounds, they can diffuse passively through the cell membrane, this process is accelerated by a rapid and selective transport system (Beltramo et al., 1997; Hillard et al., 1997). The molecular characterization of such transporter is still missing, although recent evidence points to the existence of an inactive cytosolic form of the AEA-degrading enzyme, fatty acid amide hydrolase (FAAH), named FAAH-like anandamide transporter (FLAT), that binds AEA with low affinity and facilitates its translocation inside the cell (Fu et al., 2012).

Once inside the cell, endocannabinoids are deactivated and degraded by specific enzymes. FAAH catabolizes AEA to arachidonic acid and ethanolamine (Cravatt et al., 1996), while monoacylglycerol lipase (MAGL) degrades 2-AG into arachidonic acid and glycerol (Dinh et al., 2002), although other pathways of degradation have been described (Blankman et al., 2007) (Figure 3). For example, the serine hydrolase ABHD6 (Marrs et al., 2010) and oxidizing enzymes as cyclooxygenase and lipoxygenase can utilize these substrates (Vandevoorde and Lambert, 2007) to generate biologically active endocannabinoid metabolites (Nomura et al., 2011) that are probably important in the modulation of synaptic function.

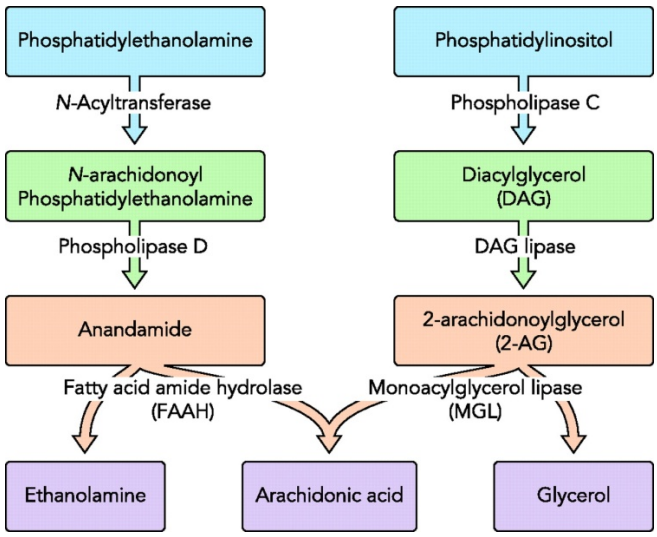

Figure 3. Main pathways of synthesis and degradation of the endocannabinoids AEA and 2-AG (taken from El Manira and Kyriakatos, 2010 Physiology, 25:230-238).

CANNABINOID RECEPTOR-COUPLED SIGNALING PATHWAYS

Cannabinoid receptors are primarily coupled to inhibitory $G_{i/o}$ proteins. Thus, activation of CB_1 and CB_2 receptors promotes the dissociation of αi and βγ subunits. The αi subunit inhibits AC, decreasing the production of cAMP and thus the activity of the cAMP-dependent protein kinase (PKA), which is involved in many biological processes and gene expression control (Howlett et al., 2002). On the other hand, βγ dimers can participate in the activation of the extracellular signal-regulated kinase (ERK) (Bouaboula et al., 1995b); (Galve-Roperh et al., 2002), the mitogen-activated protein

kinase (MAPK) p38 (Liu et al., 2000); (Rueda et al., 2000) and the c-Jun N-terminal protein kinase (JNK) (Derkinderen et al., 2001) (Rueda et al., 2000). Cannabinoids also regulate other pathways involved in the control of cell proliferation and survival, including the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (Gomez del Pulgar et al., 2000), ceramide production (Guzman et al., 2001), sphingomyelin hydrolysis through the adaptor protein FAN (Sanchez et al., 2001) and the regulation of various ion channels (Pertwee, 2005). For example, the CB_1 receptor induces the inhibition of N- and P/Q-type voltage sensitive Ca^{2+} channels (Caulfield and Brown, 1992); (Gebremedhin et al., 1999) and activation of G-protein-activated inwardly rectifying K^+ (GIRK) channels (Mackie, 2005), which hyperpolarize and thus reduces the excitability of the plasma membrane [Reviewed in (Howlett et al., 2002), *Figure 4*].

CB_1 receptors can also couple to other G proteins. Coupling to G_q proteins activates phospholipase C (PLC), which leads to the generation of inositol *1,4,5-tris*phosphate (IP_3) and DAG, and the subsequent release of Ca^{2+} from the endoplasmic reticulum, and activation of PKCs and other DAG targets (Netzeband et al., 1999). On the other hand, the activation of CB_1 can trigger the elevation in cAMP and cytosolic Ca^{2+} concentration and the reduction of K^+ currents via activation of G_s proteins in some cellular contexts (Bash et al., 2003; Hampson et al., 2000). Of note, besides THC, other constituents of *C. Sativa* that do not show high affinity for cannabinoid receptors have also been reported to produce biological effects of potential therapeutic interest. The most representative among the non-psychoactive cannabinoids is cannabidiol (CBD): it exerts, for example, anti-psychotic, anti-inflammatory, anti-convulsant and anti-cancer actions in animal models (Grotenhermen, 2004); (Mechoulam and Hanus, 2002) (Vaccani et al., 2005), while it does not bind with significant affinity to CB_1 or CB_2 receptors. Hence, its effects have been attributed to the inhibition of anandamide degradation, to its antioxidant properties or to other functions (Izzo et al., 2009), although its precise mechanism of action remains to be unraveled.

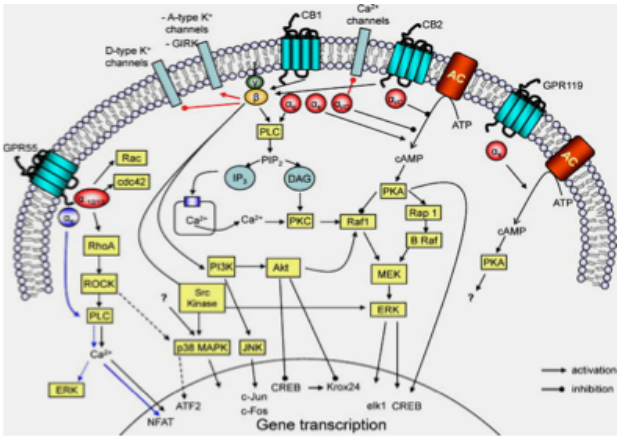

Figure 4. Cannabinoid receptor-coupled signaling pathways (adapted from André et al. Eur J Neurosci, 2010 Jan;31(1):14-28).

BIOLOGICAL FUNCTIONS OF THE ENDOCANNABINOID SYSTEM

The endocannabinoid system participates in the regulation of many different physiological processes (*Table 1*). The stimulation of cannabinoid receptors by endocannabinoids activates the aforementioned signal transduction pathways in a cell- and tissue- specific manner. Hence, the pharmacological manipulation of the endocannabinoid system constitutes a powerful tool to get a better insight into these physiological processes and may represent a potential therapeutic target. This section of the Introduction

will focus on the neuromodulatory and neuroprotective function of the endocannabinoid system, as these are the most relevant processes related to this Doctoral Thesis.

the brain region and neuronal type (*Figure 5*). A large amount of evidence has shown that inhibitory synapses generally have higher levels of CB_1 than excitatory synapses (Kano et al., 2009; Katona and

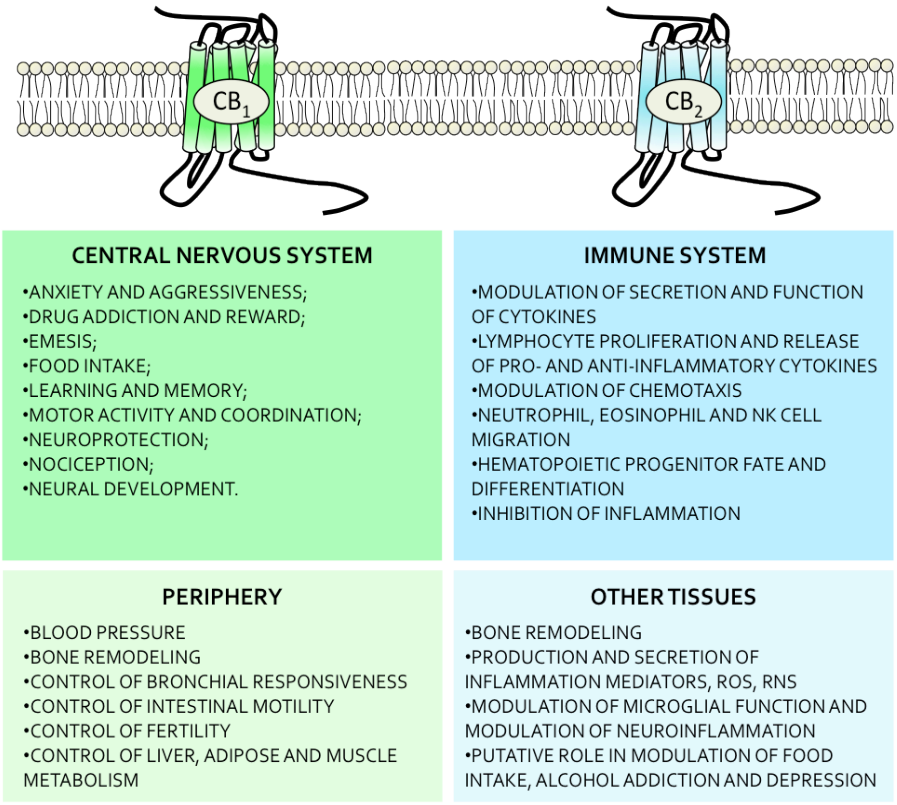

Table 1 . Physiological functions of the endocannabinoid system. The endocannabinoid system participates in the regulation of many different physiological processes. The stimulation of cannabinoid receptors by endocannabinoids activates numerous signal transduction pathways (see Introduction), in a cell- and tissue-specific manner. Hence, the pharmacological manipulation of the endocannabinoid system constitutes a powerful tool to get a better insight in these physiological processes and may have potential therapeutic applications (adapted from Pacher et al. Pharmacol Rev. 2006 Sep;58(3):389-462 ; Pacher and Kunos FEBS J. 2013 May;280(9):1918-43).

Neuromodulation

Within the central nervous system, the major function of the endocannabinoid system accepted so far is the modulation of synaptic transmission by retrograde signaling. Unlike traditional synaptic transmission, in which neurotransmitter release from the presynaptic terminal leads to postsynaptic receptor activation, retrograde signaling involves postsynaptic release of a compound that then acts on presynaptic receptors.

The CB_1 receptor is the most abundant GPCR in the mammalian brain, is mainly expressed in neurons and its expression levels vary depending on

Freund, 2008; Marsicano and Lutz, 1999). CB_1 is also found in astrocytes (Sanchez et al., 1998), oligodendrocytes (Molina-Holgado et al., 2002) and microglia (Stella, 2010). At a subcellular level, CB_1 receptor is mainly located presynaptically (Herkenham et al., 1991) whereas the synthetic enzymes for 2-AG production are found postsynaptically (Kano et al., 2009; Katona and Freund, 2008) (Figure 6). Nevertheless, the receptor has also been found on the plasma membrane of some postsynaptic neurons (Bacci et al., 2004; Kofalvi et al., 2005; Marinelli et al., 2009), and intracellularly, mainly in endosomes, owing to its constitutive recycling cycle (Leterrier et al., 2004)

and on the outer mitochondrial membrane, modulating the rate of mitochondrial respiration in hippocampal neurons (Benard et al., 2012).

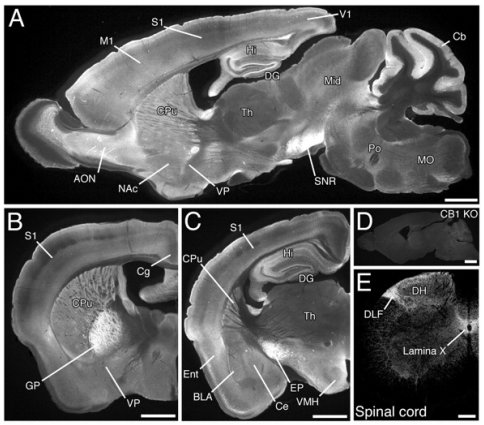

Figure 5. Distribution of CB_1 receptors in the central nervous system of adult mice.
A–D: Overall distribution in brain sections of wild-type (*A–C*) and CB_1-knockout (*D*) mice. Substantia nigra pars reticulata (SNR), globus pallidus (GP), entopeduncular nucleus (EP), hippocampus (Hi), dentate gyrus (DG), primary somatosensory cortex (S1), primary motor cortex (M1), primary visual cortex (V1), cingulate cortex (Cg), entorhinal cortex (Ent), basolateral amygdaloid nucleus (BLA), anterior olfactory nucleus (AON), caudate putamen (CPu), ventromedial hypothalamus (VMH), cerebellar cortex (Cb). *E*: CB_1 immunolabeling in the spinal cord. Dorsal horn (DH), dorsolateral funiculus (DLF), lamina X. Scale bars: 1 mm (*A–C*, *E*); 200 µm (*D*); 50 µm (adapted from Kano et al., Physiol Rev. 2009 Jan;89(1):309-80).

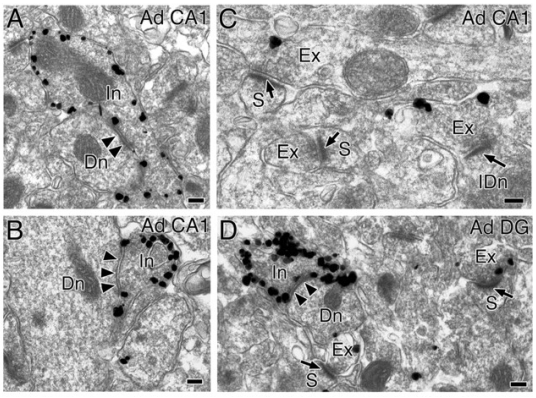

Figure 6. Presynaptic location and signaling of CB_1 receptors.
Immunoelectron microscopy showing presynaptic localization of CB_1 receptors in the hippocampus. *Stratum radiatum* of the CA1 region (A–C) and innermost molecular layer of the dentate gyrus (D). Arrowheads and arrows indicate symmetrical and asymmetrical synapses, respectively. Dn, dendrite; Ex, excitatory terminal; IDn, interneuronal dendrite; In, inhibitory terminal; S, dendritic spine. Scale bar: 100 nm. (adapted from Kano et al., Physiol Rev. 2009 Jan;89(1):309-80).

CB_1 activation leads essentially to presynaptic inhibition of neurotransmitter release (Schlicker and Kathmann, 2001): depolarization of the postsynaptic neuron induces the release of endocannabinoids, which travel retrogradely and suppress presynaptic activity (Freund et al., 2003). These seminal observations led to the hypothesis of an endocannabinoid-mediated homeostasis of synaptic plasticity in the central nervous system (Kreitzer and Regehr, 2001); (Ohno-Shosaku et al., 2001); (Wilson and Nicoll, 2001). Several successive studies eventually revealed that the endocannabinoid system is involved in two key neurophysiological processes, called short-term synaptic depression (STD) and long-term synaptic depression (LTD) (Castillo et al., 2012; Kano et al., 2009).

Depolarization of the postsynaptic membrane, as a consequence of an enhanced Ca^{2+} influx, triggers two types of STD, both mediated by CB_1 activation: depolarization-induced suppression of excitation (DSE; (Kreitzer and Regehr, 2001) and depolarization-induced suppression of inhibition [DSI; (Ohno-Shosaku et al., 2001)] depending on whether the net effect is to decrease glutamatergic excitatory or GABAergic inhibitory synaptic transmission (Figure 7). DSI and DSE phenomena have been described in many different brain regions including cerebellum, hippocampus, neocortex and basal ganglia (Kano et al., 2009). Moreover, the activation of metabotropic glutamate receptors and muscarinic acetylcholine receptors, which lead to a calcium-independent 2-AG-mediated transient inhibition of neurotransmission, is involved in STD induction

(Castillo et al., 2012; Kano et al., 2009).

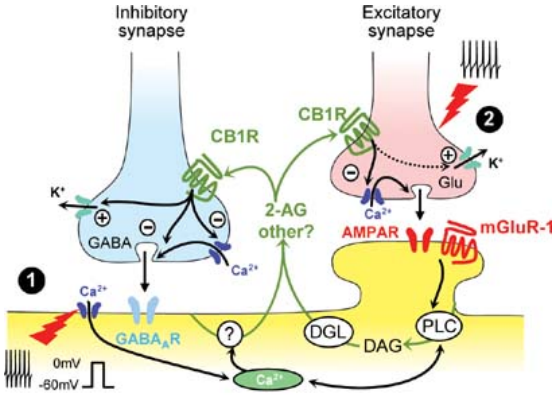

Figure 7. Two major pathways produce eCBs during STD. The first is triggered by Ca^{2+} influx through voltage-gated channels consequent to postsynaptic step depolarization or action potentials. The second pathway is triggered by a brief stimulation of excitatory afferents and group I metabotropic glutamate receptors (mGluRI) activation. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) activation may also contribute to this mechanism. Phospholipase C (PLC) and diacylglycerol lipase (DGL) are required downstream of mGluR, implicating 2-AG in this pathway. The newly synthesized endocannabinoid traverses the synaptic cleft and binds to presynaptic CB_1 receptors, resulting in Ca^{2+} channel inhibition, a direct effect on the vesicle release machinery, and/or K^+ channel activation (taken from Chevaleyre et al., Annu Rev Neurosci. 2006;29:37-76).

A second form of synaptic plasticity mediated by endocannabinoids is LTD (*Figure 8*). LTD is produced by prolonged low-frequency synaptic stimulation that leads to a CB_1-dependent, long-lasting decrease in neurotransmitter release (Chevaleyre et al., 2006); (Lovinger, 2008). It was first described in the amygdala (Marsicano et al., 2002b) and the hippocampus (Chevaleyre and Castillo, 2003), even though later evidence showed that high frequency stimulation of corticostriatal glutamatergic afferents, paired with postsynaptic depolarization, causes a LTD of excitatory inputs onto dorsolateral striatal neurons (Ronesi et al., 2004) and in the nucleus accumbens (Robbe et al., 2002), which depends on retrograde endocannabinoid transmission.

The inhibition of CB_1-mediated neurotransmitter release appears to be mediated by two mechanisms. In the case of STD, in which CB_1 receptors are activated for a few seconds, the mechanism involves direct G protein-dependent (likely via βγ subunits) inhibition of presynaptic Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs) (Brown et al., 2003; Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001). For LTD, the predominant mechanism requires inhibition of AC and downregulation of the cAMP/PKA pathway via the αi/o limb (Chevaleyre et al., 2006; Heifets and Castillo, 2009). Moreover, CB_1 receptors only need to be engaged during the induction, but not expression phase of LTD. Induction also requires combined presynaptic firing with CB_1 activation, thereby providing a mechanism for input-specificity; hence, only active synapses detecting endocannabinoids express long-term plasticity (Heifets and Castillo, 2009). The expression mechanism for LTD may involve presynaptic proteins like Rab3B/RIM1α, (Chevaleyre et al., 2007; Tsetsenis et al., 2011) or a reduction of P/Q-type VGCCs (Mato et al., 2008).

Besides these two forms of synaptic plasticity, recent studies have revealed that TRPV1 mediates a postsynaptic form of LTD. This TRPV1-LTD has been observed in dopamine receptor-2 (D2)-positive medium-sized spiny neurons (MSNs) of the nucleus accumbens (Grueter et al., 2010), in dentate granule cells (Chavez et al., 2010), and in the bed nucleus of the *stria terminalis* (Puente et al., 2011). Likewise, neocortical interneurons expressing cholecystokinin (CCK) and neocortical pyramidal neurons are able to regulate their own activity through a CB_1-mediated somatodendritic slow self-inhibition (SSI) (Bacci et al., 2004; Marinelli et al., 2009).

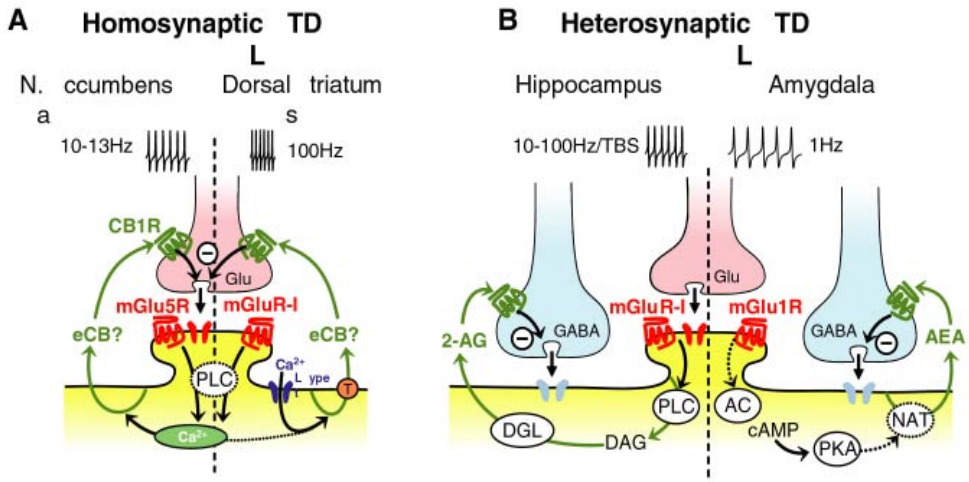

Figure 8. Schematic summary of eCB-LTD in different brain structures.
A: homosynaptic endocannabinoid-LTD. LTD in the dorsal striatum and nucleus accumbens is triggered by stimulation of excitatory inputs to medium spiny neurons and requires mGluR-I activation and increased postsynaptic Ca^{2+} for induction. Implicated in this Ca^{2+} increase are intracellular stores in n. accumbens and l-type Ca^{2+} channels in the dorsal striatum. In the dorsal striatum, D2 receptor activation is also required for induction. *B*: heterosynaptic endocannabinoid-LTD. In both the hippocampus and basolateral amygdala (BLA), LTD is initiated by glutamate release and mGluR-I activation but results in a heterosynaptic decrease in GABA release. LTD induction at these inhibitory synapses does not require increased postsynaptic Ca^{2+} (adapted from Chevaleyre et al., Annu Rev Neurosci. 2006;29:37-76).

It is also worth mentioning that endocannabinoids can activate CB_1 receptors located on astrocytes, potentiating synaptic transmission (Navarrete and Araque, 2010).

At the moment, the relative contribution of the two main endocannabinoids to synaptic transmission and plasticity is still matter of debate. Most studies have described a 2-AG-mediated DSI, STD and LTD at specific synapses (Castillo et al., 2012; Kano et al., 2009) but recent findings suggest that 2-AG and AEA can be recruited differentially from the same postsynaptic neuron depending on the type of presynaptic activity (Lerner and Kreitzer, 2012); (Maccarrone et al., 2008; Puente et al., 2011).

Neuroprotection

General mechanisms of (endo)cannabinoid-mediated neuroprotection

Besides its well-established neuromodulatory functions, an extensive amount of data has highlighted that neuronal damage activates an intrinsic urn, leads to the nstream of the mote neuronal

The first reports on the involvement of the endocannabinoid system in neuroprotection date almost two decades ago, when different groups demonstrated that cannabinoids are able to exert neuroprotection against excitotoxicity (*Box 2*) both *in vitro* (Skaper et al., 1996); (Shen and Thayer, 1998) and *in vivo* (Nagayama et al., 1999). Subsequent studies in different animal models of neurodegeneration demonstrated that the pharmacological activation of the endocannabinoid system protects neurons form death. For example, THC reduced neuronal loss and brain damage in models of excitotoxicity and ischemia (van der Stelt et al., 2001a), AEA was neuroprotective against excitotoxic damage (van der Stelt et al., 2001b); (Veldhuis et al., 2003), and 2-AG prevented neuronal death in a model of traumatic brain injury (Panikashvili et al., 2001). Since then, the interest on the neuroprotective potential of the endocannabinoid system has grown exponentially, leading to the characterization of various

mechanisms by which this system protect the CNS against insults (reviewed in (Galve-Roperh et al., 2008; Gowran et al., 2011; Shohami et al., 2011); 20230193). Studies based on the observation that the brain overproduces endocannabinoids upon damage (Marsicano et al., 2003), (Mechoulam et al., 2007) determined that the modulation of the endocannabinoid tone, by using inhibitors of transport and degradation of AEA and 2-AG, prevented behavioral alterations and memory impairment due to excitotoxicity in a CB_1-dependent manner (Karanian et al., 2005); (Coomber et al., 2008). In addition to the aforementioned pharmacological studies, the generation of CB_1 receptor knock-out (CB_1-KO) mice provided further evidence for the neuroprotective role of the endocannabinoid system. These animals are more sensitive than the wild-type to various brain insults. For instance, CB_1-KO mice show increased excitotoxic injury after brain stroke or kainic acid (KA) administration (Parmentier-Batteur et al., 2002); (Marsicano et al., 2003). Likewise, mice lacking CB_1 show an enhanced age-related loss of hippocampal neurons accompanied by a decrease in cognitive functions (Bilkei-Gorzo et al., 2005).

The neuroprotective action of endocannabinoid signaling relies mainly on the

BOX2 Excitotoxicity

Glutamate is the major excitatory neurotransmitter in the mammalian brain; it mediates both fast/ionotropic and slow/metabotropic synaptic transmission and modulates synaptic plasticity, learning and memory, and many other cognitive functions. A prolonged exposure to glutamate and/or its excessive extracellular concentration leads to uncontrolled shifts of sodium, potassium and calcium, thereby disrupting ionic homeostasis and ultimately leading to cell death (**excitotoxicity**). In order to prevent this noxious glutamate exposure, the brain has developed several mechanisms to control glutamate concentration: inside the neurons, vesicular glutamate transporters (vGluTs) confine glutamate in synaptic vesicles, and release it in the synaptic cleft only upon neuronal depolarization. On the other hand, astrocytes are majorly in charge of the uptake and recycling of this neurotransmitter via different glutamate-uptake proteins.

Almost every class of glutamate receptors has been implicated at a certain extent in excitotoxic cell death, although the major role of post-synaptic NMDARs is now unanimously accepted. NMDAR-mediated excitotoxicity is believed to contribute to the pathophysiology of several human neurodegenerative diseases, including Huntington's disease (HD), Alzheimer's disease (AD) and human immunodeficiency virus (HIV)-associated dementia. In physiological conditions, a voltage-dependent block of Mg2+ modulates the function of the receptors. Upon depolarization, Mg2+ is transiently removed from the channel, thus triggering an increase in Ca2+ influx and the subsequent downstream signaling. Under pathological conditions, the overactivation of NMDARs permanently removes the Mg2+ ion from the channel. This lead to an excessive influx of Ca2+ that activates a wide array of cytotoxic processes that can eventually lead to necrosis, apoptosis and/or dendritic/synaptic damage (Figure 1 box2)

Over the years, many mechanisms explaining NMDAR-induced neurodegeneration have been proposed, although further research is needed to fully understand the role of these receptors in excitotoxicity. Recently, several research groups have described a dual role of NMDARs dependent on their location at the synapse: while NMDARs located synaptically may promote pro-survival signaling and neuroprotection, extrasynaptic NMDAR activation could induce neuronal damage and death. This dual nature of NMDARs, depending on their subcellular location, and also on their subunit composition, represents a promising tool for the development of strategies to counteract glutamate excitotoxicity.

inhibition of excitotoxic glutamatergic neurotransmission (Katona and Freund, 2008). Functional CB_1 receptors located on glutamatergic terminals become activated by endocannabinoids upon excitatory synaptic transmission and prevent massive glutamate release (Monory et al., 2006). Nonetheless, the vast majority of CB_1 receptors in the central nervous system is located on GABAergic neurons, and thus it is also conceivable that they can control excitotoxic neurostransmission indirectly. Elucidating the precise contribution of the pools of CB_1 receptors located on excitatory or inhibitory terminals constitutes precisely one of the main objectives of this Doctoral Thesis.

In addition, CB_1 receptors can trigger cell-autonomous intracellular signal transduction events that promote cell survival. Specifically, several studies have established the coupling of CB_1 receptors to at least two important cell survival signaling routes: the PI3K/Akt and the ERK pathways (*Figure 4*). CB_1 receptor activation evokes PI3K/Akt stimulation in vitro (Gomez del Pulgar et al., 2000); (Molina-Holgado et al., 2002) (Molina-Holgado et al., 2005) and in vivo (Ozaita et al., 2007), an event that has been related with cannabinoid-mediated neuroprotection. The downstream targets by which CB_1 receptors may signal neuroprotection via Akt are as yet unclear, but one of them could be glycogen synthase kinase-3 (GSK-3), a potentially neurotoxic protein that is phosphorylated and inactivated by Akt (Gomez Del Pulgar et al., 2002; Molina-Holgado et al., 2002; Ozaita et al., 2007).

Even though it has not been demonstrated in the context of neuroprotection, mammalian target of rapamycin (mTORC1) pathway is activated upon THC treatment (Puighermanal et al., 2009) or increased endocannabinoid tone (Busquets-Garcia et al., 2011), and is involved neural progenitor cell proliferation (Diaz-Alonso et al., 2014; Palazuelos et al., 2012), and in olygodendrocyte differentiation (Gomez et al., 2011). Thus, the involvement of mTORC1 as a downstream target of the PI3K/Akt pathway in CB_1-mediated neuroprotection is quite likely.

Other proteins potentially involved in CB_1/AKt-mediated neuroprotection are Phosphoprotein Enriched in Astrocytes 15 (PEA15) and Fas-Associated protein with Death Domain (FADD), whose CB_1-induced phosphorylation confers them anti-apoptotic functions (Alvaro-Bartolome et al., 2010).

On the other hand, cannabinoid administration leads to CB_1 receptor-mediated ERK pathway activation in different areas of the brain, like hippocampus (Derkinderen et al., 2003), striatum (Valjent et al., 2001), frontal cortex (Moranta et al., 2007) and cerebellum (Tonini et al., 2006). CB_1 receptor coupling to ERK pathway may signal via different context-dependent effectors. For instance, the induction of various transcription factors such as the early-response genes *c-fos* (Porcella et al., 1998), (Zhao et al., 1998) and Krox-24 (Derkinderen et al., 2003), (Bouaboula et al., 1995a), the phosphorylation/activation of transcription factors such as Elk-1 (Valjent et al., 2001) or other targets such as p90 ribosomal S6 kinase (Gomez Del Pulgar et al., 2002) have been implicated in CB_1 receptor-evoked ERK effects in

Other studies have related the neuroprotective effects mediated by CB_1 receptors with brain

derived neurotrophic factor (BDNF) signaling system. CB_1 receptors are involved in BDNF production, and this process is associated with the anti-excitotoxic response elicited by endocannabinoids in the hippocampus and in the cortex (De March et al., 2008; Khaspekov et al., 2004; Marsicano et al., 2003). CB_1 can also trans-activate TrkB receptors (Berghuis et al., 2005) and cross-talk with bFGF and EGF signalling systems, which might be involved in the neuroregenerative response after excitotoxic injury, (Hart et al., 2004), (Aguado et al., 2007b).

Other mechanisms of (endo)cannabinoid-mediated neuroprotection

Together with the neuroprotective signaling pathaways discussed above, the endocannabinoid system may contribute to neuroprotection via other processes such as anti-inflammatory response (e.g. (Maresz et al., 2005); (Fernandez-Ruiz et al., 2008)), neurorepair (Galve-Roperh et al., 2013), and receptor-independent effects (Galve-Roperh et al., 2008).

-Modulation of neuroinflammation

The immediate inflammatory response that follows brain injury, as well as the neuroinflammatory processes observed in many neurodegenerative disorders, contributes to neuron survival. The immune response after neuronal damage is essential for the homeostasis of the central nervous system, but an excessive or prolonged inflammatory response, together with the dysregulation of the activity of the brain-resident immune cells, negatively affects neuronal survival (Block et al., 2007; (Kettenmann et al., 2011; Lobsiger and Cleveland, 2007). Thus, an excessive activation of microglial cells induces neurotoxicity in models of neurodegeneration such as ischemia (Takeuchi et al., 2008), traumatic brain injury (Jordan et al., 2008), excitotoxicity (Cho et al., 2008) and Huntington's disease (Shin et al., 2005), (Hsiao and Chern, 2010), among others.

In this context, the immunomodulation of microglia mediated by the endocannabinoid system (Table 1) plays an important role in the prevention of neurodegeneration (reviewed in (Fernandez-Ruiz et al., 2011; Stella, 2009)) and represents a potential pharmacological tool for the prevention of aberrant neuroinflammation. Specifically, CB_2 receptors are expressed on various circulating and resident immune cells, where they inhibit the release of inflammatory mediators, including nitric oxide (NO), interleukin 2 (IL-2), and tumor necrosis factor (TNF-α), reduce the activation of the cell-mediated immune processes and attenuate cell proliferation and chemotaxis (Palazuelos et al., 2008); (Maresz et al., 2007); (Romero-Sandoval et al., 2009); reviewed in (Stella, 2004). Likewise, CB_2 receptor expression increases upon microglial activation in various models of brain damage and inflammation (Docagne et al., 2007); (Fernandez-Ruiz et al., 2007); (Maresz et al., 2005); (Palazuelos et al., 2009), thus supporting the hypothesis of its participation in cannabinoid–induced neuroprotection, although the precise role of microglial CB_2 receptors in neurodegeneration still remains unclear (Miller and Stella, 2008).

-Neurorepair

Among the neuromodulatory functions of the endocannabinoid system, several studies have described its regulatory role in brain development (reviewed in (Galve-Roperh et al., 2013; Harkany et

al., 2007)). The initial finding of a CB_1-mediated regulation of neurogenesis (Rueda et al., 2002), was followed by the description of the expression of the endocannabinoid system in neural progenitors (NPs; (Jin et al., 2004); (Aguado et al., 2005) Mulder 2008 PNAS). Moreover, the endocannabinoid system participates in the control of NPs proliferation, differentiation and migration (Jiang et al., 2005); (Aguado et al., 2006); (Diaz-Alonso et al., 2012) in the developing brain (reviewed in (Galve-Roperh et al., 2013; Harkany et al., 2007)). In the adult brain, generation of new neurons is essentially restricted to two discrete areas: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus (Deng et al., 2010). Newly generated neurons have the ability to become functional and integrate into established brain circuits (Lledo et al., 2006). In addition, the observation of increased neurogenesis after brain injury has been proposed to constitute an endogenous neuroprotective response aimed at repairing brain damage (Lie et al., 2004); (Kernie and Parent, 2010).

The endocannabinoid system has been involucrated also in adult brain neurogenesis. Thus, NPs from both the adult SVZ and the dentate gyrus (DG) have been shown to express a functional endocannabinoid pro-neurogenic signaling machinery (Aguado et al., 2006), (Jiang et al., 2005), (Arevalo-Martin et al., 2007). The description of endocannabinoid-mediated adult neurogenesis suggested that the endocannabinoid system might be involved in the structural and functional repair of the brain. Indeed, cannabinoids, via CB_1 activation, ease the proliferative response of neuronal progenitors against excitotoxicity induced by KA in the hippocampus (Aguado et al., 2007a). SVZ neuronal progenitors protect MSNs from glutamatergic excitotoxicity by secreting AEA that in turn modulates corticostriatal glutamatergic currents by binding to CB_1 receptor (Butti et al., 2012). In addition, cannabinoid treatment promotes neurogenesis in SGZ of the hippocampus with anxiolytic and antidepressant effects (Jiang et al., 2005), while the reduction of the endocannabinoid tone was postulated as responsible of the age-related decline in neurogenesis (Goncalves et al., 2008).

-Cannabinoid receptor-independend neuroprotection

Some cannabinoids can also exert neuroprotective actions by receptor-independent mechanisms. Cannabinoids containing a phenol group, like THC and cannabidiol, show anti-oxidant properties (Hampson et al., 1998; Marsicano et al., 2002a), for example, in models of ischemic damage (Hayakawa et al., 2007) and Parkinson's disease (Lastres-Becker et al., 2005). On the other hand, the bioactive lipidic products of endocannabinoid metabolism may be involved in neuronal survival. For example, cyclooxygenase-2 (COX-2)-mediated metabolism of endocannabinoid substrates (Kozak et al., 2002) generates different neuroactive prostaglandins (Sang et al., 2006) and prostamides (Correa et al., 2008), whereas AEA hydrolysis by FAAH produces ethanolamine, which is protective for neuroblastoma cells (Matas et al., 2007). Moreover, in addition to an enhanced synthesis of the classical endocannabinoids AEA and 2-AG, other fatty acylethanolamides, by binding to receptors different from CB_1 and CB_2, are

overproduced upon brain insults (Mechoulam and Shohami, 2007).

Neurotoxic effects of (endo)cannabinoids

Despite the large amount of evidence describing the neuroprotective role of the endocannabinoid system, a few studies have drawn attention to a possible neurotoxic effect of cannabinoids (reviewed in (Fowler et al., 2010; Sarne et al., 2011)). Thus, *in vitro* studies demonstrated that THC and AEA are toxic in neuron primary culture, although the concentrations used were considerably higher than those needed to activate cannabinoids receptors (Chan et al., 1998; Movsesyan et al., 2004). In addition, treatment with AEA induced loss of hippocampal neurons by mechanisms dependent on TRPV1 receptor activation (Cernak et al., 2004; Maccarrone and Finazzi-Agro, 2003), while the blockade of CB_1 receptors in the postnatal brain prevented NMDA-induced excitotoxicity (Hansen et al., 2002). Moreover, in a more recent study, treatment with THC and WIN55,212-2 potentiated the neurotoxic effects of ethanol in the developing brain (Hansen et al., 2008).

Conversely, treatment with the CB_1 antagonist/inverse agonist rimonabant exerted neuroprotection in mouse models of excitotoxic/ischemic damage (Hansen et al., 2002; Muthian et al., 2004; Pegorini et al., 2006), ethanol-induced neurotoxicity (Hansen et al., 2008), and Parkinson's disease (van der Stelt et al., 2005). In line with these results, CB_1-KO mice showed less neuronal loss after ethanol exposure (Hansen et al., 2008).

Various signaling mechanisms have been proposed for the cannabinoid-mediated enhanced susceptibility to neurotoxicity. Among them, it is worth mentioning prostanoid synthesis and generation of free radicals by ciclooxigenases (Chan et al., 1998), stimulation of the pro-apoptotic JNK and p38 MAPK cascades (Derkinderen et al., 2001; Downer et al., 2003), activation of calpains (Cernak et al., 2004; Movsesyan et al., 2004) and increase of p53-dependent lysosomal permeability (Gowran et al., 2011).

This dual neuroprotective/neurotoxic effect of cannabinoids remains still controversial. Some authors have postulated that the differential ability of cannabinoids to engage either CB_1 or TRPV1 would be responsible for neuroprotective or neurotoxic effects, respectively (Bari et al., 2005). The differential activation of CB_1 on either glutamatergic or GABAergic neurons, in different brain areas, could also explain this dual effect of cannabinoids. Moreover, the dose of the cannabinoid seems a critical factor as neuroprotective effects are normally observed at low doses, while neurotoxic effects can occur at higher doses. Likewise, the immature brain is usually more susceptible to being altered by cannabinoids that the adult, mature brain.

HUNTINGTON'S DISEASE

Huntington's disease (HD) is an autosomal dominant inherited neurodegenerative disorder, with a prevalence of ~5 to 10 per 100,000 persons (Vonsattel and DiFiglia, 1998). This genetic disease is caused by an abnormal CAG repeat expansion in the exon 1 of the huntingtin (HTT) gene; therefore, the encoded protein carries a polyglutamine (polyQ) tract in its N-terminal domain (1993). HD is characterized by several symptoms that include

BOX3 Basal ganglia and corticostriatal circuitry

Cortico-basal ganglia circuits arise from basically all parts of the cortex and project in a topographical manner to the striatum (both caudate-putamen and nucleus accumbens); from there, via basal ganglia output nuclei and the thalamus, they project back to the cortex. Sensory and motor areas of the cortex project to the dorsal striatum through glutamatergic corticostriatal afferents. Simultaneously, the striatum receives glutamatergic inputs from thalamic nuclei and dopaminergic projections from the substantia nigra *pars compacta*. The main function of the striatum is to process the information originating in different cortical regions and send this information back to the cortex in order to complete the cortico-basal ganglia-thalamo-cortical loop. Hence, the correct functioning of the striatum is essential in the control and modulation of goal-directed behavior, like voluntary movement, decision-making and reward.

Cortical and thalamic inputs primarily target GABAergic medium-sized spiny neurons (MSNs). These cells constitute approximately 95% of the striatal neuronal population, and their function is to integrate and deliver the information to other nuclei of the basal ganglia. MSNs are usually classified in two populations on the basis of their respective projection targets and neurochemical composition. MSNs that preferentially express D_1-type dopamine receptors, substance P and dynorphin, and project to the output nuclei of the basal ganglia (e.g., the internal segment of the globus pallidus and substantia nigra *pars reticulata*), constitute the direct pathway. In contrast, indirect pathway MSNs preferentially express D_2-type dopamine receptors and encephalin, and project to the output nuclei of the basal ganglia through a network that includes the external segment of the globus pallidus and subthalamic nucleus. In the classical model of basal ganglia-circuit function, the direct and indirect pathways act in opposite manners to control motor behavior. Activation of the direct pathway results in the disinhibition of thalamocortical projections and the facilitation of motor routines, while activation of the indirect pathway results in the inhibition of thalamocortical projections and attenuation of movement-related processing. The direct and indirect pathways also differ in their cortical innervations, as indirect-pathway MSNs have stronger synaptic coupling with cortical inputs than direct neurons.

The critical filtering role played by the basal ganglia network also relies on the close interactions between MSNs and several subtypes of interneurons. Interneurons constitute the remaining 5% of the striatal neurons, and have been traditionally classified in four subtypes: fast-spiking GABAergic, cholinergic, nitric oxide synthase-positive and calretinin-positive interneurons. All of them receive powerful excitatory inputs from the cortex and thalamus, and exert a modulatory action on striatal synaptic transmission through pre- and postsynaptic mechanisms, thus regulating the function of the corticostriatal glutamatergic system. Despite representing a minority of the total striatal neuronal population, interneurons play a crucial role in the adecuate modulation of striatal function, thereby contributing to the correct processing of corticostriatal information .

Alterations at any level of this fine-tuned neuronal network can differentially influence basal ganglia processing. In fact, pathophysiological changes in the corticostriatal and basal ganglia circuitry are characteristic of many neurological diseases, including Huntington's disease, Parkinson's disease, schizophrenia, obsessive-compulsive disorder, autistic spectrum disorder and other pathologies.

progressive emotional and psychiatric disturbances, generalized motor dysfunctions and gradual cognitive decline. Disease onset generally occurs between 35 and 50 years of age, progressing inexorably over 15-20 years until death (Bates G, 2002; Roos et al., 1991).

Normal individuals have 35 or fewer CAG repeats (Kremer et al., 1994). The presence of 36 or more CAG repeats leads to the eventual development of the disease ((Rubinsztein et al., 1996)). However, the expanded CAG regions are relatively unstable,

particularly when passed via the paternal germline. This phenomenon, in which the CAG repeat number tends to increase in subsequent family generations, is known as anticipation ((Myers et al., 1982) (Duyao et al., 1993)). The age of onset of symptoms correlate inversely with the length of the CAG expansion (Andrew et al., 1993) (Brinkman et al., 1997; Stine et al., 1993), and so most adult onset cases have CAG repeat lengths of 40 to 50, while juvenile onset cases have longer CAG expansions (>60) (Brandt et al., 1996; Conneally, 1984).

The neuropathological hallmark of HD is mainly brain specific, with extreme but selective neurodegeneration in the striatum (caudate nucleus and putamen). The striatum processes the information originating in different cortical regions and sends this information back to the cortex to complete the cortico-basal ganglia-thalamo-cortical loop (Graybiel et al., 1994). (For an overview of the corticostriatal pathways see *box 3*). GABAergic MSNs constitute 95% of the striatal cells and are the main and earliest striatal cell type affected in HD, whereas striatal interneurons remain unaffected or only mildly affected at late stages of the disease (Ferrante et al., 1985; Reiner et al., 1988; Vonsattel et al., 1985). Moreover, extensive studies on port-mortem HD brains show a differential vulnerability between MSNs subtypes (Cicchetti et al., 2000). In the early stages of HD, the subpopulation of MSNs that expresses enkephalin and projects to the external segment of the globus pallidus (indirect pathway) is the first to degenerate. This is followed by the degeneration of the substance P-expressing MSNs that project to the internal segment of the globus pallidus and substantia nigra pars reticulata (direct pathway) (Albin et al., 1992; Reiner et al., 1988; Richfield et al., 1995). *(Figure 9)* The abnormalities observed in the early stages in the indirect pathway have been associated with development of choreic movements in HD (Crossman et al., 1988). On the other hand, the late degeneration of the MSNs of the direct pathway manifests as rigidity and bradykinesia (Berardelli et al., 1999).

Although the striatum experiences the greatest extent of neuronal loss, in subsequent stages of the disease, other brain regions are affected: different

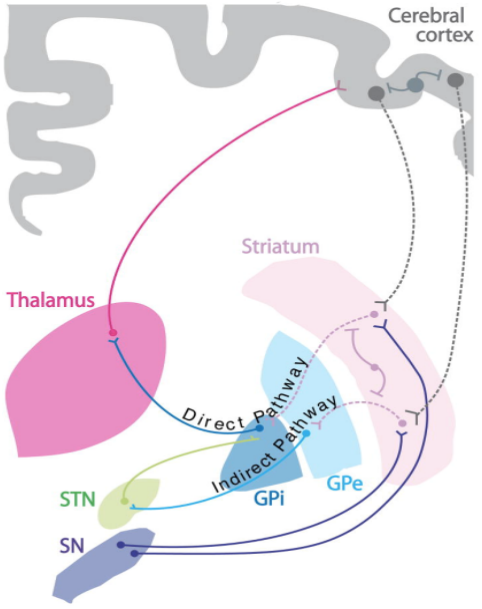

Figure 9. Overview of corticostriatal circuitry alterations in HD pathology.
A subset of projection neurons in the striatum and the cortex (represented by dashed lines) are particularly vulnerable in HD. These include medium-sized spiny neurons (MSNs, pink dashed lines) of the striatum and large pyramidal projection neurons in cortical layers V, VI and III of the cerebral cortex (gray dashed lines). MSNs in the 'indirect pathway' of the basal ganglia project to the external segment of the globus pallidus (GPe) and are affected early in the course of the disease. As HD progresses, MSNs projecting to the internal segment of the globus pallidus (GPi) via the 'direct pathway' and cortical pyramidal cells projecting to the striatum are also impaired. STN, subthalamic nucleus; GPe, external globus pallidus; GPi, internal globus pallidus; SN, substantia nigra (taken from Han et al., J Neurochem. 2010 Jun;113(5):1073-91).

studies have described neuronal atrophy and neuronal loss in the substantia nigra, globus pallidus, thalamus and hippocampus (Cowan and Raymond, 2006; de la Monte et al., 1988). Likewise, a decrease in cortical volume and the death of cortical neurons occur in more advanced cases of HD, with the remarkable loss of the large pyramidal neurons in layers III, V, and VI that project directly to the striatum (Cudkowicz and Kowall, 1990; Raymond et al., 2011; Rosas et al., 2005; Vonsattel and DiFiglia, 1998). In this advanced stage, the disease turns systemic, leading to a wide spectrum of endocrine, metabolic, and skeletal alterations (van der Burg et al., 2009).

HUNTINGTIN IN HEALTH AND DISEASE

Huntingtin is widely distributed throughout many tissues of the body and throughout most brain regions (Ferrante et al., 1997; Landwehrmeyer et al., 1995; Strong et al., 1993; Trottier et al., 1995). Interestingly, cortical pyramidal neurons that project to striatal neurons, (layers III and V) express high levels of the protein (Fusco et al., 1999). Huntingtin is involved in many cellular processes although not all of them have been elucidated yet. Several studies have demonstrated its essential role during embryonic development for the complete formation of the nervous system (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). There is also evidence that indicates its anti-apoptotic function -both *in vitro* and *in vivo*- and its regulatory role in balancing neuronal death/survival (Ho et al., 2001; Leavitt et al., 2001; Rigamonti et al., 2000) and in neuronal sensitivity to excitotoxic neurodegeneration (Leavitt et al., 2006; Nasir et al., 1995).

Since the cloning of the HTT gene in 1993, a very large amount of molecular dysfunctions have been elucidated that seem to contribute to the explanation of the early deterioration of the MSNs in the striatum (reviewed in (Zuccato et al., 2010)). However, the exact mechanism by which mutant Huntingtin (mHTT) causes such specific neuronal degeneration, despite its ubiquitous expression, is still unclear. It is widely behaved that the single mutation in the HTT gene provides the protein with new toxic functions that are deleterious for the cells (gain of function hypothesis). At the same time, the loss of the normal protein physiological activities can also contribute to disease pathogenesis and, in particular, to its selectivity (loss of function hypothesis (Cattaneo et al., 2001)). As a consequence of the mutation, reduced wild-type Huntingtin physiological activity may cause the striatal neurons to become particularly vulnerable [(Cattaneo et al., 2005; Zuccato et al., 2003). *Figure 10*].

In recent years, progresses have been made in elucidating the etiology of HD. Furthermore a growing body of evidence suggests the involvement of multiple pathogenic pathways in this disease. Examples of this would be oxidative stress, transcriptional deregulation, alteration in protein degradation, mitochondrial dysfunction, alteration in the regulation of neurotrophic factors -e.g. BDNF- and excitotoxicity (reviewed in (Zuccato et al., 2010)). Unfortunately, little is known about mechanism underlying the increased vulnerability of selected neuronal populations in HD, even though several investigations show that the pathophysiology of HD may arise both from cell autonomous processes within vulnerable neurons

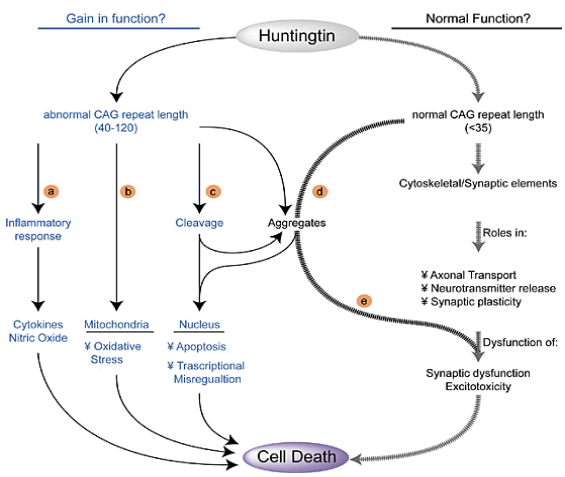

Figure 10. Huntingtin gain/loss of function.
Mutant huntingtin may incur a toxic gain-of-function through a variety of mechanisms: *a*, it may activate an inflammatory response leading to the release of cytokines and nitric oxide which at high levels exert a toxic effect; *b*, it may interfere with energy metabolism triggering oxidative stress; *c*, it may be abnormally cleaved creating fragments which may induce apoptosis; *d*, it may form aggregates which may induce apoptosis and/or transcriptional dysregulation; *e*, it may sequester other proteins including normal huntingtin, thereby creating a dominant negative effect while also conferring a loss-of-function (adapted from Di Prospero and Tagle Nature Medicine 2000; 6, 1208 - 1209).

and dysfunction of neuron-neuron interactions, specifically at the level of the corticostriatal afferents (Cepeda et al., 2007; Fan and Raymond, 2007; Imarisio et al., 2008; Zuccato and Cattaneo, 2007).

EXCITOTOXICITY HYPOTHESIS AND CORTICAL DYSFUNCTION IN HD

Excitotoxicity *(see box 2)* is the pathological process by which neurons are damaged by the overactivation for the excitatory neurotransmitter glutamate receptors, especially NMDARs. It results in neurodegeneration and a consequent dysfunction of neuronal interaction and circuitries. The striatum receives a large number of cortical and thalamic glutamatergic afferents, hence it is more susceptible to suffering excitotoxic damage in a situation whereby an excess of glutamate is observed (Cepeda et al., 2007). Throughout the years, among all the hypotheses formulated about the pathogenesis of HD, several line of evidence emphasized the role of excitotoxicity and corticostriatal dysfunction (Schwarcz et al., 2010). Numerous studies demonstrated that the expression of mHtt impairs corticostriatal glutamate release by interrupting axonal transport, vesicular release, vesicular reuptake and presynaptic receptor regulation (Fan and Raymond, 2007; Hansson et al., 1999; Morton and Leavens, 2000). They also highlighted a decrement of glutamate uptake at the synaptic cleft in both mice models (Behrens et al., 2002; Estrada-Sanchez et al., 2009) and post-mortem brain tissues from HD patients (Arzberger et al., 1997; Hassel et al., 2008), as a result of an altered expression of the glial transporter of glutamate GLT-1. Furthermore, due to the loss of function of WT Htt, a decreased production of BDNF is observed (Zuccato et al., 2001; Zuccato et al., 2003). BDNF is a neurotrophic factor, essential for survival of striatal neurons and the control of glutamate release from cortical pyramidal neurons (Altar et al., 1997; Shouman et al., 2006). The reduced levels of BDNF might severely affect the activity of the corsticostriatal synapse, contributing to the selective vulnerability of MSNs.

Evidence in the postmortem analyses of HD brain tissues brought to attention abnormalities in the glutamatergic neurotransmission: researchers observed a reduction in glutamate receptor levels and a reduced NMDAR binding at pre- and early symptomatic stages of HD (Albin et al., 1990; DiFiglia, 1990; London et al., 1981; Young et al., 1988). Further analyses in rodent models showed that intrastriatal injection of glutamate receptors

agonists, particularly those acting on NMDAR, caused the selective loss of MSNs while sparing interneurons, as observed in human HD brains (Beal et al., 1991; Ferrante et al., 1993; Schwarcz et al., 1984). Similarly, alteration in the kynurenyne pathway (the major catabolic route of tryptophan in mammals), (Kita et al., 2002; Schwarcz et al., 2010) with consequent altered levels of one of its downstream metabolites, the NMDAR agonist quinolinic acid (QA), may contribute, at least in part, to excitotoxicity (Guidetti et al., 2006; Guidetti et al., 2004).

Studies in rodent models of HD showed pre-symptomatic electrophysiological alterations, increased sensitivity of NMDAR to NMDA and a lower sensitivity to Mg^{2+} (Fan and Raymond, 2007; Starling et al., 2005), together with impaired synaptic plasticity in the prefrontal cortex (Cummings et al., 2006; Miller et al., 2008; Walker et al., 2008). These findings suggest the presence of a constitutive abnormal NMDAR signaling, which may contribute to the development of the HD phenotype.

Many hypotheses have been proposed on the possible mechanisms responsible for the aberrant NMDAR activity in HD. Firstly, results from (Chen et al., 1999), demonstrated that mHtt increases the response on NMDAR containing NR1/NR2B subunits. Whereas, receptors containing NR1/NR2A subunits were not differentially affected by the presence of the mutant protein. These results were confirmed by a consecutive work from (Zeron et al., 2001) which showed that mHtt enhanced apoptotic cell death in cells co-transfected with NR1/NR2B subunits and not NR1/NR2A, giving rise to the NR2B-selective hypothesis. This subunit is particularly abundant in the striatum, compared to other brain regions. Thus, the presence of NR2B-containing NMDAR complexes, with pathological characteristics, together with mHtt may help to explain the preferential vulnerability of MSNs in HD (Cowan et al., 2008; Zeron et al., 2002). Secondly, several evidences highlighted a dual role of NMDAR. Depending on the localization of the receptor to synaptic or extra-synaptic sites it can exert pro-survival or pro-apoptotic function, respectively (Papadia and Hardingham, 2007). Two parallel studies revealed a disruption of the existing balance between these two subset of receptors in animal models of HD, that show increased extrasynaptic NMDAR signaling as a consequence (Milnerwood et al., 2010; Okamoto et al., 2009).

In addition, the polyQ expansion inhibits the ability of WT-Htt to interact with post-synaptic density 95 (PSD95; a scaffolding protein of the postsynapse) resulting in the sensitization of NMDA receptors (Sun et al., 2001), and therefore increased the vulnerability of neurons to glutamate mediated excitotoxicity (Song et al., 2003). NMDAR function is also modulated by posttranslational modifications, such as phosphorylation. mHtt-induced phosphorylation of NR2B subunit contributes to promoting overactivation of NMDAR (Song et al., 2003). NMDAR trafficking is also impaired in HD by the destabilization of the clathrin-mediated endocytic complex that involves NMDAR, Huntingtin and Huntingtin interacting protein 1 [HIP1; (Fan et al., 2007; Metzler et al., 2007) *Figure 11*].

Recently, an elegant work from Marco et al 2013 described other mechanisms of NMDARs aberrant activity in HD. The levels of NMDARs containing

N3A subunits are increased in mouse models and human samples of HD. Physiologically, these receptors prevent premature synapse plasticity and stabilization during early stages of postnatal brain development, but are down-regulated in adult brain (Das et al., 1998); (Roberts et al., 2009) by the endocytic adaptor PACSIN1. In HD, mHtt sequesters PACSIN1, promoting the maintenance this form of NMDARs, at the synapses. This reactivates, in the adult brain, pruning mechanisms normally restricted to developmental stages, yielding a higher proportion of small, immature synapses. The authors showed that the suppression of the expression of the NMDAR subunit N3A, rescue the NMDAR dysfunction, synapse loss and cognitive decline observes in HD.

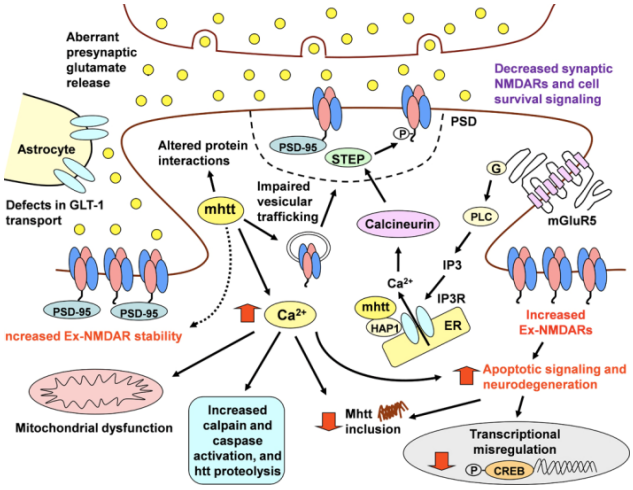

Figure 11. Model of mutant huntingtin-induced synaptic dysfunction in HD.
Aberrant glutamate release from cortical and thalamic afferents stimulates postsynaptic and extrasynaptic NMDARs on MSNs. Overactivation of extrasynaptic NMDARs also may be facilitated by deficits in glutamate uptake by the transporter GLT-1 on astrocytes. The mutant huntingtin protein affects multiple cellular processes such as Ca^{2+} homeostasis, mitochondrial function, transcriptional regulation, protein-protein interactions and vesicular transport of proteins including neurotransmitter receptors. Mutant huntingtin also affects group 1 metabotropic receptor signaling, with the consequent increase of Ca^{2+}. HD is also associated with decreased synaptic NMDAR stability and increased expression, function and signaling of extrasynaptic-NMDARs. It is postulated that enhanced extrasynaptic-NMDAR stability may be mediated by increased binding to scaffolding proteins such as PSD-95. estrasynaptic NMDAR stimulation leads to downregulation of pro-survival signaling such as CREB-mediated gene transcription, decreased mutant huntingtin inclusion formation, and increased activation of pro-apoptotic signaling that facilitates further neuronal dysfunction and neurodegeneration (taken from Neuroscience. 2011 Dec 15;198:252-73).

Glutamate transmission dysfunction and aberrant NMDAR activity in HD translates into an altered Ca^{2+} homeostasis. Resting Ca^{2+} levels are elevated in MSNs in animal models of HD (Hannson, 2001), and its levels upon NMDAR stimulation are increased in MSNs expressing mHtt (Zeron et al., 2004). Also, the probability of Ca^{2+} release from intracellular stores seems to be increased (Tang et al., 2003). As a consequence of the altered NMDAR function, the elevated Ca^{2+} intracellular concentration and the presence of mHtt, mitochondrial functionality is also affected. Expression of mHtt reduces the ability of mitochondria to re-establish baseline membrane potential (Oliveira et al., 2006), increasing the risk of apoptotic neuronal death (Schinder et al., 1996). Excessive Ca^{2+} concentration leads to a pathological activation of a conductance known as the mitochondrial permeability transition (Crompton, 1999; Dubinsky and Levi, 1998; White and Reynolds, 1996), which is associated with apoptotic neuronal death processes (Brustovetsky et al., 2002; Marchetti et al., 1996; Nicholls and Budd, 1998). Together these changes are responsible for the increased vulnerability of striatal MSNs to excitotoxic cell death.

ENDOCANNABINOID SYSTEM IN HD

As detailed earlier in this Introduction, CB_1 receptors are the most abundant GPCRs in the brain. They are highly expressed in the basal ganglia, mainly in striatal neurons, where they play a pivotal role in the control of motor behavior (Katona and Freund, 2008; Pazos et al., 2008). Moreover, they are located, for example, on terminals at the target nuclei of striatal projection

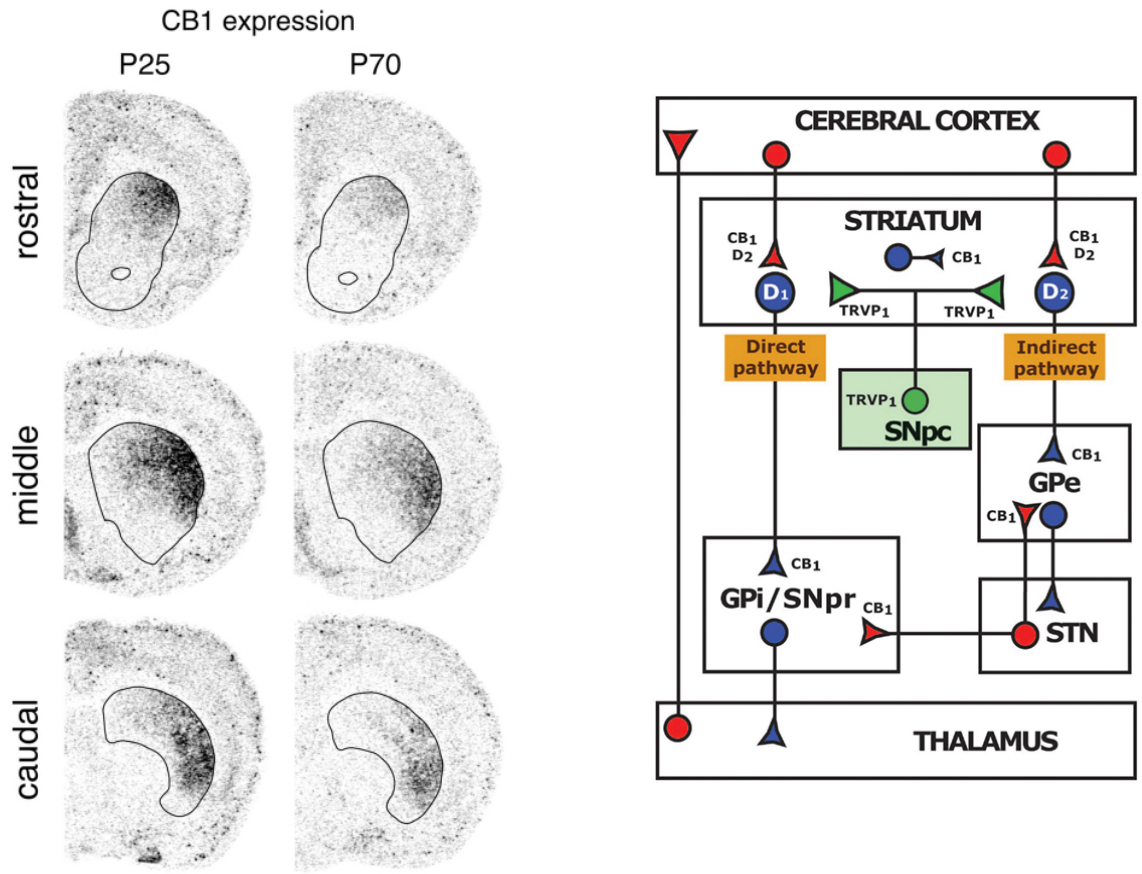

Figure 13. CB_1 cannabinoid receptor expression at basal ganglia.
A: Regional distribution of CB_1 receptor expression in the striatum, autoradiograms show distribution of CB_1 mRNA in coronal sections from the rostral, middle and caudal striatum in juvenile (P25) and adult (P70) rats (from Van Waes et al., 2012); *B:* Distribution of CB_1 receptor and its coexpression with dopaminergic D1 and D2 receptors in a simplified diagram of the basal ganglia circuits. GABAergic inhibitory pathways are represented in blue and glutamatergic excitatory pathways in red. Modulatory dopaminergic connections are indicated in green. GPe, external globus pallidus; GPi, internal globus pallidus; STN, subthalamic nucleus; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata (taken from Morera-Herreras et al., Front Pharmacol. 2012 ; Jun 12;3:110).

neurons, the substantia nigra pars reticulate and the globus pallidus (Herkenham et al., 1991; Herkenham et al., 1990), and on terminals of the neurons that project from the cortex to the striatum (Uchigashima et al., 2007) (*Figure 13*). This, together with the important global neuromodulatory and neuroprotective functions of the endocannabinoid system have suggested its implication in the pathophysiology and/or its validity as a therapeutic target in many of the disorders associated with these circuits, including Parkinson's and Huntington's disease, (Fernandez-Ruiz et al., 2011; Maccarrone et al., 2007; Pazos et al., 2008). In particular, a down-regulation of CB_1 receptor levels has been described as one of the most characteristic neurochemical alterations observed in the MSNs of both HD patients (Glass et al., 2000) and HD animal models (e.g. (Casteels et al., 2011; Denovan-Wright and Robertson, 2000; Lastres-Becker et al., 2002; McCaw et al., 2004)). This loss occurs at early stages of the disease, prior to the onset of the overt clinical symptoms, and is specific for the MSNs of the dorsal and medial striatum, although a recent report (Horne et al.,

2013) has shown that CB_1 receptor levels are also reduced in NPY/nNOS-expressing interneurons in basal ganglia. Furthermore, endocannabinoid levels were also markedly reduced in the brain of HD mouse models (Bari et al., 2013; Bisogno et al., 2008; Lastres-Becker et al., 2001), while CB_2 is up-regulated in the striata of HD individuals and HD-like mouse models (Palazuelos et al., 2009; Sagredo et al., 2009), consistent with marked microgliosis in this region.

The role of the early down-regulation of CB_1 receptor in the pathophysiology of HD still remains unclear. Several reports have endorsed a CB_1-mediated neuroprotection in HD mouse models (Lastres-Becker et al., 2004; Mievis et al., 2011; Pintor et al., 2006; Valdeolivas et al., 2012), while others have not (Dowie et al., 2010; Lastres-Becker et al., 2003). Despite the large amount of data available, the mechanisms responsible for the possible CB_1-mediated neuroprotection in HD are still unclear; likewise the molecular mechanisms responsible for the reduced expression of the receptor from presymtpomatic stages of the disease are unknown. These issues will be studied in this Doctoral Thesis.

AIMS

In the last two decades a very large amount of studies has demonstrated the involvement of the endocannabinoid system in a plethora of physiological processes that control the homeostatic regulation of the mammalian body. As explained in the Introduction, many of those studies have shown that the endocannabinoid system finely modulates the functioning of the central nervous system. Aside from this well-established neuromodulatory role, it has become evident that the endocannabinoid system participates in the control of neuronal survival and represents an endogenous system that confers cytoprotection against neuronal damage. However, despite the widely reported neuroprotective role of the CB_1 receptor, there is still a need to thoroughly understand the mechanisms underlying this process.

In this context, the global AIM of this THESIS is to investigate in further detail the neuroprotective activity of the CB_1 receptor and unravel its physiological relevance and therapeutic potential in neurodegeneration, with especial emphasis on the striatum and Huntington's disease.

This main objective can be divided into 3 specific aims:

1. To elucidate the molecular mechanism by which mutant huntingtin downregulates CB_1 receptor gene expression.

2. To clarify the possible neuroprotective role of the CB_1 receptor in the neurodegenerative processes that occur in Huntington's disease.

3.To assess the precise relevance of two differently-located CB_1 receptor pools, namely those situated on GABAergic neurons (medium-sized spiny neurons) or glutamatergic neurons (corticostriatal projection neurons), in Huntington's disease-associated excitotoxicity.

RESULTS AND DISCUSSION

Chapter 1

In this first chapter we will study the pathophysiological relevance of the CB_1 receptor in the context of a neurodegenerative disease (Huntington's disease). We will first focus on the consequences of CB_1 receptor genetic ablation on the Huntington's disease-like symptomatology, neuropathology and molecular pathology in a well-established model of the disease, namely the R6/2 mouse. We will subsequently analyze the effect of the pharmacological activation of CB_1 receptors in these mice, as well as the neuroprotective activity of the receptor in conditionally-immortalized murine striatal neuroblasts ($STHdh^{Q7/Q7}$, $STHdh^{Q7/Q111}$ and $STHdh^{Q111/Q111}$ cells). Lastly, we will try to elucidate the molecular mechanism by which mutant huntingtin downregulates CB_1 receptor gene expression.

Capítulo 1

En este primer capítulo se evaluará la relevancia fisiopatológica del receptor CB_1 en el contexto neurodegenerativo de la enfermedad de Huntington. Se estudiarán en primer lugar las consecuencias de la deleción genética del receptor en la sintomatología, la neuropatología y la patología molecular de ratones R6/2, que constituyen un modelo animal bien establecido de la enfermedad. A continuación, en este mismo modelo murino, se analizarán los efectos de la activación farmacológica del receptor CB_1. Además, se estudiará el papel neuroprotector de dicho receptor en neuroblastos estriatales murinos condicionalmente inmortalizados (células $STHdh^{Q7/Q7}$, $STHdh^{Q7/Q111}$ y $STHdh^{Q111/Q111}$). Por último, trataremos de desvelar el mecanismo molecular por el cual la huntingtina mutada inhibe la expresión del gen del receptor CB_1.

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Loss of striatal type 1 cannabinoid receptors is a key pathogenic factor in Huntington's disease

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Endocannabinoids act as neuromodulatory and neuroprotective cues by engaging type 1 cannabinoid receptors. These receptors are highly abundant in the basal ganglia and play a pivotal role in the control of motor behaviour. An early downregulation of type 1 cannabinoid receptors has been documented in the basal ganglia of patients with Huntington's disease and animal models. However, the pathophysiological impact of this loss of receptors in Huntington's disease is as yet unknown. Here, we generated a double-mutant mouse model that expresses human mutant huntingtin exon 1 in a type 1 cannabinoid receptor-null background, and found that receptor deletion aggravates the symptoms, neuropathology and molecular pathology of the disease. Moreover, pharmacological administration of the cannabinoid Δ^9-tetrahydrocannabinol to mice expressing human mutant huntingtin exon 1 exerted a therapeutic effect and ameliorated those parameters. Experiments conducted in striatal

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cells show that the mutant huntingtin-dependent downregulation of the receptors involves the control of the type 1 cannabinoid receptor gene promoter by repressor element 1 silencing transcription factor and sensitizes cells to excitotoxic damage. We also provide *in vitro* and *in vivo* evidence that supports type 1 cannabinoid receptor control of striatal brain-derived neurotrophic factor expression and the decrease in brain-derived neurotrophic factor levels concomitant with type 1 cannabinoid receptor loss, which may contribute significantly to striatal damage in Huntington's disease. Altogether, these results support the notion that downregulation of type 1 cannabinoid receptors is a key pathogenic event in Huntington's disease, and suggest that activation of these receptors in patients with Huntington's disease may attenuate disease progression.

Keywords: cannabinoid; receptor; Huntington's disease; neuroprotection; experimental therapeutics

Abbreviations: BDNF = brain-derived neurotrophic factor; CAT = chloramphenicol acetyltransferase; CB_1 = type 1 cannabinoid; FAAH = fatty acid amide hydrolase; GABA = gamma-aminobutyric acid; GAD67 = glutamic acid decarboxylase 67 KDa isoform; GFP = green fluorescent protein; NMDA = *N*-methyl-D-aspartate; PSD95 = post-synaptic density protein 95; RE1 = repressor element 1; REST = repressor element 1 silencing transcription factor; THC = Δ^9-tetrahydrocannabinol

Introduction

Endocannabinoids are a family of neural retrograde messengers that act by engaging type 1 cannabinoid (CB_1) receptors, the same receptors targeted by Δ^9-tetrahydrocannabinol (THC), the major active component of marijuana (Gaoni and Mechoulam, 1964; Piomelli, 2003). Endocannabinoid generation occurs by on-demand synthesis and cleavage of plasma membrane lipid precursors and is tightly controlled by neuronal activity. Endocannabinoid signalling serves as a major feedback mechanism to prevent excessive presynaptic activity, and thus tunes the functionality and plasticity of many synapses (Piomelli, 2003; Katona and Freund, 2008). In concert with this well-established neuromodulatory function, studies in various animal models support that CB_1 receptor activation promotes neuron survival upon acute brain injury and neuroinflammatory insults (Nagayama *et al.*, 1999; Panikashvili *et al.*, 2001; Parmentier-Batteur *et al.*, 2002; Marsicano *et al.*, 2003; Pryce *et al.*, 2003). This neuroprotective action of endocannabinoid signalling relies on the inhibition of excitotoxic glutamatergic neurotransmission as well as on other mechanisms, and is supported by the observation that the brain overproduces endocannabinoids upon damage (Mechoulam *et al.*, 2002; Marsicano *et al.*, 2003; Galve-Roperh *et al*., 2008).

CB_1 is the most abundant G protein-coupled receptor in the brain and, specifically, is very highly expressed in the neocortex, hippocampus, cerebellum and basal ganglia (Katona and Freund, 2008). In the latter, CB_1 receptors are mostly localized at synapses established by neurons containing gamma-aminobutyric acid (GABA; e.g. striatal projection neurons and some striatal interneuron subpopulations) and glutamate (e.g. corticostriatal and subthalamonigral neurons) as transmitters, and play a pivotal role in the inhibitory control of motor behaviour (Katona and Freund, 2008; Pazos *et al.*, 2008). Of possible clinical importance, alterations in CB_1 receptor expression have been reported in various pathologies affecting the basal ganglia (Maccarrone *et al.*, 2007; Pazos *et al.*, 2008). Specifically, a significant downregulation of CB_1 receptor binding and messenger RNA levels has been documented in the basal ganglia of patients (Glass *et al.*, 2000) and animal models (Denovan-Wright and Robertson, 2000; Lastres-Becker *et al.*, 2002; McCaw *et al.*, 2004) of Huntington's disease, a devastating neurodegenerative disorder that is primarily caused by a degeneration of medium-sized spiny striato-efferent GABAergic neurons and that is clinically characterized by a variety of movement disturbances, including chorea, dystonia and Parkinson's disease-like symptoms, as well as by cognitive and behavioural impairment (Walker, 2007). Of interest, CB_1 receptors are abundant in the great majority of medium-sized spiny neurons of the striatum (Marsicano and Lutz, 1999; Hohmann and Herkenham, 2000; Hermann *et al.*, 2002), but their loss in mutant huntingtin transgenic mice is brain region-specific, as it occurs in the lateral striatum and, to a lesser extent, in the medial striatum, but not in the cortex (Denovan-Wright and Robertson, 2000; McCaw *et al.*, 2004). Moreover, the downregulation of CB_1 receptor expression observed in patients with Huntington's disease and animal models seems to occur at early stages of the disease and prior to the appearance of overt clinical symptoms, neurodegeneration and changes in other neurochemical parameters (Maccarrone *et al.*, 2007; Pazos *et al.*, 2008). Although Huntington's disease has long been known to be caused by a single-gene mutation, specifically a CAG repeat expansion in exon 1 of the huntingtin gene that translates into an expanded polyglutamine tract in the N-terminal domain of the huntingtin protein (The Huntington's Disease Collaborative Research Consortium, 1993), the mechanisms by which mutant huntingtin produces the progressive degeneration of striatal neurons are extremely complex and as yet incompletely understood (Walker, 2007; Imarisio *et al.*, 2008). Hence, this work was undertaken to evaluate the potential contribution of the loss of CB_1 receptors to Huntington's disease pathogenesis and the molecular mechanism underlying this event.

Materials and methods

Animals

Hemizygous male mice transgenic for exon 1 of the human huntingtin gene with a greatly expanded CAG repeat (R6/2 mice) (Mangiarini *et al.*, 1996) and wild-type littermates were purchased from The Jackson Laboratory [Bar Harbor, ME; code

B6CBA-Tg(HDexon1)62Gpb/1J); 155–175 CAG repeats] or kindly provided by Gill Bates (King's College London School of Medicine, London, UK). The colony was maintained by back-crossing R6/2 males with (CBA × C57BL/6J) F1 females. Animals were housed and maintained in groups of mixed genotypes (Hockly *et al.*, 2003) with free access to food and water and on a 12-h light/dark cycle. From Week 10 of age, animals were provided with extra in-cage food and water. Due to welfare considerations based on the 3Rs (replacement, reduction and refinement) principle, animals were not allowed to die naturally (Olsson *et al.*, 2008). Instead, they were routinely sacrificed for brain samples for biochemical and histological analyses. Some experiments were conducted on hemizygous male R6/1 mice, which were maintained and handled as described (Canals *et al.*, 2004). Animal handling procedures were approved by Complutense University Animal Research Committee in accordance with Directive 86/609/EU of the European Commission.

To obtain double-mutant mice that express human mutant huntingtin exon 1 and are deficient in CB_1 cannabinoid receptors, we first cross-mated wild-type CBA female mice with $CB_1^{-/-}$ (C57BL/6J) male mice (Marsicano *et al.*, 2002). The $CB_1^{+/-}$ (CBA × C57BL/6J) F1 females were crossed with R6/2 males (Mangiarini *et al.*, 1996), and the resulting R6/2:$CB_1^{+/-}$ (CBA × C57BL/6J) F2 males were back-crossed with the aforementioned $CB_1^{+/-}$ F1 females to generate the $CB_1^{+/+}$, $CB_1^{-/-}$, R6/2:$CB_1^{+/+}$ and R6/2:$CB_1^{-/-}$ (CBA × C57BL/6J) animals. All experiments were performed with male littermates from this population to avoid strain and sex differences. These animals were not treated with any vehicle or drug. The uniformity of the CBA × C57BL/6J background in our mouse colony was routinely assessed by Illumina Bead Scanner-based profiling of 256 representative single-nucleotide polymorphisms of the CBA and the C57BL/6J backgrounds. Analyses were conducted at Centro Nacional de Genotipado (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain).

Cannabinoid administration to animals

On the basis of their basal RotaRod performance and body weight, wild-type and R6/2 mice were matched into the different treatment groups and injected daily (intraperitoneally) with vehicle [1% (v/v) dimethyl sulphoxide in 100 µl Tween/saline (1:18, v/v)] or THC (The Health Concept, Richelbach, Germany) at 2 mg/kg body weight per day. Behaviour tests were conducted prior to injections.

Behaviour analyses

Motor coordination (RotaRod) analysis was conducted with acceleration from 4 to 40 r.p.m. over a period of 570 s in an LE8200 device (Harvard Apparatus, Barcelona, Spain). Any mice remaining on the apparatus after 600 s were removed and their time scored as 600 s. For basal RotaRod performance, mice were tested on four consecutive days, for three trials per day with a rest period of ~30 min between trials. At each successive age analysed, mice were tested on three consecutive days, for three trials per day with a rest period of ~30 min between trials. Data from the three trials per day were averaged for each animal, and the mean value of each day averaged for each animal. Data from the first day (or the first 2 days in the basal test) were not used in statistical analyses.

Motor activity and exploration analyses were conducted in an automated actimeter (ActiTrack; Panlab, Barcelona, Spain). This consisted of a 22.5 × 22.5-cm area with 16 surrounding infrared beams coupled to a computerized control unit. Activity was recorded for a period of 10 min, and total distance travelled, resting time and movements >5 cm/s were recorded for each animal.

Limb-clasping analysis was conducted in animals that were tail-suspended and video-recorded for 45 s. We evaluated total clasping time (in at least one limb) of each animal.

Magnetic resonance imaging

Striatal volume was calculated by magnetic resonance imaging. 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. Mice were anaesthetized with oxygen:isofluorane and subsequently placed in prone position inside a cradle. The animal's 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, saggital and coronal directions were acquired (time to repetition/echo time = 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: time to repetition = 3000 ms, effective echo time = 86.5 ms, number of averages = 2, field of view = 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.

Real-time quantitative polymerase chain reaction

RNA was isolated using Trizol Reagent or RNeasy (Invitrogen, Carlsbad, CA). Complementary DNA was obtained with Transcriptor (Roche, Basel, Switzerland). Real-time quantitative polymerase chain reaction assays were performed using the FastStart Master Mix with Rox (Roche) and probes were obtained from the Universal Probe Library Set (Roche). Amplifications were run in a 7900 HT-Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Each value was adjusted to β-actin levels as reference. Relative gene expression data were determined by the $2^{-\Delta\Delta Ct}$ method. The 18S RNA levels were routinely used as an additional control to further validate the data. Probes and primers used are shown in Supplementary Table 1.

Microscopy analyses

Cells were cultured on coverslips and fixed in 4% paraformaldehyde. Coronal free-floating sections were obtained from paraformaldehyde-perfused mouse brains (Aguado *et al.*, 2006). Samples were incubated with anti-CB_1 receptor [raised against a glutathione *S*-transferase fusion protein containing the first 77 residues of the CB_1 receptor (Twitchell *et al.*, 1997); 1:500; kindly provided by Ken Mackie, Indiana University, Bloomington, IN, USA], anti-brain-derived neurotrophic factor (BDNF; 1:500; generated at Michael Sendtner's laboratory, University of Würzburg, Germany), anti-glutamic acid decarboxylase 67 KDa isoform (GAD67; 1:250; Chemicon, Temecula, CA; cat. no. MAB5406), anti-synaptophysin (1:250; Synaptic Systems; cat. no. 101 002), anti-post-synaptic density protein 95 (PSD95; 1:1000, Abcam, Cambridge, UK; cat. no. ab2723) or anti-NeuN (1:400; Chemicon; cat. no. MAB377) antibodies, followed by staining with the corresponding highly cross-adsorbed Alexa Fluor 488, 594 or 647 antibodies (1:500; Molecular Probes, Leyden, The Netherlands).

After washing, samples were incubated with Hoescht 33342 (1:2000; Invitrogen) to stain cell nuclei and subsequently mounted in Mowiol solution. Immunofluorescence images of cells were obtained with an Axioplan 2 microscope (Carl-Zeiss, Oberkochen, Germany). Confocal fluorescence images were acquired using TCS-SP2 software and a SP2 AOBS microscope (Leica, Wetzlar, Germany). Pixel quantification and co-localization were analysed with Metamorph-Offline software (Universal Imaging, Downingtown, PA).

For quantification of huntingtin aggregates, 30 μm coronal sections were pre-treated with 1% bovine serum albumin, 5% foetal bovine serum and 0.2% Triton X-100, and then incubated with anti-human huntingtin antibody (1:500; Chemicon; cat. no. MAB5374). Samples were subsequently incubated in avidin–biotin complex using the mouse Elite Vectastain kit (Vector Laboratories) and chromogen reactions were performed with 0.05% diaminobenzidine (Sigma-Aldrich, St. Louis, MO) and 0.01% H_2O_2. Sections were mounted with Mowiol, analysed in an Olympus BX-41 microscope (Barcelona, Spain) with a CCD ColorView IIIu camera and quantified using Metamorph-Offline software. Specifically, counting of huntingtin inclusions was conducted in the caudate-putamen area of both hemispheres in a 1-in-10 series per animal, ranging from bregma +1.18 mm to −0.46 mm coronal coordinates. Sections were analysed at a magnification of ×40 and spots sized 5–2000 pixels were recorded. Data are presented as number of huntingtin aggregates relative to the control animal group.

Western blot

Western blot analysis was conducted with antibodies against CB_1 receptors (see characteristics of the antibody above; 1:1000), fatty acid amide hydrolase (FAAH; 1:1000; Chemicon; cat. no. AB5644P) or α-tubulin (1:4000; Sigma-Aldrich; cat. no. T9026) following standard procedures. Specifically, samples were lysed in a buffer containing 50 mM Tris, 0.1% Triton X-100, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 50 mM NaF, 10 mM sodium β-glicerophosphate, 5 mM sodium pyrophosphate and 1 mM sodium orthovanadate (pH 7.5) supplemented with a protease inhibitor cocktail (Roche; cat. no. 11697498001), 0.1 mM phenylmethanesulphonylfluoride, 0.1% β-mercaptoethanol and 1 μM microcystin. The running buffer consisted of 200 mM glycine, 25 mM Tris and 0.1% sodium dodecyl sulphate (pH 8.3), and the transfer buffer contained 200 mM glycine, 25 mM Tris and 20% methanol (pH 8.3). Blots were incubated with Tris-buffered saline (20 mM Tris and 0.5 mM NaCl, pH 7.5)/Tween-20 (0.1%) supplemented with 1% bovine serum albumin. Densitometric analysis was performed with Quantity One software (Bio-Rad, Hercules, CA).

Cell and slice culture

Conditionally immortalized striatal neuroblasts obtained from wild-type mice ($STHdh^{Q7/Q7}$ cells) or knock-in mice expressing one copy ($STHdh^{Q7/Q111}$ cells) or two copies ($STHdh^{Q111/Q111}$ cells) of a mutant huntingtin allele, thus expressing endogenous levels of full-length huntingtin with only seven glutamines, 7 and 111 glutamines or only 111 glutamines in the protein N-terminal domain, respectively, were used (Trettel *et al.*, 2000). Cell infection with a defective retrovirus transducing the temperature-sensitive A58/U19 large T antigen, selection of geneticin-resistant colonies at the permissive temperature of 33°C and analysis of colonies by immunostaining has been previously described (Trettel *et al.*, 2000; Paoletti *et al.*, 2008). Cells were grown at 33°C in Dulbecco's modified eagle's medium supplemented with 10% foetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine and 400 μg/ml geneticin (Paoletti *et al.*, 2008).

Adult striatal slices were obtained from wild-type and R6/2 mice. Brains were dissected and cut coronally with a vibratome. Slices (300 μm thick) were cultivated for 20 h in semidry conditions in wells containing Neurobasal medium supplemented with B27, N2 and 2.5 mM L-glutamine.

Cell viability

Cells were transferred to serum-free Dulbecco's modified eagle's medium for 24 h and incubated for a further 5 h in Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 5 mM Hepes, 20 mM glucose and 10 μM glycine) supplemented or not with *N*-methyl-D-aspartate (NMDA) and cannabinoid receptor agonists (THC, HU-210, WIN-55,212-2), the CB_1 cannabinoid receptor antagonist SR141716 (kindly provided by Sanofi-Aventis, Montpellier, France) or the respective vehicle [dimethyl sulphoxide, 0.1–0.2% (v/v) final concentration]. The medium was subsequently replaced by NMDA/serum-free Dulbecco's modified eagle's medium and cell viability was determined after 24 h by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide test.

Cell transfection

Cells were transfected transiently with constructs expressing human wild-type huntingtin exon 1 with 17 glutamines fused to green fluorescent protein (GFP) (17Q-GFP), human mutant huntingtin exon 1 with 72 glutamines fused to GFP (72Q-GFP) (kindly provided by Montserrat Arrasate and Steven Finkbeiner, The Gladstone Institute of Neurological Disease, San Francisco, CA, USA), human full-length wild-type huntingtin with 17 glutamines (17Q-FL), human full-length mutant huntingtin with 75 glutamines (75Q-FL) (kindly provided by Frédéric Saudou, Institut Curie, Orsay, France), mouse pcDNA3-CB_1 cannabinoid receptor complementary DNA (generated at Beat Lutz's laboratory, Johannes Gutenberg University Mainz, Germany) or with their respective empty vectors, using Lipofectamine 2000 (Invitrogen). In other experiments, cells were transfected with small interfering RNA duplexes corresponding to mouse huntingtin (5′-GAACGUACCCAG UUUGAAA-3′) or a non-targeted control (5′-UGGUUUACAUGUC GACUAA-3′) using the DharmaFECT 1 transfection reagent (Dharmacon, Lafayette, CO), and/or with double-stranded repressor element 1 (RE1) decoy oligonucleotides (5′-GCCCCGAGGGCGGAGG ACAGGTG-3′) or a non-targeted control (5′-CTCCGAACGTGTCACGT CTCGAAT-3′) using Lipofectamine 2000.

CB_1 cannabinoid receptor gene promoter activity

Cells were transfected transiently with the aforementioned huntingtin-expressing plasmids together with a construct encoding the −3016 to +142 sequence (referring to the first nucleotide of exon 1) of the human CB_1 receptor gene promoter fused to the chloramphenicol acetyltransferase (CAT) reporter gene (phCB_1-3016-CAT) (Borner *et al.*, 2008). All reporter gene constructs were based on the pBLCAT2/pBLCAT3 system, in which the thymidine kinase minimal promoter was replaced for the human CB_1 receptor promoter upstream of *CAT*. The 5′ deletion constructs of this plasmid were generated either by site-specific restriction enzyme deletion (−2420: AfeI; −1880: SpeI; −1583: EagI; −648: SphI) or by a deletion strategy using the sequence-unspecific enzyme Bal31 (−1099; −898; −559; −223).

The reporter plasmids phCB$_1$-962/-934-tk-CAT and pRE1-tk-CAT were constructed by ligation of double-stranded oligonucleotides (Metabion, Martinsried, Germany) encoding the −962/−934 fragment of the human CB_1 receptor promoter or a consensus RE1 site, respectively, into the BamHI site of pBLCAT2 upstream of the herpes simplex thymidine kinase promoter. The sequences (sense strands) used were 5′-GATCCGCCCCGAGGGCGGAGGACAGGTG GCCGC-3′ for the −962/−934 plasmid, and 5′- GATCCTTCAG CGCCACGGACAGCGCC-3′ for the RE1 plasmid. The correct insertion of the sequences and the deletions of all the plasmids were verified by DNA sequencing.

Human samples

Human caudate-putamen samples were obtained from patients with Huntington's disease or without neurological disease (controls) according to the standardized procedures of the Banco de Tejidos para Investigación Neurológica (Madrid, Spain). Briefly, both the patients with Huntington's disease and the control subjects, according to the Declaration of Helsinki, had signed during their life a donation protocol that was in custody of their relatives and the brain bank. After death, the corpses were immediately stored at 4°C until autopsy, which was performed within a time interval ranging from 2 to 12 h post mortem. After removal of the brain, the quality of the samples was checked by their pH. The brain was split in two parts by a saggital section through the midline: the right hemibrain was used for histopathological studies and the left hemibrain for Western blot and other biochemical analyses. Both hemibrains were dissected in coronal sections (~1 cm thick) to evaluate the presence of additional lesions, such as cerebral infarctions. The right hemibrain was immersed in formalin, and the slices of the left hemibrain were frozen in a metal plate cooled at −80°C. The frozen samples were stored at −80°C in freezers with continuous recording of temperature and a double temperature control (liquid CO_2-backup connection and alarm telephone). All protocols were approved by the institutional ethics committee.

Statistical analyses

Data are presented as mean ± SEM. Statistical comparisons were made by ANOVA with *post hoc* Student–Neuman–Keuls test or by unpaired Student's *t*-test, as appropriate.

Results

Genetic deletion of CB_1 cannabinoid receptors aggravates Huntington's disease-like symptomatology, neuropathology and molecular pathology in R6/2 mice

To evaluate the pathophysiological relevance of CB_1 receptor loss in Huntington's disease, we first generated double-mutant mice expressing human mutant huntingtin exon 1 [R6/2 mice, which recapitulate the Huntington's disease-associated decrease of striatal CB_1 receptors (Denovan-Wright and Robertson, 2000; McCaw *et al.*, 2004)] in a CB_1 receptor-null background. These R6/2:$CB_1^{-/-}$ mice showed a significant motor-coordination impairment phenotype—as assessed by RotaRod performance—at Week 4, an age at which R6/2:$CB_1^{+/+}$ animals are overtly normal (Fig. 1A). Moreover, the subsequent decline in motor coordination evidenced by R6/2:$CB_1^{+/+}$ mice was exacerbated in R6/2:$CB_1^{-/-}$ littermates (Fig. 1A). CB_1 receptor genetic ablation in R6/2 mice induced the appearance of other phenotypic alterations such as impairment of general motor and exploratory behaviour (decreased ambulation, activity and speed; Fig. 1B) and limb clasping (Fig. 1C). Moreover, striatal atrophy, as determined by MRI (Fig. 1D), and accumulation of huntingtin aggregates (Fig. 1E), two hallmarks of Huntington's disease neuropathology, were exacerbated upon CB_1 receptor deletion in R6/2 mice. Body weight from Week 4 to Week 10 was not significantly different in wild-type, $CB_1^{-/-}$, R6/2:$CB_1^{+/+}$ and R6/2:$CB_1^{-/-}$ mice (n = 20–30 animals per group; data not shown), indicating that CB_1 receptor ablation does not affect the general health status of the animals.

We next evaluated the expression of various molecular markers of neuronal integrity in the double-mutant mice. A remarkable decrease in striatal messenger RNA levels and immunoreactivity of the GABAergic neuron marker GAD67 was evident in R6/2:$CB_1^{-/-}$ mice (Fig. 2A). Likewise, the expression of the pre-synaptic marker synaptophysin (Fig. 2B) and the post-synaptic marker PSD95 (Fig. 2C) was reduced in the striata of R6/2:$CB_1^{-/-}$ mice when compared with R6/2:$CB_1^{+/+}$ littermates.

Pharmacological activation of CB_1 cannabinoid receptors ameliorates Huntington's disease-like symptomatology, neuropathology and molecular pathology in R6/2 mice

The worsening of the Huntington's disease-like phenotype shown by R6/2 mice upon genetic loss of CB_1 receptors suggests that pharmacological activation of CB_1 receptors could have a therapeutic impact on disease progression. To address this issue we treated R6/2 mice and wild-type littermates with vehicle or THC starting at Week 4 of life and found that cannabinoid treatment attenuated the motor coordination deficits of R6/2 mice, as evaluated in the RotaRod test (Fig. 3A). THC administration also ameliorated the impairment of motor and exploratory behaviour (Fig. 3B) and the limb clasping (Fig. 3C) that appeared in R6/2 mice at later stages of the disease—Weeks 8–10. Striatal atrophy (Fig. 3D) and huntingtin aggregate accumulation (Fig. 3E) were also attenuated by THC delivery to R6/2 mice. THC treatment did not significantly affect body weight from Week 4 to Week 10 in wild-type or R6/2 mice (n = 20–30 animals per group; data not shown).

As CB_1 receptor deficiency downregulated the expression of molecular markers of neuronal integrity in R6/2 mice, we reasoned that pharmacological receptor activation would have the opposite effect, thereby improving the molecular pathology profile of the animals. Thus, THC administration was able to normalize the decline of GAD67 (Fig. 4A), synaptophysin (Fig. 4B) and PSD95 (Fig. 4C) expression observed in vehicle-treated R6/2 mice.

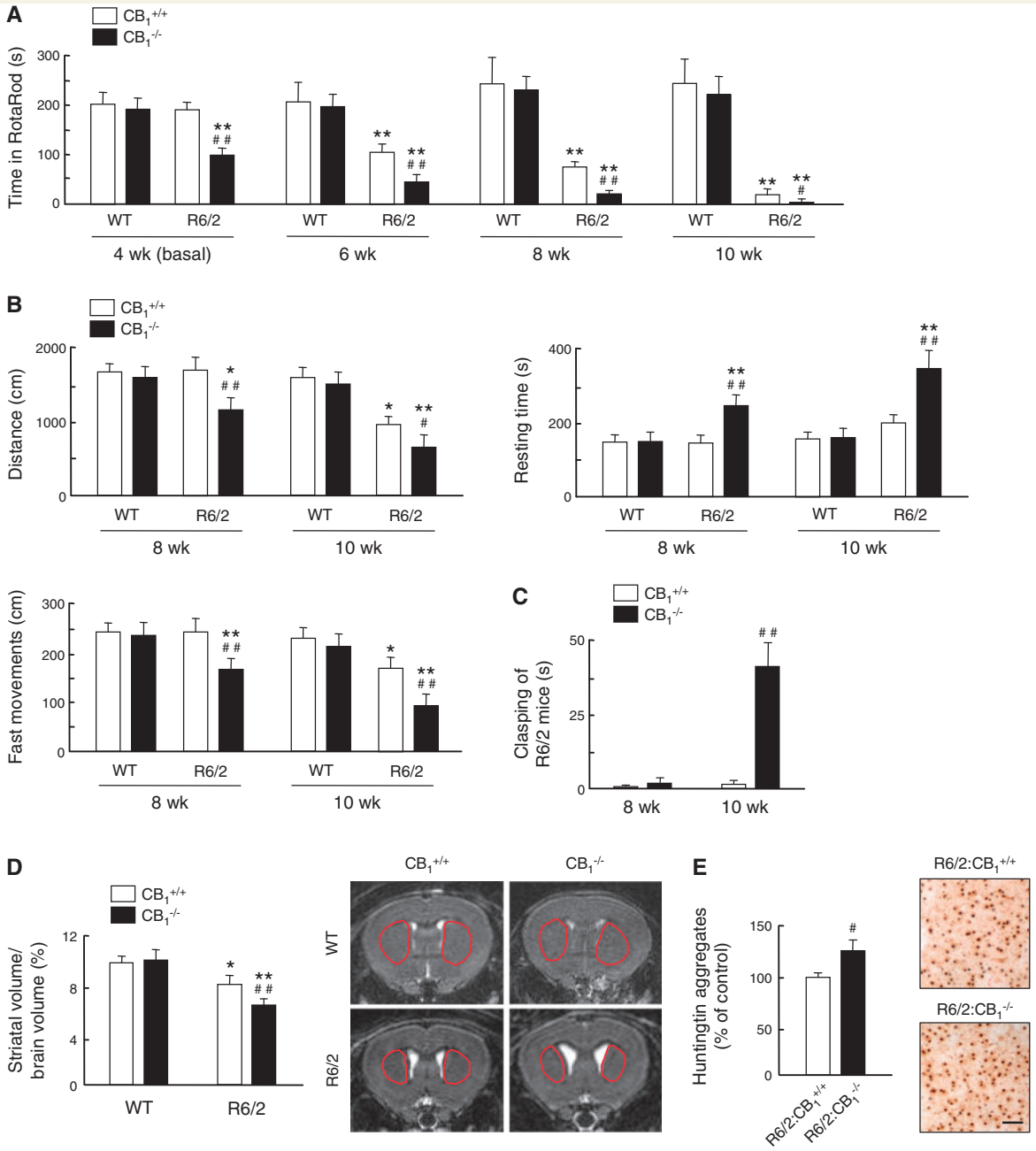

Figure 1 Genetic deletion of CB_1 cannabinoid receptors aggravates Huntington's disease-like symptomatology and neuropathology in R6/2 mice. (**A**) RotaRod performance of $CB_1^{+/+}$, $CB_1^{-/-}$ (WT), R6/2:$CB_1^{+/+}$ and R6/2:$CB_1^{-/-}$ (R6/2) mice at the indicated ages ($n = 10$–12 animals per group). (**B**) Motor activity at Weeks 8 and 10 as determined by total distance, resting time, and fast movements ($n = 14$–18 animals per group). (**C**) Clasping of R6/2 mice at Weeks 8 and 10 ($n = 16$–20 animals per group). (Clasping was not observed in wild-type littermates at those ages.) (**D**) Striatal volume relative to total brain volume at Week 8 ($n = 10$–12 animals per group). (**E**) Huntingtin aggregates in the striatum at Week 8 ($n = 8$–10 animals per group). (Aggregates were not detected in wild-type littermates at that age.) In all panels $^{*}P < 0.05$, $^{**}P < 0.01$ from the corresponding wild-type group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ from the corresponding $CB_1^{+/+}$ group. Representative images are shown in panels **D** and **E** (scale bar 50 μm).

CB_1 cannabinoid receptors protect striatal cells from excitotoxic damage

We next conducted a series of experiments aimed at unravelling the mechanism and consequences of the mutant huntingtin-evoked loss of CB_1 receptors in striatal cells. To address this question we first made use of striatal neuroblasts obtained from wild-type mice ($STHdh^{Q7/Q7}$ cells) and their mutant huntingtin knock-in counterparts ($STHdh^{Q111/Q111}$ cells), which express endogenous levels of huntingtin with 7 and 111 glutamines in the protein N-terminal domain, respectively. We exposed these cells to the ionotropic glutamate receptor agonist NMDA and

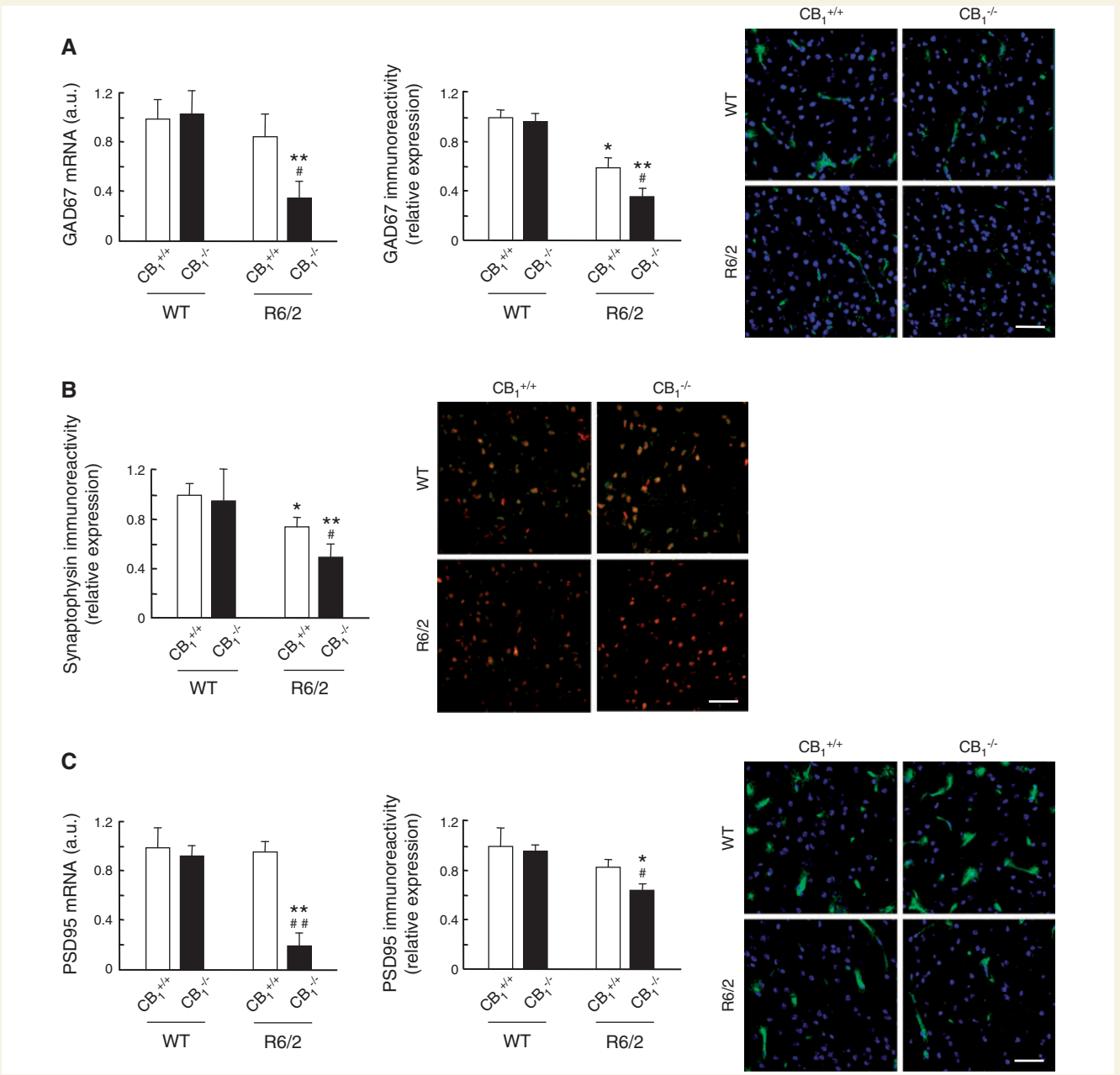

Figure 2 Genetic deletion of CB_1 cannabinoid receptors aggravates Huntington's disease-like molecular pathology in R6/2 mice. (**A**) Striatal GAD67 mRNA levels and immunoreactivity [given as relative values of $GAD67^+$ area (in green)/total cell number (nuclei in blue)]. (**B**) Striatal synaptophysin immunoreactivity [given as relative values of synaptophysin$^+$ intensity (in red)/$NeuN^+$ area (in green)]. (Striatal synaptophysin messenger RNA levels were not significantly different in wild-type, $CB_1^{-/-}$ (WT), R6/2:$CB_1^{+/+}$ and R6/2:$CB_1^{-/-}$ (R6/) mice; data not shown.) (**C**) Striatal PSD95 messenger RNA levels and immunoreactivity [given as relative values of $PSD95^+$ area (in green)/total cell number (nuclei in blue)]. In all panels samples were taken at Week 8 of life ($n = 6$–8 animals per group; $^*P < 0.05$, $^{**}P < 0.01$ from the corresponding wild-type group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ from the corresponding $CB_1^{+/+}$ group). Representative confocal microscopy images are shown. Scale bar 50 μm.

found that THC rescued $STHdh^{Q7/Q7}$ cells from death. In contrast, $STHdh^{Q111/Q111}$ cells, which express significantly lower levels of CB_1 receptors than do $STHdh^{Q7/Q7}$ cells (see below), showed an enhanced basal sensitivity to death and an impaired THC-mediated protective response (Fig. 5A). A pivotal role for CB_1 receptors in promoting cell survival was supported by the observation that THC-induced protection of $STHdh^{Q7/Q7}$ cells was mimicked by the synthetic cannabinoid agonists HU-210 and WIN-55,212-2 (data not shown) and was prevented by the CB_1 receptor-selective antagonist SR141716 (Fig. 5A). Moreover, ectopic expression of CB_1 receptors in $STHdh^{Q111/Q111}$ cells decreased their basal sensitivity to NMDA-induced death and

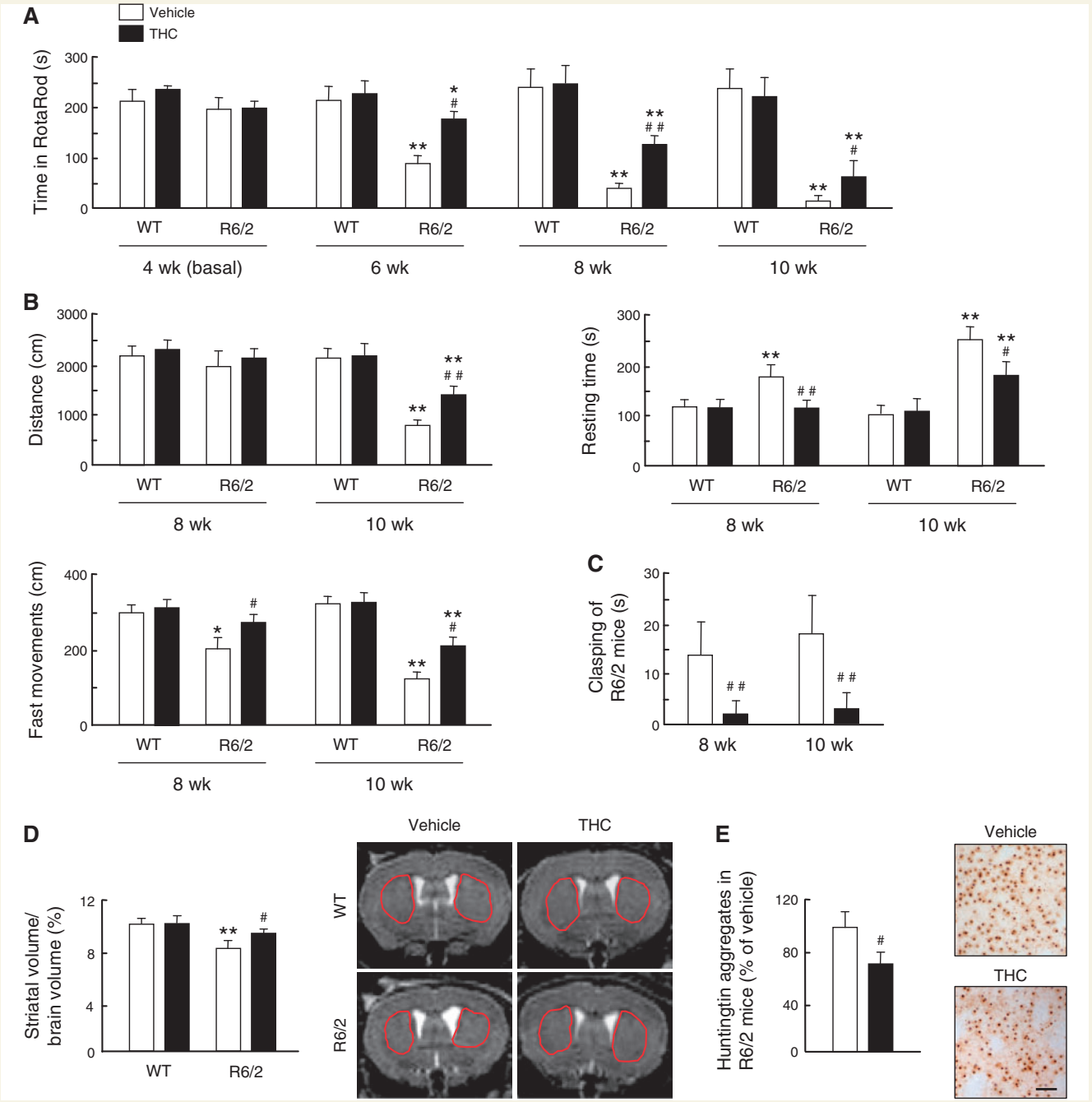

Figure 3 Pharmacological activation of CB_1 cannabinoid receptors ameliorates Huntington's disease-like symptomatology and neuropathology in R6/2 mice. R6/2 mice and wild-type (WT) littermates were treated daily with vehicle (white bars) or THC (2 mg/kg body weight per day; black bars) from Week 4. (**A**) RotaRod performance at the indicated ages ($n = 10$–14 animals per group). (**B**) Motor activity at Weeks 8 and 10 as determined by total distance, resting time and fast movements ($n = 14$–18 animals per group). (**C**) Clasping of R6/2 mice at Weeks 8 and 10 ($n = 16$–20 animals per group). (Clasping was not observed in wild-type littermates at those ages.) (**D**) Striatal volume relative to total brain volume at Week 8 ($n = 10$–12 animals per group). (**E**) Huntingtin aggregates in the striatum at Week 8 ($n = 8$–10 animals per group). (Aggregates were not detected in wild-type littermates at that age.) In all panels, $^{*}P < 0.05$, $^{**}P < 0.01$ from the corresponding wild-type group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ from the corresponding vehicle-treated group. Representative images are shown in panels **D** and **E** (scale bar 50 μm).

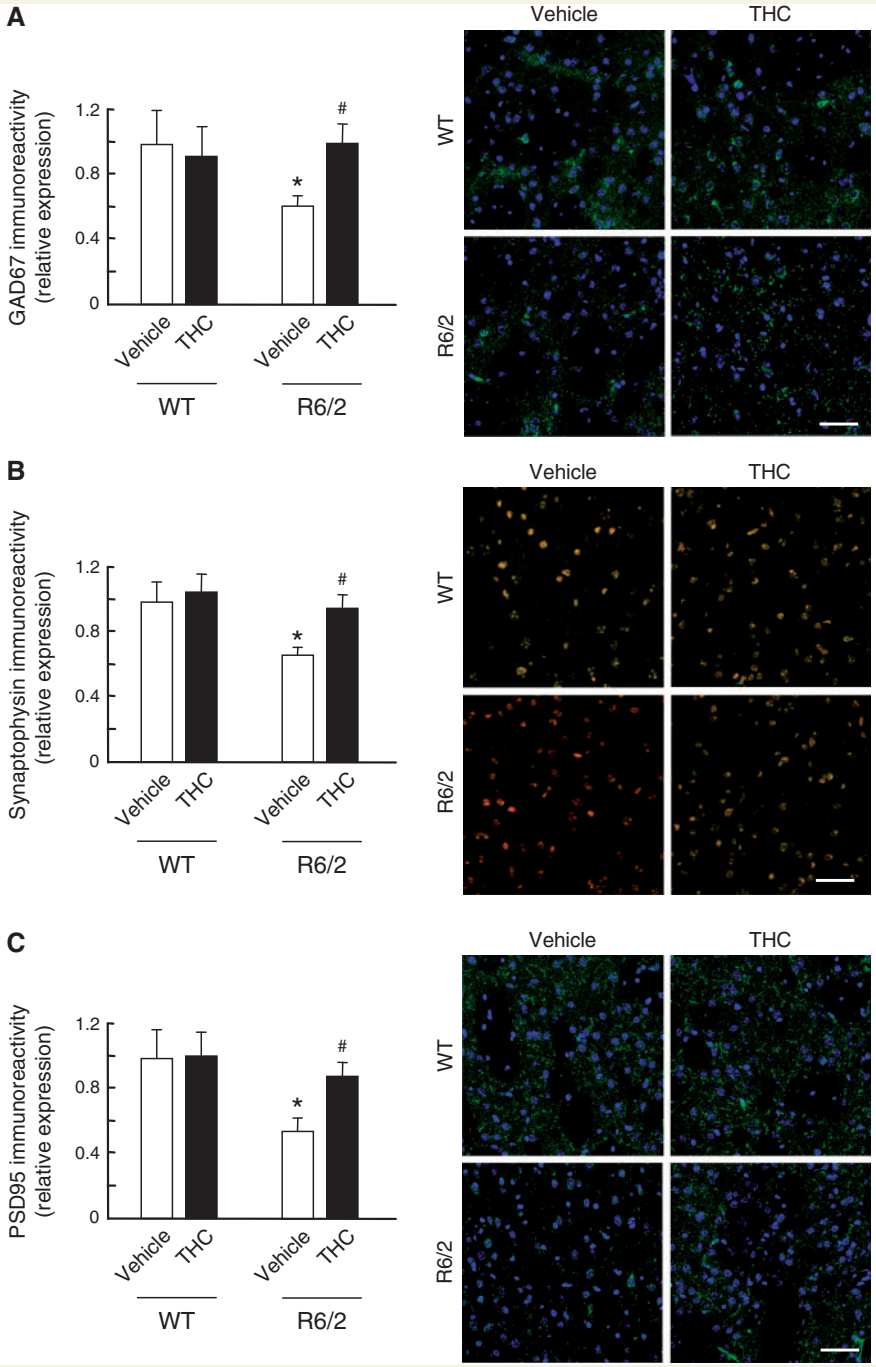

Figure 4 Pharmacological activation of CB_1 cannabinoid receptors ameliorates Huntington's disease-like molecular pathology in R6/2 mice. R6/2 mice and wild-type (WT) littermates were treated daily with vehicle (white bars) or THC (2 mg/kg body weight per day; black bars) from Week 4 of life. (**A**) Striatal GAD67 immunoreactivity [given as relative values of $GAD67^+$ area (in green)/total cell number (nuclei in blue)]. (**B**) Striatal synaptophysin immunoreactivity [given as relative values of $synaptophysin^+$ intensity (in red)/$NeuN^+$ area (in green)]. (**C**) Striatal PSD95 immunoreactivity [given as relative values of $PSD95^+$ intensity (in green)/total cell number (nuclei in blue)]. (Striatal GAD67, synaptophysin and PSD95 messenger RNA levels were not significantly different in wild-type or R6/2 mice treated with vehicle or THC; data not shown.) In all panels samples were taken at Week 8 of life ($n = 6$–8 animals per group; $^*P < 0.05$, $^{**}P < 0.01$ from the corresponding wild-type group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ from the corresponding vehicle-treated group). Representative confocal microscopy images are shown. Scale bar 50 μm.

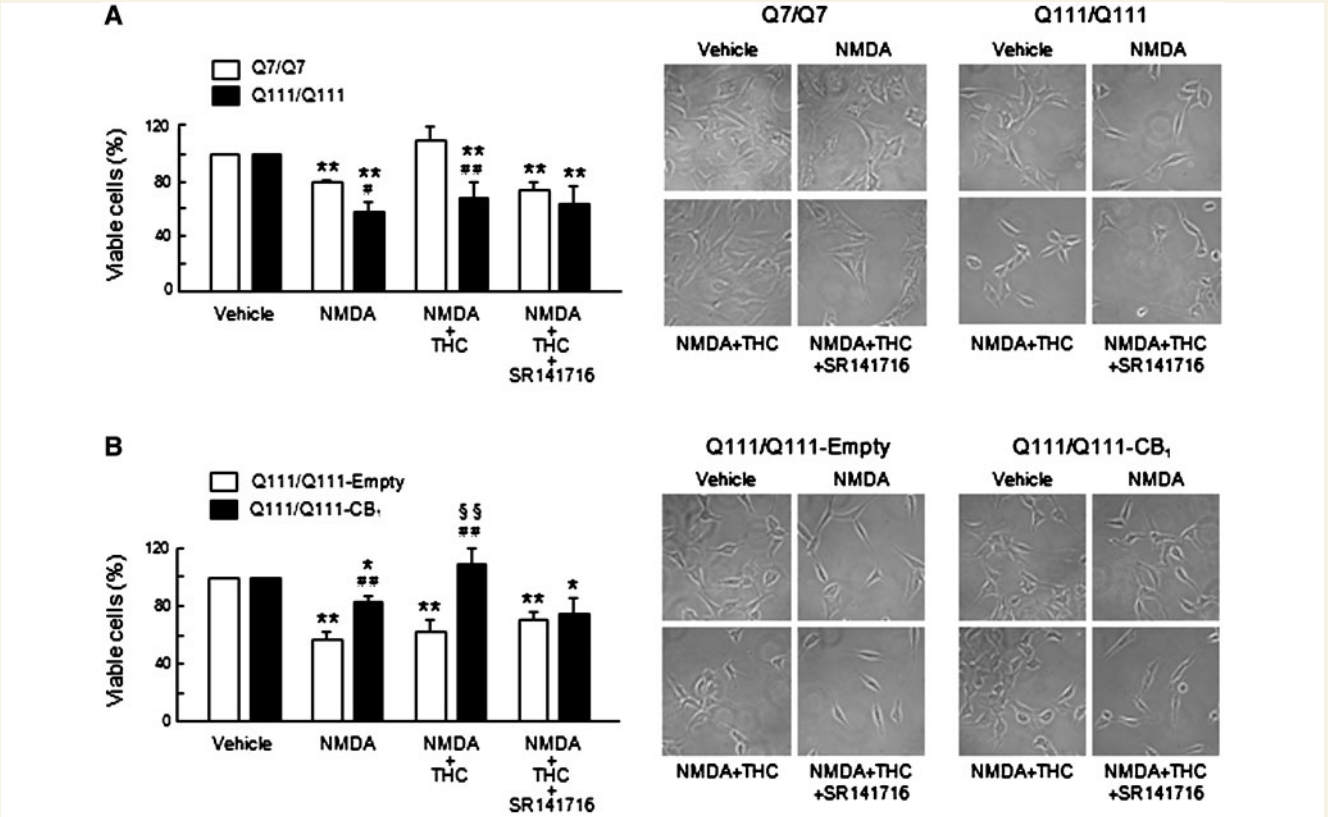

Figure 5 CB_1 cannabinoid receptors protect striatal cells from excitotoxic damage. (**A**) $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells were preincubated for 5 h in Locke's solution with or without 1 mM NMDA together with vehicle, 0.5 μM THC and/or 0.25 μM SR141716, and subsequently incubated for 24 h in NMDA-free medium. Relative numbers of viable cells are shown ($n = 6$ experiments; $^{**}P < 0.01$ from the corresponding vehicle-treated cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ from the corresponding $STHdh^{Q7/Q7}$ cells). (THC alone or SR141716 alone did not exert any significant effect on $STHdh^{Q7/Q7}$ or $STHdh^{Q111/Q111}$ cell viability; data not shown.) (**B**) $STHdh^{Q111/Q111}$ cells were transfected with a mouse CB_1 receptor-expressing vector or with empty vector and subsequently treated with or without NMDA, THC and/or SR141716 as above ($n = 6$ experiments; $^{*}P < 0.05$, $^{**}P < 0.01$ from the corresponding vehicle-treated cells; $^{\#\#}P < 0.01$ from the corresponding empty vector-transfected cells; $^{\S\S}P < 0.01$ from NMDA alone or from NMDA + THC + SR141716). (THC alone or SR141716 alone did not exert any significant effect on $STHdh^{Q111/Q111}$-Empty or $STHdh^{Q111/Q111}$-CB_1 cell viability; data not shown.) Representative micrographs of the different experimental conditions are shown.

rendered them as responsive as $STHdh^{Q7/Q7}$ cells to cannabinoid-mediated protection (Fig. 5B).

Transient regulation of huntingtin expression controls CB_1 cannabinoid receptor gene promoter activity via repressor element 1 silencing transcription factor

To study how mutant huntingtin affects CB_1 receptor expression, we transfected wild-type mouse striatal neuroblasts ($STHdh^{Q7/Q7}$ cells) with constructs expressing human wild-type huntingtin exon 1 with 17 glutamines fused to GFP or human mutant huntingtin exon 1 with 72 glutamines fused to GFP, or with constructs expressing human full-length wild-type huntingtin with 17 glutamines or human full-length mutant huntingtin with 75 glutamines, together with a construct that encodes a 3 kb human CB_1 receptor promoter fused to the CAT reporter gene. Promoter activity was enhanced by wild-type huntingtin exon 1 (Fig. 6A, left panel) and full-length wild-type huntingtin (Fig. 6B, left panel), but was not affected by their respective mutant huntingtin counterparts. This indicates that, although mutant huntingtin usually dysregulates gene transcription by gain-of-function mechanisms (Walker, 2007; Imarisio *et al.*, 2008), the huntingtin mutation in our system is—at least in part—associated with a loss-of-function process. To further support this notion, we knocked-down endogenous huntingtin with a huntingtin-directed small interfering RNA (which diminished huntingtin messenger RNA levels to 30 ± 3% of control small interfering RNA-transfected cells; $n = 4$ experiments, $P < 0.01$) and found that CB_1 receptor promoter activity decreased to 68 ± 9% of control small interfering RNA-transfected cells (Fig. 6C, left panel).

We next aimed to characterize promoter regions involved in the control of CB_1 receptor gene transcription. Cells were thus transfected with reporter constructs containing sequential 5′ deletions of the receptor promoter (Fig. 6D). An increase in reporter activity

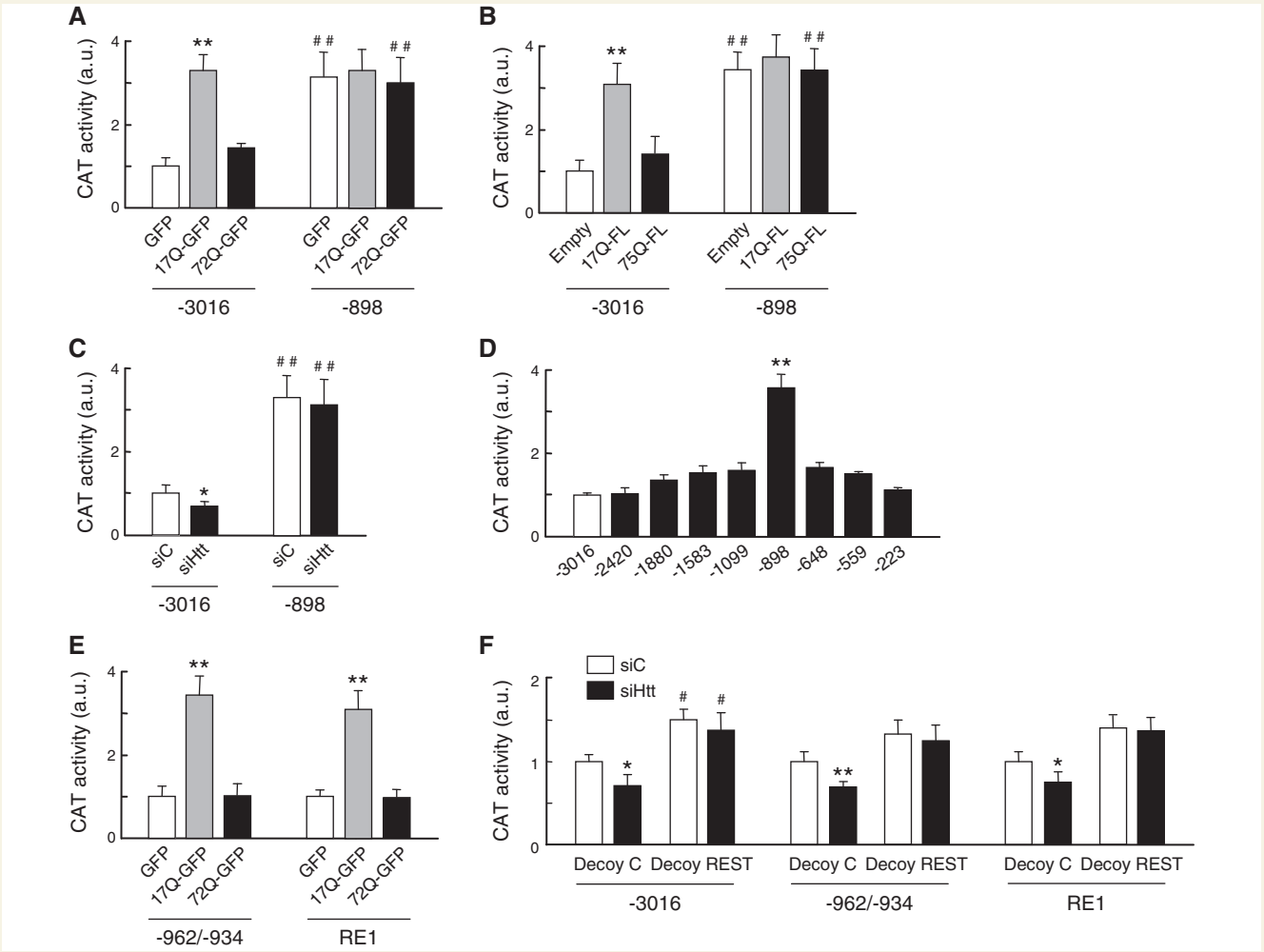

Figure 6 Transient regulation of huntingtin expression controls CB_1 cannabinoid receptor gene promoter activity via repressor element 1 silencing transcription factor. (**A** and **B**) CB_1 receptor promoter activity in STHdh$^{Q7/Q7}$ cells transfected with GFP, 17Q-GFP or 72Q-GFP (**A**), or with empty vector, 17Q-FL or 75Q-FL (**B**) and CAT reporter constructs encoding a 3016- or an 898-bp human CB_1 receptor promoter ($n = 4$ experiments; $^{**}P < 0.01$ from empty construct; $^{\#\#}P < 0.01$ from the corresponding −3016 construct). (**C**) CB_1 receptor promoter activity in STHdh$^{Q7/Q7}$ cells transfected with control small interfering RNA (siC) or huntingtin-directed small interfering RNA (siHtt) and the aforementioned reporter constructs ($n = 4$ experiments; $^{*}P < 0.05$ from siC; $^{\#\#}P < 0.01$ from the corresponding −3016 construct). (**D**) CB_1 receptor promoter activity in STHdh$^{Q7/Q7}$ cells transfected with reporter constructs encoding sequential 5′-promoter deletions ($n = 4$ experiments; $^{**}P < 0.01$ from −3016 construct). (**E**) Promoter activity in STHdh$^{Q7/Q7}$ cells transfected with GFP, 17Q-GFP or 72Q-GFP, and reporter constructs encoding the CB_1 receptor promoter −962/−934 sequence or a RE1 consensus sequence ($n = 4$ experiments; $^{**}P < 0.01$ from the corresponding empty construct). (**F**) Promoter activity in STHdh$^{Q7/Q7}$ cells transfected with siC or siHtt, control (C) or REST-directed decoy oligonucleotides, and reporter constructs encoding a 3016-bp CB_1 receptor promoter, the CB_1 receptor promoter −962/−934 sequence or a RE1 consensus sequence ($n = 4$ experiments; $^{*}P < 0.05$, $^{**}P < 0.01$ from siC; $^{\#}P < 0.05$ from the corresponding decoy C). a.u = arbitrary units.

was observed upon deletion of the promoter sequence from nucleotide −1099 to nucleotide −898, indicating that it contains negative regulatory elements. On the other hand, the sequence comprising nucleotide −898 to nucleotide −648 may contain enhancer elements as its deletion decreased reporter activity. Of interest, the −898 promoter was insensitive to wild-type or mutant huntingtin ectopic expression (Fig. 6A and B, right panel) as well as to huntingtin downregulation (Fig. 6C, right panel), supporting the involvement of negative regulatory elements at the 5′ side of the −898 position but not of positive regulatory elements at the 3′ side of that position in the huntingtin-mediated control of CB_1 receptor gene expression.

To date, the best-established factor that participates in the huntingtin-mediated control of neuronal gene expression and whose transcriptional activity changes upon loss of wild-type huntingtin function is repressor element 1 silencing transcription factor (REST) (Zuccato *et al.*, 2003; Cattaneo *et al.*, 2005). We therefore considered whether REST is involved in CB_1 receptor promoter regulation. Computer-aided analysis of the CB_1 receptor promoter allowed us to identify three potential REST-binding RE1 sites

(Bruce *et al.*, 2004) at positions −2522 to −2506, −1569 to −1553 and −958 to −942 (Supplementary Fig. 1), the latter of which could be a candidate for huntingtin-dependent control of CB_1 receptor gene expression. To test this possibility, we cloned a small portion of the CB_1 receptor promoter harbouring the −958/−942 sequence (specifically the −962/−934 fragment) in a CAT reporter construct, and found that wild-type but not mutant huntingtin increased the reporter activity of that sequence to the same extent as that of a control RE1 consensus sequence (Fig. 6E). Moreover, sequestering REST by using RE1-targeted decoy oligonucleotides prevented the decrease of reporter activity induced by endogenous huntingtin silencing on the −3016 CB_1 receptor promoter, the CB_1 receptor promoter −962/−934 sequence and the RE1 consensus sequence (Fig. 6F).

Endogenous huntingtin controls CB_1 cannabinoid receptor gene promoter activity via repressor element 1 silencing transcription factor

To evaluate the huntingtin-mediated control of the CB_1 receptor promoter in a huntingtin constitutive expression setting and to search for possible dose-dependent effects of huntingtin on CB_1 receptor expression, we used striatal neuroblasts from wild-type mice ($STHdh^{Q7/Q7}$ cells) and from knock-in mice expressing one copy ($STHdh^{Q7/Q111}$ cells) or two copies ($STHdh^{Q111/Q111}$ cells) of a mutant huntingtin allele. We first observed that CB_1 receptor expression, as determined by real-time quantitative PCR (Fig. 7A, left panel), western blot (Fig. 7B, middle panel) and immunofluorescence (Fig. 7C, right panel), followed the relative order $STHdh^{Q7/Q7}$ cells > $STHdh^{Q7/Q111}$ cells > $STHdh^{Q111/Q111}$ cells. We then transfected those cells with the CB_1 receptor promoter construct and found that reporter activity displayed the same sequential order as receptor expression (Fig. 7B, left panel). Likewise, the reporter activity of the CB_1 receptor promoter −962/−934 sequence (Fig. 7B, middle panel), as well as that of a RE1 consensus sequence (Fig. 7B, right panel), was higher under wild-type huntingtin expression conditions, pointing again to an important role of the RE1 site in the huntingtin-mediated control of the CB_1 receptor promoter. Further support for this notion was provided by the observation that delivery of RE1-targeted decoy oligonucleotides to mutant huntingtin-expressing cells recovered CB_1 receptor promoter activity to values close to those found in $STHdh^{Q7/Q7}$ cells (Fig. 7C).

CB_1 cannabinoid receptors control striatal brain-derived neurotrophic factor expression

CB_1 receptors can confer neuroprotection by cross-talking to neurotrophic-factor signalling systems (Galve-Roperh *et al.*, 2008). Specifically, CB_1 receptors have been reported to upregulate BDNF expression, which may play a key mechanistic role in cannabinoid-evoked neuroprotection from excitotoxic damage (Marsicano *et al.*, 2003; Khaspekov *et al.*, 2004). Of interest, the downregulation of this particular neurotrophin is critically involved in Huntington's disease neurodegeneration (Canals *et al.*, 2004; Cattaneo *et al.*, 2005; Zuccato and Cattaneo, 2007). We therefore evaluated how modulation of CB_1 receptor function affects BDNF expression in R6/2 mice. The messenger RNA levels and immunoreactivity of striatal BDNF were lower in $R6/2{:}CB_1^{-/-}$ mice than in their $R6/2{:}CB_1^{+/+}$ littermates (Fig. 8A). Moreover, THC administration was able to prevent the decline of striatal BDNF expression observed in vehicle-treated R6/2 mice (Fig. 8B). The messenger RNA levels of the BDNF receptor TrkB were not significantly different in the striata of 8-week-old wild-type, $CB_1^{-/-}$, $R6/2{:}CB_1^{+/+}$ and $R6/2{:}CB_1^{-/-}$mice, or of 8-week-old wild-type or R6/2 mice treated with vehicle or THC (data not shown).

To provide further support for the direct involvement of huntingtin/CB_1 receptors in the control of striatal BDNF expression, we exposed striatal cells to THC. We found that cannabinoid challenge upregulated BDNF expression in $STHdh^{Q7/Q7}$ cells, an effect that was prevented by CB_1 receptor blockade (Fig. 8C). In contrast, $STHdh^{Q111/Q111}$ cells showed a reduced basal expression of BDNF [in line with previous data (Zuccato *et al.*, 2001)] that was insensitive to CB_1 receptor agonism or antagonism (Fig. 8C). Next, we conducted experiments in striatal organotypic cultures obtained from wild-type and R6/2 mice. THC increased BDNF expression in slices from wild-type mice of 6 and 10 weeks of age, as well as in slices from 6-week-old R6/2 mice (Fig. 8C). However, BDNF expression in 10-week-old R6/2 mouse slices—in which CB_1 receptors are severely downregulated—was low and refractory to cannabinoid challenge (Fig. 8C).

Striatal fatty acid amide hydrolase expression increases in R6 mice and patients with Huntington's disease

The experimental evidence described above strongly supports that CB_1 receptor downregulation plays a pivotal role in Huntington's disease-like pathology in R6/2 mice. Nonetheless, the possible participation of other endocannabinoid system elements in progression of the disease may also be considered. Specifically, the levels of anandamide and other endocannabinoids have been shown to decline in the striatum of symptomatic (10-week-old) R6/2 mice (Bisogno *et al.*, 2008). Therefore, our next question was whether the expression of the endocannabinoid-deactivating enzyme FAAH is altered in the disease. CB_1 receptor expression was always monitored in parallel as a functionally related, well-established control. We found that striatal FAAH messenger RNA levels were higher in symptomatic (8- to 12-week-old) R6/2 mice than in their wild-type littermates (Fig. 9A). Striatal FAAH upregulation was also evident at late stages of Huntington's disease-like progression in the R6/1 mouse line, a slow-course transgenic model of Huntington's disease (Fig. 9B). Likewise, western blot analysis of post-mortem samples showed an increase of FAAH expression in the caudate-putamen of patients with Huntington's disease compared to control subjects (Fig. 9C). In contrast to FAAH, the expression of monoacylglycerol lipase, the major enzyme involved in the breakdown of the endocannabinoid

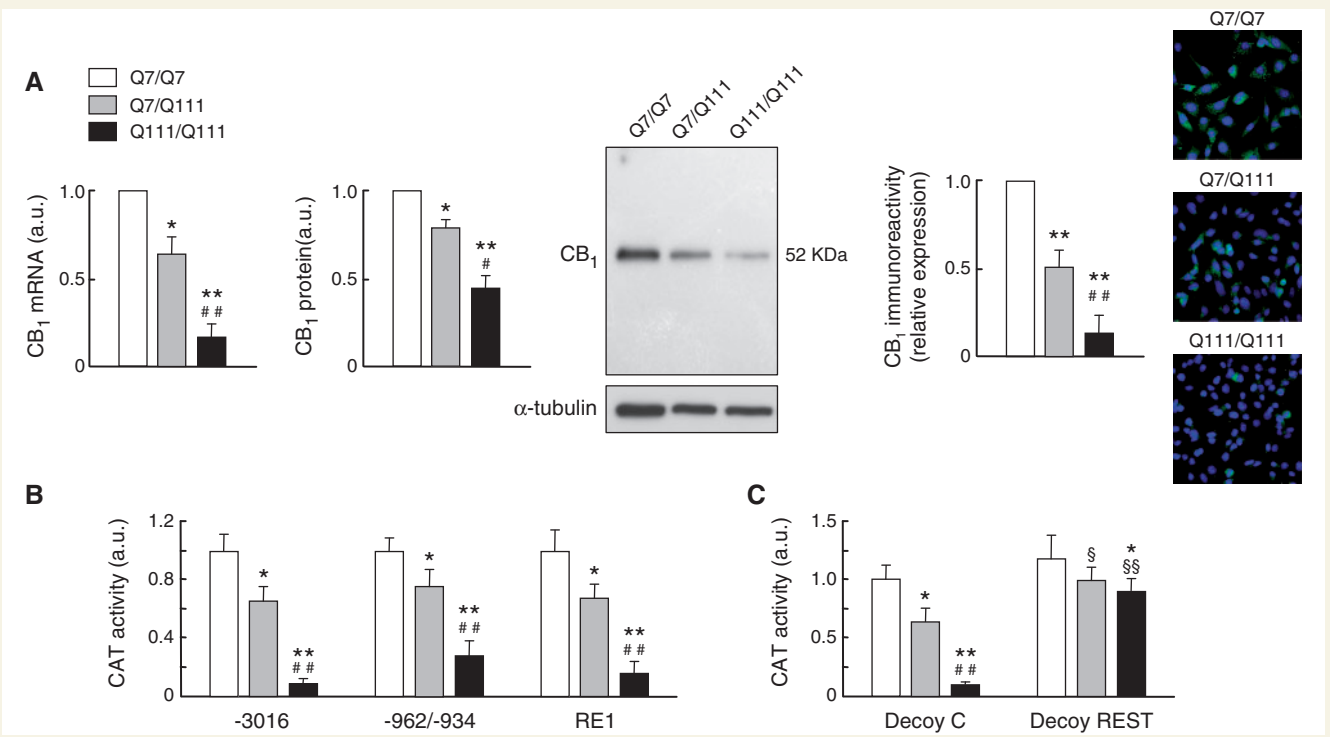

Figure 7 Endogenous huntingtin controls CB_1 cannabinoid receptor gene promoter activity via repressor element 1 silencing transcription factor. (**A**) CB_1 receptor expression in wild-type huntingtin striatal neuroblasts ($STHdh^{Q7/Q7}$ cells, white bars) and mutant huntingtin knock-in striatal neuroblasts [both heterozygous ($STHdh^{Q7/Q111}$ cells, grey bars) and homozygous ($STHdh^{Q111/Q111}$ cells, black bars)] as determined by real-time quantitative PCR (left), western blot [middle; quantification of optical density values relative to those of loading controls (α-tubulin) as well as a representative blot with the Mr of the selected protein bands are shown] and immunofluorescence [right; given as relative values of CB_1^+ area (in green)/total cell number (nuclei in blue), $n = 4$ experiments; $^*P < 0.05$, $^{**}P < 0.01$ from $STHdh^{Q7/Q7}$ cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ from $STHdh^{Q7/Q111}$ cells]. (**B**) Promoter activity in STHdh cells transfected with CAT reporter constructs encoding a 3016-bp human CB_1 receptor promoter, the CB_1 receptor promoter −962/−934 sequence or a RE1 consensus sequence ($n = 6$ experiments; $^*P < 0.05$, $^{**}P < 0.01$ from $STHdh^{Q7/Q7}$ cells; $^{\#\#}P < 0.01$ from $STHdh^{Q7/Q111}$ cells). (**C**) Promoter activity in STHdh cells transfected with control (C) or REST-directed decoy oligonucleotides and a reporter construct encoding a 3016-bp CB_1 receptor promoter ($n = 4$ experiments; $^*P < 0.05$, $^{**}P < 0.01$ from the corresponding $STHdh^{Q7/Q7}$ cells; $^{\#\#}P < 0.01$ from $STHdh^{Q7/Q111}$ cells; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ from the corresponding decoy C). a.u = arbitrary units.

2-arachidonoylglycerol, remained unchanged in the striata of R6/2 or R6/1 mice along disease progression as determined by real-time quantitative PCR (data not shown).

Discussion

One of the most widely reported effects of mutant huntingtin is the alteration of gene expression, and thus transcriptional dysregulation has emerged as a central pathogenic feature of Huntington's disease (Cha, 2007; Imarisio *et al.*, 2008). However, the functional impact of most of these mutant huntingtin-evoked gene expression changes on Huntington's disease pathogenesis remains unclear. Here we show that the loss of striatal CB_1 cannabinoid receptors that occurs in an animal model of Huntington's disease is caused by a mutant huntingtin-associated impairment of CB_1 receptor gene expression, and that this event may constitute a key pathogenic factor of the disease. Thus, CB_1 receptor genetic ablation in mice aggravates Huntington's disease symptoms and pathology, while CB_1 receptor pharmacological activation attenuates them. Likewise, CB_1 receptor downregulation sensitizes striatal cells to excitotoxic damage, while enforced CB_1 receptor expression renders striatal cells more resistant to excitotoxic damage. Besides this pivotal role of CB_1 receptors, the participation of other endocannabinoid system elements in Huntington's disease pathology might also be considered. Specifically, the striatal expression of the anandamide-degrading enzyme FAAH is upregulated in symptomatic Huntington's disease-like mice as well as in patients with Huntington's disease, most likely reflecting—like in other neuropathologies—a process of astroglial activation (Benito *et al.*, 2003, 2007). Accordingly, the levels of anandamide and palmitoylethanolamide (another FAAH substrate) have been shown to decline in the striata of symptomatic—but not pre-symptomatic—R6/2 mice (Bisogno *et al.*, 2008). This decrease in endocannabinoid and endocannabinoid-like messengers might contribute to the aggravation of Huntington's disease symptomatology at late stages of the disease. In contrast to these findings in striatal specimens, FAAH activity has been reported to decrease—and endocannabinoid levels to increase—in peripheral lymphocytes

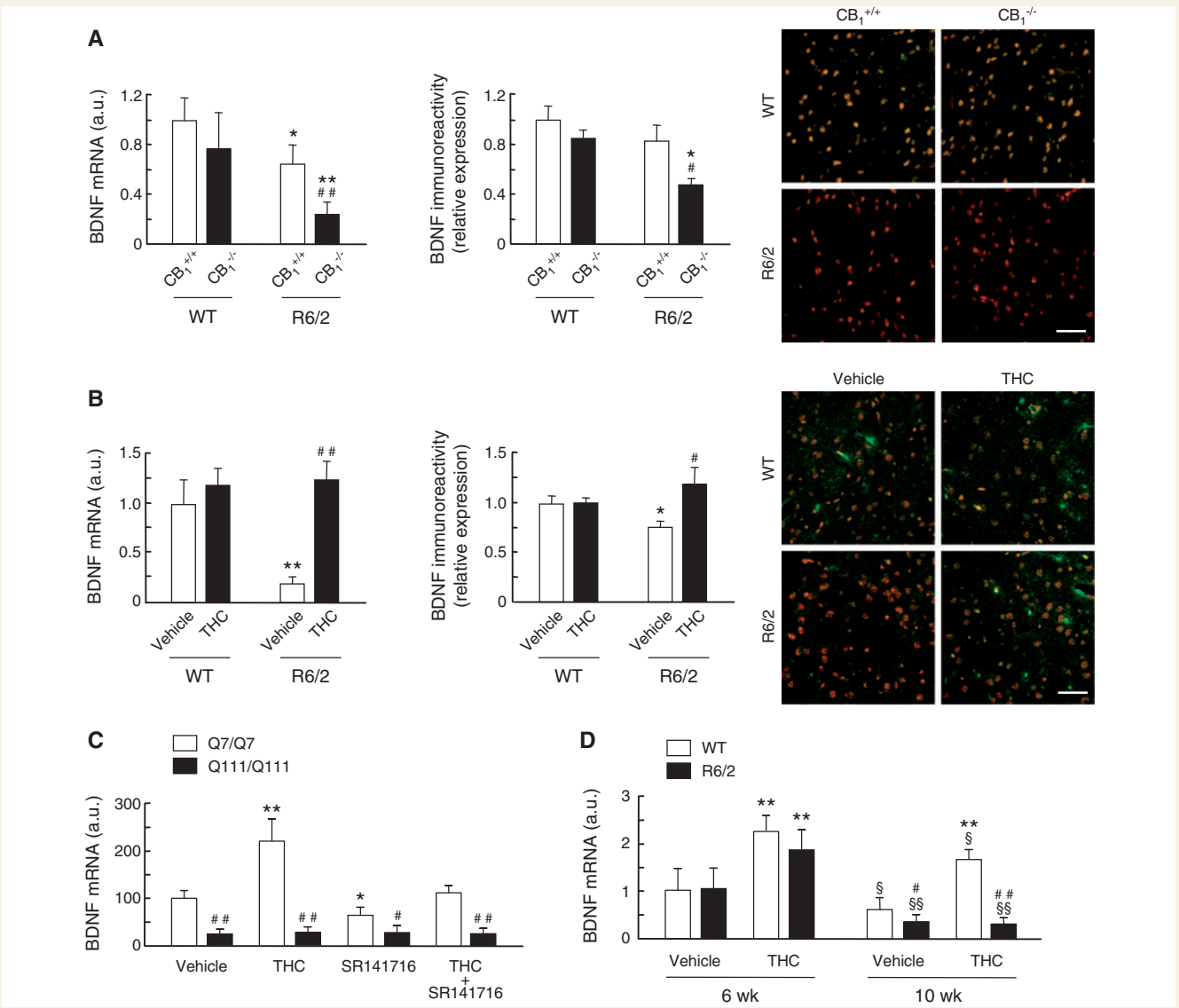

Figure 8 CB_1 cannabinoid receptors control striatal brain-derived neurotrophic factor expression. (**A**) Striatal BDNF messenger RNA levels and immunoreactivity [given as relative values of $BDNF^+$ area (in red)/$NeuN^+$ area (in green)] in 8-week-old wild-type (WT), $CB_1^{-/-}$, R6/2:$CB_1^{+/+}$ and R6/2:$CB_1^{-/-}$ mice ($n = 6$–8 animals per group; $^*P < 0.05$, $^{**}P < 0.01$ from the corresponding wild-type group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ from the corresponding $CB_1^{+/+}$ group). Representative confocal microscopy images are shown. Scale bar 50 μm. (**B**) Striatal BDNF messenger RNA levels and immunoreactivity [given as relative values of $BDNF^+$ area (in red)/$NeuN^+$ area (in green)] in 8-week-old R6/2 mice and wild-type (WT) littermates treated daily with vehicle (white bars) or THC (2 mg/kg body weight/day; black bars) from Week 4 of life ($n = 6$–8 animals per group; $^*P < 0.05$, $^{**}P < 0.01$ from the corresponding wild-type group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ from the corresponding vehicle-treated group). Representative confocal microscopy images are shown. Scale bar 50 μm. (**C**) BDNF expression in wild-type huntingtin striatal neuroblasts ($STHdh^{Q7/Q7}$ cells) and mutant huntingtin knock-in striatal neuroblasts ($STHdh^{Q111/Q111}$ cells), as determined by real-time quantitative PCR, after incubation for 12 h with vehicle, 0.5 μM THC and/or 0.25 μM SR141716 ($n = 6$ experiments; $^*P < 0.05$, $^{**}P < 0.01$ from the corresponding vehicle-treated cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ from the corresponding $STHdh^{Q7/Q7}$ cells). (**D**) BDNF expression in striatal slices from wild-type (WT) and R6/2 mice of the indicated ages, as determined by real-time quantitative PCR, after incubation for 24 h with vehicle or 1 μM THC ($n = 4$ animals per group; $^{**}P < 0.01$ from the corresponding vehicle-treated slices; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ from the corresponding wild-type group; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ from the corresponding 6-week-old group). a.u = arbitrary units.

from patients with Huntington's disease compared to healthy subjects (Battista *et al.*, 2007). As shown in the present study, the expression of monoacylglycerol lipase, the major enzyme involved in the breakdown of the endocannabinoid 2-arachidonoylglycerol, remains however unchanged in the striata of R6/2 or R6/1 mice along disease progression. On the other hand, microglial CB_2 cannabinoid receptors are induced upon various neuroinflammatory conditions, in which they are believed to inhibit the production

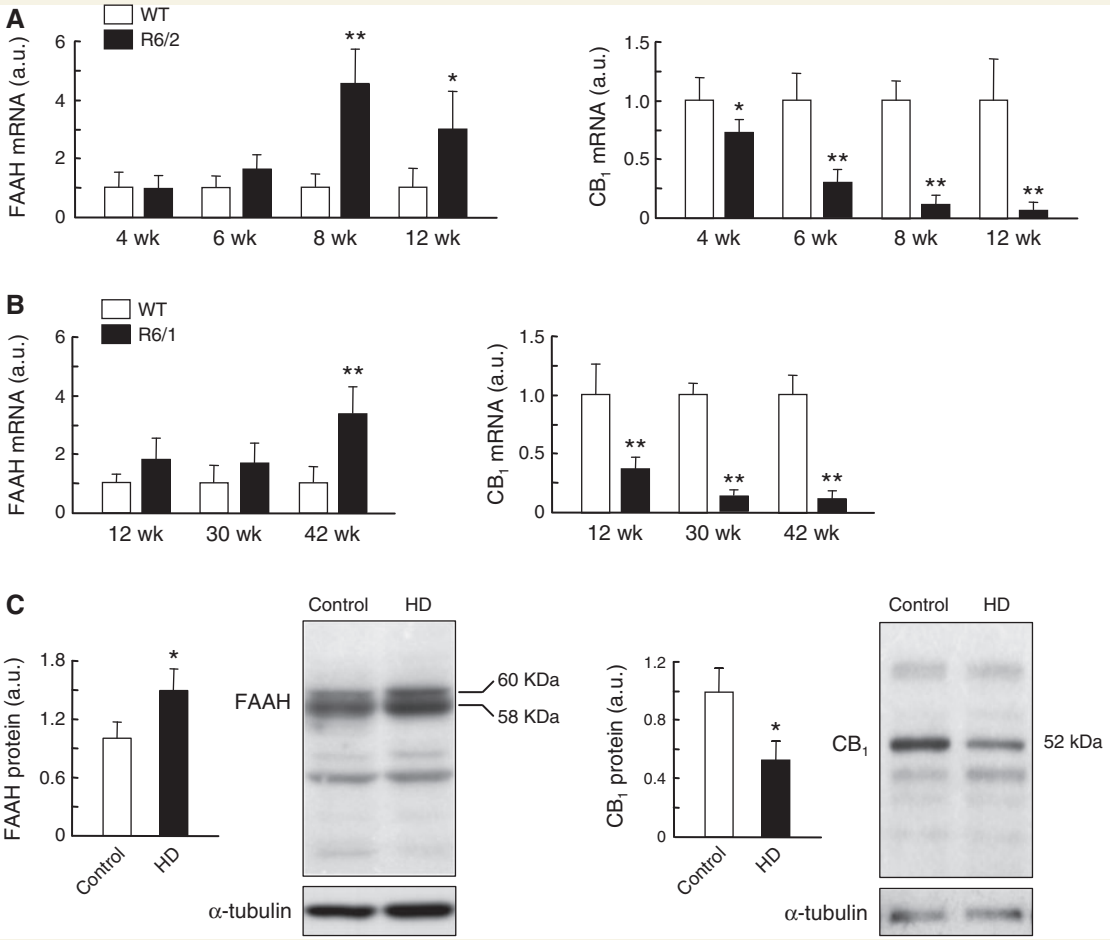

Figure 9 Striatal fatty acid amide hydrolase expression increases in R6 mice and patients with Huntington's disease. (**A**, **B**) Striatal FAAH (left) and CB_1 receptor (right) messenger RNA levels in R6/2 (**A**) and R6/1 (**B**) mice at different ages as determined by real-time quantitative PCR ($n = 6$–8 animals per group; $^*P < 0.05$, $^{**}P < 0.01$ from the corresponding wild-type (WT) group). (**C**) Western blot analysis of FAAH (left) and CB_1 receptor (right) expression in caudate-putamen specimens from patients with Huntington's disease (HD) and control subjects. Quantification of optical density values relative to those of loading controls (α-tubulin) as well as representative blots with the relative molecular mass (M^r) of the selected protein bands are shown ($n = 6$ patients with Huntington's disease and $n = 6$ control subjects; $^*P < 0.05$ from control subjects). a.u = arbitrary units.

of pro-inflammatory cytokines and reactive oxygen species (Fernandez-Ruiz *et al.*, 2007). Thus, the recently described upregulation of CB_2 receptors in striatal microglia of Huntington's disease patient samples and transgenic and neurotoxin-induced Huntington's disease animal models (Palazuelos *et al.*, 2009, Sagredo *et al.*, 2009) might constitute a defensive response aimed at attenuating microglial overactivation in late stages of Huntington's disease. We cannot rule out that activation of CB_2 receptors participates in the beneficial effects of THC reported here. However, the implication of microglial overactivation selectively in advanced stages of the disease, the strong impact of CB_1 receptor genetic ablation at early stages of the disease and the indispensable involvement of CB_1 receptors in cannabinoid-induced neuroprotection and BDNF upregulation found in our striatal cell/tissue culture experiments strongly support that CB_1 receptors make a major contribution to the observed effects of THC as administered—as in the present study FAAH to 4- to 10-week-old animals. On the other hand, the finding that the modulation of CB_1 receptor (the present work) or CB_2 receptor (Palazuelos *et al.*, 2009) activity in R6/2 mice affects the immunoreactivity of the pre-synaptic terminal marker synaptophysin—besides that of the post-synaptic marker PSD95 and the GABAergic neuron marker GAD67—supports the possibility that the endocannabinoid system confers protection not only to striatal medium-sized spiny neurons, the cells that degenerate primarily in Huntington's disease, but also to other types of neurons that are targeted by the disease such as those projecting the striatum (e.g. corticostriatal neurons and nigrostriatal neurons) and striatal interneurons.

Huntington's disease is usually envisaged as a gain-of-function disease (Walker, 2007; Imarisio *et al.*, 2008). However, although the cellular functions of wild-type huntingtin are still not completely clear, it has been proposed that loss of wild-type huntingtin function also contributes to Huntington's disease (Cattaneo *et al.*, 2005). Our data support that the impact of CB_1 receptor downregulation on Huntington's disease pathology is associated,

at least in part, to a loss of wild-type huntingtin function process, and that the huntingtin-mediated control of CB_1 receptor gene expression relies on REST, a transcriptional repressor that regulates the expression of a large network of neuronal proteins (Johnson and Buckley, 2009). It was previously shown that wild-type huntingtin sequesters REST in the cytoplasm, thereby preventing its gene-silencing action (Zuccato *et al.*, 2003). A subsequent report supported that this interaction is not direct, so that huntingtin binds to REST through two intermediate proteins, dynactin $p150^{Glue}$ and REST/neuron restrictive silencer factor-interacting LIM domain protein (Shimojo, 2008). The latter study further suggested that mutant huntingtin binds to that multi-protein complex and alters its conformation, thus permitting REST to translocate to the nucleus and repress gene expression. Our data fit well with this current model of huntingtin/REST action. Nonetheless, it cannot be ruled out that the huntingtin-mediated control of CB_1 receptor expression is a more complex issue, as, for example, mutant huntingtin is well known to impact gene/protein expression by a plethora of different transcriptional and post-transcriptional mechanisms (Benn *et al.*, 2008; Imarisio *et al.*, 2008; Johnson and Buckley, 2009).

Of note, REST also participates in the huntingtin-mediated transcriptional control of BDNF, a particular neurotrophin that is critically involved in Huntington's disease pathophysiology (Cattaneo *et al.*, 2005; Zuccato and Cattaneo, 2007). In addition, several reports support that CB_1 receptors confer neuroprotection by enhancing BDNF expression, although the molecular basis of this connection remains unknown (Galve-Roperh *et al.*, 2008). It is thus conceivable that the decrease of BDNF levels concomitant with CB_1 receptor loss contributes significantly to striatal damage in Huntington's disease, for which our findings support that BDNF is a *bona fide* marker of Huntington's disease neurodegeneration (Zuccato and Cattaneo, 2007) and CB_1 receptor-evoked neuroprotection (Galve-Roperh *et al.*, 2008). Striatal BDNF can be produced *in situ* (Timmusk *et al.*, 1995; Canals *et al.*, 1998; Aid *et al.*, 2007; Hasbi *et al.*, 2009). Additionally, striatal GABAergic projections receive BDNF from the cortex (Altar *et al.*, 1997; Mufson *et al.*, 1999), indicating that impaired anterograde BDNF transport in corticostriatal neurons may contribute to the decreased BDNF protein expression found in the striata of Huntington's disease mice (Cattaneo *et al.*, 2005). Nonetheless, mutant huntingtin has been shown to affect axonal transport of BDNF in striatal neurons but not in cortical neurons (Her and Goldstein, 2008), and CB_1 receptor loss or gain of function does not affect cortical BDNF expression in R6/2 mice (Supplementary Fig. 2).

Potential clinical implications

Previous studies on the potential role of CB_1 receptors in Huntington's disease have been undertaken on simpler experimental systems and have provided contradictory data. Thus, screening of a large library of compounds for their ability to protect cultured PC12 pheochromocytoma cells from mutant huntingtin-induced toxicity unveiled THC and other plant-derived cannabinoids as very efficient agents (Aiken *et al.*, 2004). However, this was not replicated in a similar study (Wang *et al.*, 2005). Likewise, administration of THC and other cannabinoid receptor agonists reduced (Lastres-Becker *et al.*, 2004; Pintor *et al.*, 2006) or increased (Lastres-Becker *et al.*, 2003) neuronal loss in rat models of neurotoxin-induced acute striatal damage. Here we used a well-established genetic model of Huntington's disease, the R6/2 mouse, which recapitulates many of the features of human Huntington's disease, including motor and cognitive impairments, weight loss, striatal atrophy, mutant-protein aggregates, neurochemical alterations, gene expression dysregulation, metabolic and neuroendocrine changes and premature death (Mangiarini *et al.*, 1996; Hockly *et al.*, 2003; Gil and Rego, 2009). Although this model displays potential limitations such as an accelerated phenotype—which may mimic juvenile-onset Huntington's disease rather than adult-onset Huntington's disease—and the expression of a truncated form of mutant huntingtin, a recent study that has compared different transgenic and knock-in models of Huntington's disease using standardized conditions has confirmed the relevance of the R6/2 line for the study of the disease (Menalled *et al.*, 2009). Our experiments of CB_1 receptor pharmacological activation in R6/2 mice, as well as the phenotypic analyses of R6/2:$CB_1^{-/-}$ mice, to the best of our knowledge the first double-mutant animals generated so far in which CB_1 receptors are ablated in a neuropathology genetic-model background, provide strong evidence for the protective role of CB_1 receptors, and may open possibilities for similar studies on other neuropathologies (such as Alzheimer's disease) in which CB_1 receptor levels fall (Benito *et al.*, 2003, Ramirez *et al.*, 2005).

Pharmacological activation of CB_1 receptors in patients with early-stage Huntington's disease might thus be beneficial in attenuating disease progression in these subjects. A first controlled trial conducted with a cannabis component (cannabidiol) reported no effect on chorea severity in 15 patients with Huntington's disease (Consroe *et al.*, 1991). However, cannabidiol, although structurally similar to THC, is not a cannabinoid receptor agonist. Two subsequent uncontrolled, single-patient studies using nabilone, a synthetic 9-keto derivative of THC that activates CB_1 receptors, reported contradictory outcomes on Huntington's disease-associated chorea—either worsening (Muller-Vahl *et al.*, 1999) or improvement (Curtis and Rickards, 2006). The only double-blind, placebo-controlled, cross-over study of a CB_1 receptor agonist (specifically nabilone) in Huntington's disease has been recently reported (Curtis *et al.*, 2009). This 44-patient trial has shown improvements in total motor score, chorea, cognition, behaviour and neuropsychiatric inventory upon cannabinoid treatment, which was safe and well tolerated. Although it is clear that there is a need for further and more exhaustive trials to establish the use of cannabinoids in Huntington's disease, this clinical study—for which our work provides strong preclinical support—opens a new therapeutic avenue for the management of this devastating disease. In this respect, THC and other cannabinoids have a favourable drug-safety profile and are already used in clinical practice as anti-emetic, appetite-stimulating and analgesic compounds (Pertwee, 2009). Additionally, approaches aimed at preventing CB_1 receptor loss (e.g. by environmental stimulation; Glass *et al.*, 2004) might be also envisaged. Finally, our results

support the potential use of CB_1 receptors as biomarkers for monitoring the onset and progression of Huntington's disease.

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Supplementary material

Supplementary material is available at *Brain* online.

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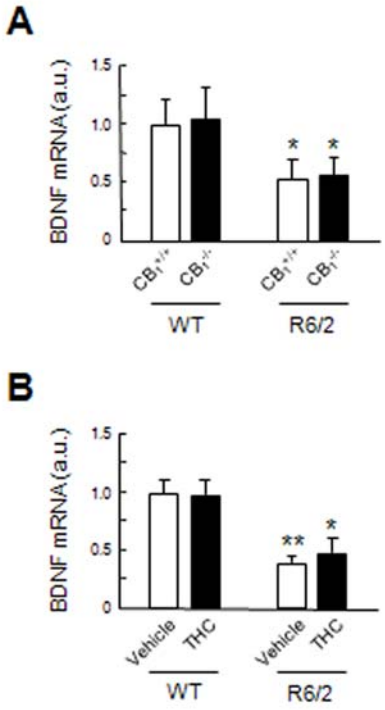
Supplementary Figure 1

Potential RE1 sites in the human CB_1 cannabinoid receptor gene promoter

Five-prime flanking region of human CB_1 receptor exon 1. Exon 1 starts at the underlined sequence; the first G thus denotes nt +1, and the first nt of the entire sequence shown (T) corresponds to position -3061. Potential RE1 sites are highlighted in red.



Supplementary Figure 2
Modulation of CB_1 cannabinoid receptor activity does not affect cortical BDNF expression in R6/2 mice

(**A**) Cortical BDNF mRNA levels in 8 wk-old $CB_1^{+/+}$, $CB_1^{-/-}$, R6/2:$CB_1^{+/+}$ and R6/2:$CB_1^{-/-}$ mice. (**B**) Cortical BDNF mRNA levels in 8 wk-old R6/2 mice and WT littermates treated daily with vehicle or THC (2 mg/kg body weight/d) from wk 4. In both panels mRNA levels were determined by real-time quantitative PCR (n=6-8 animals per group; **P*<0.05, ***P*<0.01 from the corresponding WT group). Data are expressed as mean ± SEM.

Gene	Probe	Primer
mCB1	79F	GGGCAAATTTCCTTGTAGCA
mCB1	79R	GGCTAACGTGACTGAGAAA
mHTT	67F	TGGTCTCCATGCTGTTACGA
mHTT	67R	GCTGCAGGACAAGGATGAAC
mBDNF	31F	AGTCTCCAGGACAGCAAAGC
mBDNF	31R	AAGGATGGTCATCACTCTTCTCA
mGAD67	89F	TGGAGATGCGAACCATGAG
mGAD67	89R	GAAGGGTTCCTGGTTTAGCC
mSYP	83F	CTGGCAGACATGGACGTG
mSYP	83R	CTTGACCACCCGGAACTG
mPSD95	10F	CGCTACCAAGATGAAGACACG
mPSD95	10R	CAATCACAGGGGGAGAATTG
mTRKB	15F	TTCTGCCTGCTGGTGATGT
mTRKB	15R	TCCAGTGGGATCTTATGAAACA
mFAAH	21F	CCTGCTCTGGATTTGAACG
mFAAH	21R	TCCAGGCAGTTATAGAGAACAGTG
mMAGL	58F	TCTTCCTCCTGGGCCACT
mMAGL	58R	AAAGTAGGTTGGCCTCTCTGC
mACTB	56F	AAGGCCAACCGTGAAAAGAT
mACTB	56R	GTGGTACGACCAGAGGCATAC
mrh18SRNA	55F	AAATCAGTTATGGTTCCTTTGGTC
mrh18SRNA	55R	GCTCTAGAATTACCACAGTTATCCAA

Supplementary Table 1
Probes and primers used for real-time quantitative PCR analyses

Chapeter 2

To further dissect the neuroprotective activity of the CB_1 receptor, in this second chapter we will analyze the physiological relevance and therapeutic potential in neurodegeneration of the CB1 receptor pools located on GABAergic or glutamatergic neurons. We will first induce excitotoxic damage in the mouse brain by administering quinolinic acid to conditional mutant animals lacking CB_1 receptors in either GABAergic or glutamatergic neurons. Subsequently, we will enhance corticostriatal glutamatergic transmission with a *designer receptor exclusively activated by designer drug* pharmacogenetic approach. We will next examine whether the detrimental consequences of knocking-out CB_1 receptors is due to the de-inhibition of glutamatergic and/or GABAergic transmission, (i) by the pharmacological modulation of $GABA_A$ receptors or NMDA receptors in R6/2 mice that do not express CB_1 receptors, and (ii) by the selective deletion in those mice of the CB_1 receptor gene in either corticostriatal glutamatergic or striatal GABAergic neurons.

Capitulo 2

Para diseccionar con mayor detalle el papel neuroprotector del receptor CB_1 en modelos de neurodegeneración, en este segundo capítulo se estudiará la relevancia fisiológica y el potencial terapéutico en modelos de neurodegeneración de las poblaciones de receptores CB_1 localizadas en neuronas GABAérgicas o glutamatérgicas. Con este fin, se usará en primer lugar un modelo de excitotoxicidad, la inyección intraestriatal de acido quinolínico, en ratones mutantes condicionales que carecen de receptores CB_1 en neuronas GABAérgicas o glutamatérgicas. A continuación, se empleará un modelo farmacogenético (*designer receptor exclusively activated by designer drug*) para sobreactivar la trasmisión excitadora en neuronas corticoestriatales. Finalmente, se evaluará si las consecuencias deletéreas de la deleción genética del receptor CB1 en ratones R6/2 se deben a una de-inhibición de la transmisión GABAergica o glutamatergica. Este objetivo se abordará con dos aproximaciones experimentales: por un lado, en ratones R6/2 que carecen de receptor CB_1, se modulará farmacológicamente la activación de los receptores $GABA_A$ o NMDA; por otro lado, en ese mismo modelo animal, se delecionará específicamente el gen del receptor CB_1 en neuronas GABAérgicas o glutamatérgicas.

A restricted population of CB_1 cannabinoid receptorswith neuroprotective activity

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The CB_1 cannabinoid receptor, the main molecular target of endocannabinoids and cannabis active components, is the most abundant G protein-coupled receptor in the mammalian brain. Of note, CB_1 receptors are expressed at the synapses of two opposing (*i.e.* GABAergic/inhibitory and glutamatergic/excitatory) neuronal populations, so the activation of one and/or another receptor population may conceivably evoke different effects. Despite the widely-reported neuroprotective activity of the CB_1 receptor in animal models, the precise pathophysiological relevance of those two CB_1 receptor pools in neurodegenerative processes is unknown. Here, we first induced excitotoxic damage in the mouse brain by (i) administering quinolinic acid to conditional mutant animals lacking CB_1 receptors selectively in either GABAergic or glutamatergic neurons, and (ii) manipulating corticostriatal glutamatergic projections remotely with a *designer receptor exclusively activated by designer drug* pharmacogenetic approach. We next examined the alterations that occur in the R6/2 mouse, a well-established model of Huntington's disease, upon (i) fully knocking-out CB_1 receptors, and (ii) deleting CB_1 receptors selectively in either corticostriatal glutamatergic or striatal GABAergic neurons. The data unequivocally identify the restricted population of CB_1 receptors located on glutamatergic terminals as an indispensable player in the neuroprotective activity of (endo)cannabinoids, therefore suggesting that this precise receptor pool constitutes a promising target for neuroprotective therapeutic strategies.

Cannabinoid receptor | neuroprotection | neuromodulation

Introduction

Endocannabinoids are a family of neuron-communication messengers that act by engaging CB_1 cannabinoid receptors, which are also targeted by Δ^9-tetrahydrocannabinol (THC), the main bioactive component of cannabis. Endocannabinoid signaling serves as a pivotal feedback mechanism to prevent excessive presynaptic activity, thereby tuning the functionality and plasticity of many synapses (1, 2). The CB_1 receptor is the most abundant G protein-coupled receptor in the brain, and is highly expressed in GABAergic terminals of the forebrain (particularly in cholecystokinin-positive and parvalbumin-negative interneurons) (3), where it inhibits GABA release. Functional CB_1 receptors reside as well on terminals of glutamatergic neurons in several brain regions, where they inhibit glutamate release (4). In concert with this well-established neuromodulatory function, the CB_1 receptor protects neurons in many different animal models of acute brain damage and chronic neurodegeneration, which, during the last years, has raised hope about the possible clinical use of cannabinoids as neuroprotective drugs, especially in still unexplored conditions such as Alzheimer's disease, Huntington's disease (HD), amyotrophic lateral sclerosis and stroke (5-7). However, the assessment of the physiological relevance and therapeutic potential of the CB_1 receptor in neurological diseases is hampered, at least in part, by the lack of knowledge of the neuron-population specificity of CB_1 receptor action. Here, by using various genetic models of CB_1 receptor loss of function, together with pharmacological and pharmacogenetic tools, we show that a unique population of CB_1 receptors, namely that located on glutamatergic terminals, plays an indispensable role in the neuroprotective activity of the endocannabinoid system in the mouse brain. This finding opens a new conceptual view on how the CB_1 receptor evokes neuroprotection and provides preclinical support for improving the development of cannabinoid-based neuroprotective therapies.

Results

CB_1 cannabinoid receptors located on glutamatergic but not GABAergic neurons protect against excitotoxic damage. To evaluate the neuroprotective role of CB_1 receptors located on glutamatergic (excitatory) or GABAergic (inhibitory) terminals we first used conditional mutant mice lacking CB_1 in either glutamatergic neurons (Glu-$CB_1^{-/-}$ mice) or GABAergic neurons

Significance

Cannabinoids and their endogenous counterparts, the so-called endocannabinoids, promote neuroprotection in laboratory animals by engaging CB_1 cannabinoid receptors, one of the most abundant types of receptors in the brain. However, the assessment of the physiological relevance and therapeutic potential of the CB_1 receptor in neurological diseases is hampered, at least in part, by the lack of knowledge of the neuron-population specificity of CB_1 receptor action. This study shows that a unique and well-defined population of CB_1 receptors, namely that located on glutamatergic terminals, plays a key neuroprotective role in the mouse brain. This finding opens a new conceptual view on how the CB_1 receptor evokes neuroprotection, and provides preclinical support for improving the development of cannabinoid-based neuroprotective therapies.

Reserved for Publication Footnotes

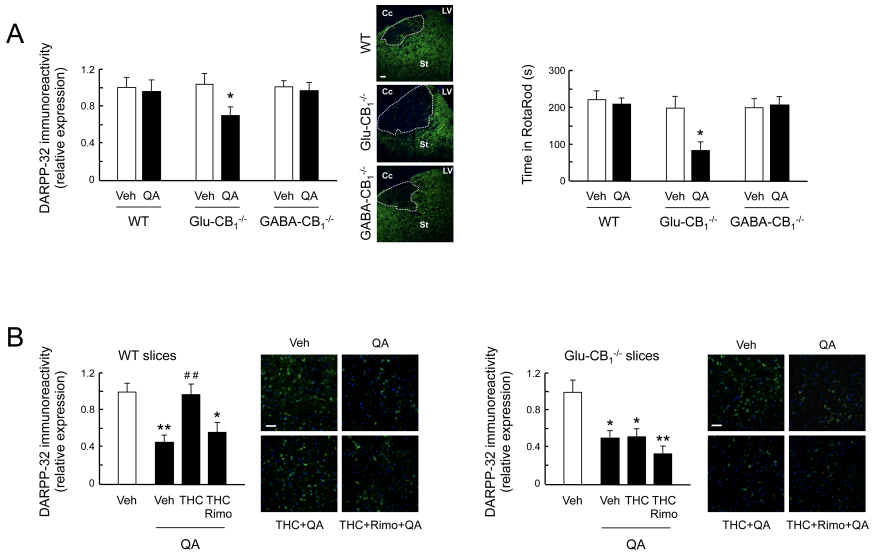

Fig. 1. CB_1 cannabinoid receptors located on glutamatergic but not GABAergic neurons protect against excitotoxic damage. (*A*) Glu-$CB_1^{-/-}$ and GABA-$CB_1^{-/-}$ mice, as well as WT ($CB_1^{floxed/floxed}$) littermates, were injected intrastriatally with vehicle (Veh) or quinolinic acid (QA; 50 nmol in 1 µL PBS, unilaterally) (n=6-8 animals per group). RotaRod performance was evaluated along the following 3 days, and the day after animals were sacrificed for determination of DARPP-32 immunoreactivity in the dorsolateral striatum (data expressed as relative values from the Veh-treated WT group). (*B*) Corticostriatal slices from WT ($CB_1^{floxed/floxed}$) mice and Glu-$CB_1^{-/-}$ littermates were incubated for 24 hours with Veh or QA (50 µM), alone or with Veh, THC (1 µM) and/or rimonabant (Rimo; 5 µM), and DARPP-32 immunoreactivity in the dorsolateral striatum was determined (data expressed as relative values from the corresponding Veh-treated group; n=4-6 preparations per condition). Representative images of DARPP-32 staining (DARPP-32, green; DAPI, blue) are shown in *A* (the area of apparent DARPP-32 loss is outlined; Cc, corpus callosum; LV, lateral ventricle; St, striatum) and *B*. Scale bars, 100 µm (*A*); 50 µm (*B*). **P*<0.05, ***P*<0.01 from the corresponding Veh-treated group. $^{\#\#}P$<0.01 from the QA-Veh-treated group.

(GABA-$CB_1^{-/-}$ mice). These animals were injected in the striatum with quinolinic acid, a widely-used agonist of ionotropic *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, at a dose (50 nmol in 1 µL PBS, unilaterally) that, in our hands, does not exert overt deficits in wild-type (WT) mice (Fig. 1*A* and Fig. S1). Glu-$CB_1^{-/-}$ mice were sensitive to excitotoxic damage, as determined by (i) the loss of dopamine- and cAMP-regulated phosphoprotein of 32 kDa [DARPP-32; a paradigmatic marker of medium-sized spiny neurons (MSNs), the cells that constitute ~90% of total striatal neurons], and (ii) the decline in RotaRod performance (a well-established motor paradigm that relies, at least in part, on striatal function) (Fig. 1*A*). In contrast, no significant neurotoxicity was observed in quinolinic acid-treated GABA-$CB_1^{-/-}$ animals (Fig. 1*A*).

To prove the direct anti-excitotoxic activity of CB_1 receptors located on glutamatergic terminals we prepared organotypic cultures of corticostriatal slices from WT mice, and found that the loss of DARPP-32 immunoreactivity produced by incubation with quinolinic acid (50 µM) was prevented by the cannabinoid receptor agonist THC (1 µM; Fig 1*B*). This neuroprotective effect of THC was (i) impaired by the CB_1 receptor-selective antagonist rimonabant (5 µM), and (ii) absent in slices from Glu-$CB_1^{-/-}$ mice (Fig 1*B*).

Cannabinoid receptor agonist prevents excitotoxic damage induced by selective activation of corticostriatal glutamatergic neurons. To further support the anti-excitotoxic activity of glutamatergic-terminal CB_1 receptors we selectively manipulated corticostriatal glutamatergic terminals *in vivo* by the *designer receptor exclusively activated by designer drug* (DREADD) pharmacogenetic technique. This is a newly developed tool based on the molecular evolution of muscarinic acetylcholine receptors, leading to a G_q protein-coupled receptor with negligible affinity for the native agonist (acetylcholine) but to which the orally bioavailable, pharmacologically inert agonist clozapine-*N*-oxide (CNO) binds with high potency and efficacy (8). Importantly, DREADDs lack detectable constitutive activity, thus allowing the remote control of neuronal activity in specific cell populations *in vivo* (9). Here, we injected stereotactically WT mice with a recombinant adeno-associated viral vector encoding an engineered G_q protein-coupled DREADD fused to mCherry (or only mCherry as control) into the motor cortex, where the somata of the glutamatergic afferents projecting onto the dorsolateral (motor) striatum reside. The expression of the transgene was driven by the calcium/calmodulin-dependent protein kinase II-α (CaMKIIα) promoter in order to confine it to principal (glutamatergic) neurons and to avoid other neuronal populations such as GABAergic interneurons. Animals were subsequently treated with CNO (or vehicle as control) in conditions that are known to evoke sustained neuronal activation (10 mg/kg body weight/day for 4 weeks) (10). This procedure triggered excitotoxic damage in the striatum by enhancing glutamatergic transmission, as evidenced by (i) the CNO-induced reduction of DARPP-32 immunoreactivity and RotaRod performance, and (ii) the abrogation of CNO action by the NMDA receptor-selective antagonist MK-801 (0.03 mg/kg body weight/day; Fig. 2*A* and *B*). Of note, treatment with THC (2 mg/kg body weight/day) prevented the striatal damage evoked by DREADD-G_q-mediated activation of corticostriatal projections (Fig. 2*A* and *B*).

Genetic deletion of CB_1 cannabinoid receptors aggravates HD-like striatal neurodegeneration by altering glutamatergic but not GABAergic transmission. To assess the functional impact of the CB_1 receptor on glutamatergic and GABAergic signaling in a neurodegenerative-disease context we conducted experiments in the R6/2 mouse, a well-established model of HD. This devastating disease constitutes so far the best paradigm to study the specific role of CB_1 receptors located on glutamatergic or GABAergic terminals because CB_1 receptors are expressed in the striatum at synapses established by neurons containing GABA (especially MSNs, the cells that primarily degenerate in HD) or glutamate (especially corticostriatal projecting neurons, which critically control MSN function) as transmitters, and play a key role in the control of motor behavior, one of the processes that is

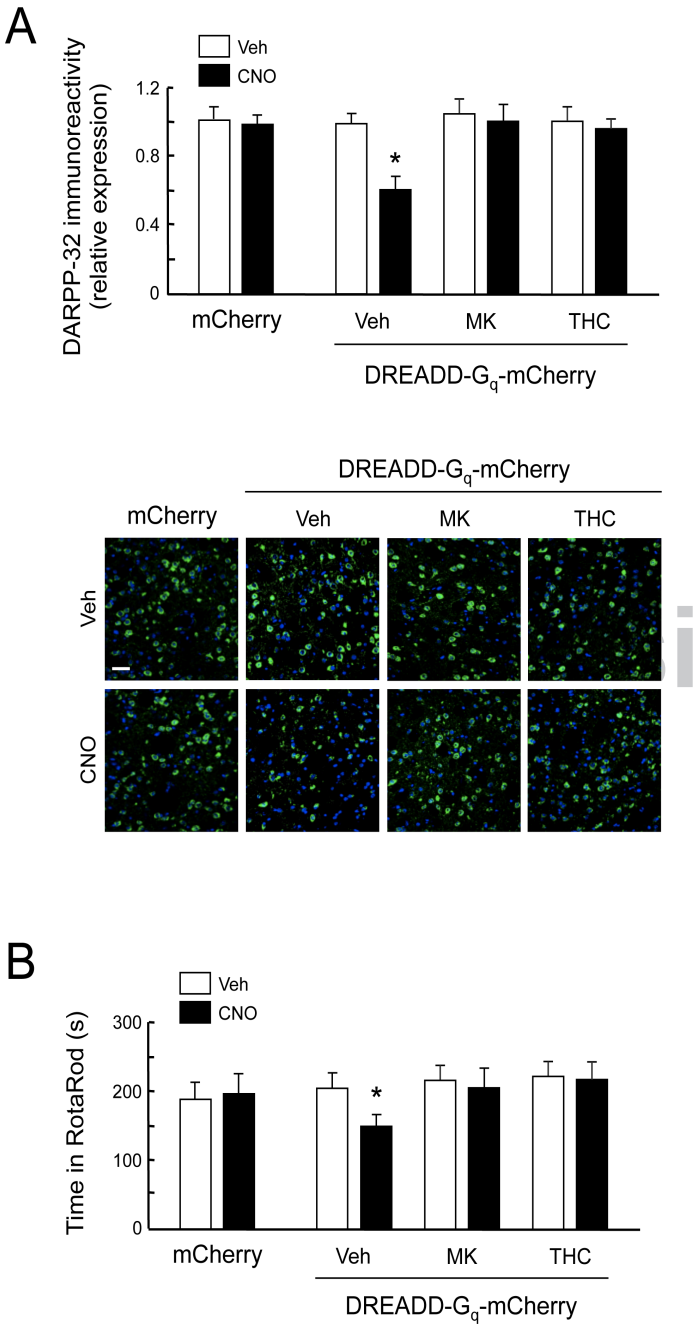

Fig. 2. Cannabinoid receptor agonist prevents excitotoxic damage induced by selective activation of corticostriatal glutamatergic neurons. (*A*, *B*) WT (C57BL/6N) mice were injected stereotactically into the motor cortex with a recombinant adeno-associated virus encoding DREADD-G_q-mCherry (or mCherry) under the control of the CaMKIIα promoter (n=8-10 animals per group). Six weeks later, mice received daily i.p. injections of Veh or CNO (10 mg/kg body weight), alone or in combination with Veh, MK-801 (0.03 mg/kg body weight) or THC (2 mg/kg body weight), for 4 weeks. RotaRod performance was evaluated along the last 3 days of treatment, and the day after animals were sacrificed for histological analyses. (*A*) DARPP-32 immunoreactivity in the dorsolateral striatum (data expressed as relative values from the Veh-treated mCherry group).Representative images of DARPP-32 staining are shown (DARPP-32, green; DAPI, blue). Scale bar, 50 µm. (*B*) RotaRod performance (time to fall). **P*<0.05 from the corresponding Veh-treated group.

most typically affected in HD (11, 12). Moreover, a remarkable down-regulation of CB_1 receptors has been documented as one

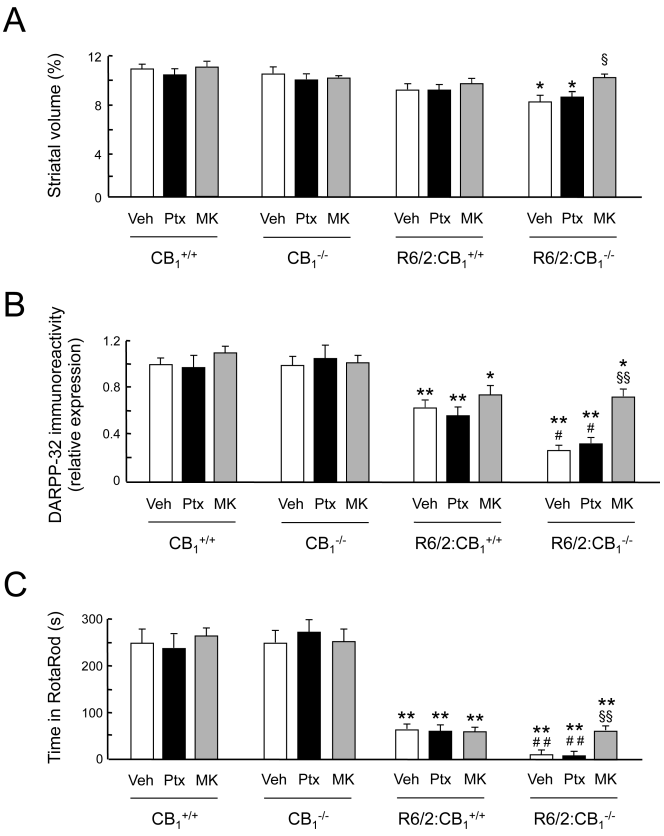

Fig. 3. Genetic deletion of CB_1 cannabinoid receptors aggravates HD-like striatal neurodegeneration by altering glutamatergic but not GABAergic transmission. (*A-C*) $CB_1^{+/+}$, $CB_1^{-/-}$, R6/2:$CB_1^{+/+}$ and R6/2:$CB_1^{-/-}$ mice were treated i.p. with vehicle (Veh), picrotoxin (Ptx; 0.3 mg/kg body weight/day) or MK-801 (MK; 0.03 mg/kg body weight/day) from week 4 to week 8 of age. (*A*) Striatal volume (percentage of total brain volume). (*B*) DARPP-32 immunoreactivity in the dorsolateral striatum (relative values from the Veh-treated $CB_1^{+/+}$ group). (*C*) RotaRod performance (time to fall). Data in *A-C* correspond to 8 week-old mice at the end of the treatments (n=8-12 animals per group). **P*<0.05, ***P*<0.01 from the corresponding $CB_1^{+/+}$ or $CB_1^{-/-}$ group; #*P*<0.05, ##*P*<0.01 from the corresponding R6/2:$CB_1^{+/+}$ group; §*P*<0.05, §§*P*<0.01 from the vehicle-treated R6/2:$CB_1^{-/-}$ group.

of the earliest and most characteristic neurochemical alterations found in the MSNs of HD animal models (13, 14) and HD patients (15, 16). In striking contrast, CB_1 receptors located on glutamatergic terminals are fully preserved in (i) the striatum of symptomatic R6/2 mice (17) (Fig. S2) and (ii) the striatum (caudate-putamen) of HD patients (Fig. S3).

We (18) and others (19) have recently reported that double-mutant mice expressing mutant huntingtin in a $CB_1^{-/-}$ background show an overt HD-like phenotype at earlier ages than their single-mutant littermates expressing mutant huntingtin in a normal $CB_1^{+/+}$ background. To test whether this detrimental consequence of knocking-out CB_1 receptors is evoked by the de-inhibition of glutamatergic and/or GABAergic transmission, we generated R6/2:$CB_1^{-/-}$ mice and their control littermates, and evaluated the effect of $GABA_A$ receptor or NMDA receptor-selective antagonists (picrotoxin and MK-801, respectively) at an early stage of the disease (4-8 weeks) in which CB_1 receptor deletion is known to precipitate HD-like alterations (18). Picrotoxin administration (0.3 mg/kg body weight/day) to R6/2:$CB_1^{-/-}$ mice was unable to counteract the deleterious effect of CB_1 genetic ablation on striatal volume (Fig. 3*A*), striatal DARPP-32 expression (Fig. 3*B*) or RotaRod performance (Fig. 3*C*). Likewise, despite the remarkable loss of CB_1 receptors in the MSNs of R6/2

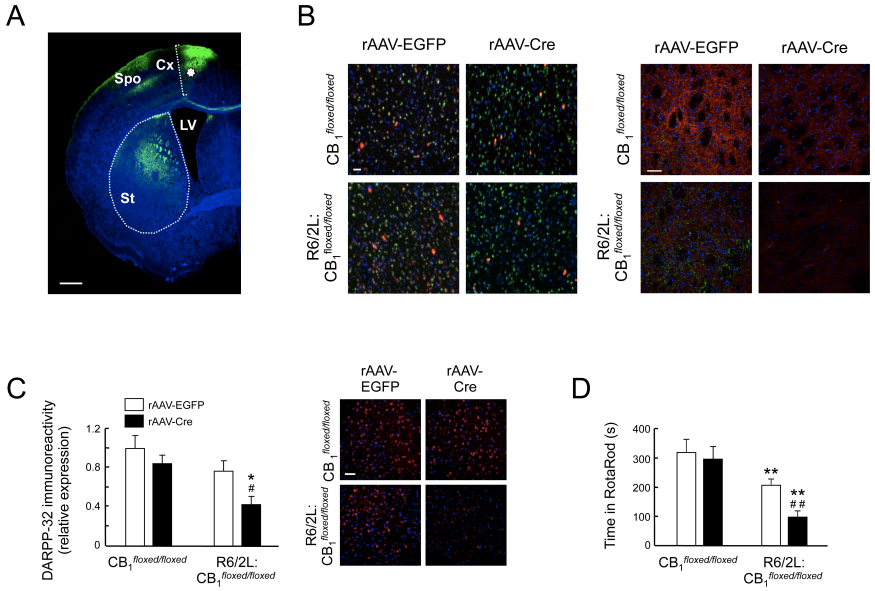

Fig. 4. Cre recombinase-driven deletion of CB_1 cannabinoid receptors in corticostriatal neurons aggravates HD-like neurodegeneration. (*A-D*) Four week-old R6/2L:$CB_1^{floxed/floxed}$ mice and $CB_1^{floxed/floxed}$ littermates were injected stereotactically into the motor cortex with a recombinant adeno-associated virus (rAAV) encoding Cre recombinase (or EGFP) under the control of the CaMKIIα promoter (n=8-12 animals per group). At week 20 of age RotaRod performance was evaluated, and the day after animals were sacrificed for histological analyses. (*A*) Example of a brain hemisphere injected with rAAV-CaMKIIα promoter-EGFP (EGFP, green; DAPI, blue). Note the striatal EGFP labelling. Cx, cortex; St, striatum; LV, lateral ventricle; Spo, injection spillover; asterisk, approximate site of injection. Scale bar, 500 µm. (*B*) *Left*: Representative images of CB_1 receptor and vesicular glutamate transporter-1 (vGluT-1) mRNA *in situ* hybridization in the motor cortex (CB_1, red; vGluT-1, green; DAPI, blue). Note the Cre-mediated reduction of CB_1 mRNA expression. The few spotted CB_1 highly-labeled cells are interneurons. Scale bar, 100 µm. *Right*: Representative images of CB_1 receptor immunostaining in the dorsolateral striatum (CB_1, red; EGFP, green; DAPI, blue). Note the Cre-mediated reduction of CB_1 protein expression. Scale bar, 50 µm. (*C*) DARPP-32 immunoreactivity in the dorsolateral striatum (relative values from the corresponding rAAV-EGFP-injected $CB_1^{floxed/floxed}$ group). Representative images of DARPP-32 staining are shown (DARPP-32, red; DAPI, blue). Scale bar, 50 µm. (*D*) RotaRod performance (time to fall). **P*<0.05, ***P*<0.01 from the corresponding $CB_1^{floxed/floxed}$ group. $^{\#}P$<0.05, $^{\#\#}P$<0.01 from the rAAV-EGFP-injected R6/2L:$CB_1^{floxed/floxed}$ group.

mice, picrotoxin did not prevent striatal neurodegeneration in our early-symptomatic (4-8 week-old; Fig. 3) or symptomatic (8-12 week-old; Fig. S4) R6/2:$CB_1^{+/+}$ mice. In contrast, MK-801 administration (0.03 mg/kg body weight/day) rescued all these HD-like neuropathological and behavioral alterations of R6/2:$CB_1^{-/-}$ mice up to the levels of their R6/2:$CB_1^{+/+}$ littermates (Fig. 3).

Cre recombinase-driven deletion of CB_1 cannabinoid receptors in corticostriatal but not striatal neurons aggravates HD-like neurodegeneration. To substantiate the selective neuroprotective activity of CB_1 receptors located on glutamatergic terminals in HD we crossed R6/2 mice with CB_1 receptor-floxed mice, thus generating a HD-like mouse line that allows the spatiotemporally-controlled excision of the *loxP*-flanked CB_1 receptor gene by Cre recombinase. Because this excision process can take several weeks in the mouse brain (20-22), we generated a new R6/2 mouse line (designated as R6/2L) that expresses a longer mutant tract (~250 CAG repeats) and has a longer survival (~30 week) than "normal" R6/2 mice [similar to other CAG tract-expanded R6/2 mouse-derived lines previously reported (23, 24)]. We therefore injected sterotactically these R6/2L:$CB_1^{floxed/floxed}$ mice (and $CB_1^{floxed/floxed}$ control littermates) with a recombinant adeno-associated viral vector encoding Cre (or EGFP as control) into either the dorsolateral striatum or the motor cortex. Cre expression was driven by the CaMKIIα promoter, and so it was confined to MSNs (injections into the striatum) or principal neurons (injections into the cortex). Cre-mediated excision of the *loxP*-flanked CB_1 receptor gene in dorsolateral MSNs of R6/2L:$CB_1^{foxed/floxed}$ mice (Fig. S5*A* and *B*) had no significant effect on DARPP-32 expression (Fig. S5*C*) or RotaRod performance (Fig. S5*D*). In contrast, inactivation of the CB_1 receptor gene in the motor cortices of R6/2L:$CB_1^{floxed/floxed}$ mice (Fig. 4*A* and *B*) worsened those two hallmarks of striatal integrity (Fig. 4*C* and *D*).

Discussion

In this report we show that a restricted population of CB_1 receptors, namely that located on glutamatergic terminals, plays an indispensable role in the neuroprotective activity of the endocannabinoid system. The size of this pool of glutamatergic-terminal CB_1 receptor molecules seems to be much smaller than that of GABAergic-terminal CB_1 receptors (4, 5). However, CB_1 receptors located on glutamatergic terminals are strongly coupled to heterotrimeric G-protein signaling (25) and, in fact, participate in the control of important neurobiological processes such as neuronal excitability (22), motor activity (26), feeding behavior (27) and anxiety (28). Our present findings support that this specific pool of CB_1 receptors should be considered a new key player in the excitotoxicity hypothesis of neural disease (29, 30). On mechanistic grounds, it is very plausible that, upon intense activation of a glutamatergic projection, glutamate spillover out of the synapse would trigger in the target neuron the activation of the perisynaptic machinery of endocannabinoid generation (5), composed of type 1 metabotropic glutamate receptors (mostly mGluR5), $G_{q/11}$ proteins, phospholipase C-β and diacylglycerol lipase-α, thus producing the endocannabinoid 2-arachidonoylglycerol, which would engage pre-synaptic CB_1 receptors located on the glutamatergic terminal, thereby inhibiting excess excitatory transmission (5) and buffering the potential neurototoxic effects of extra-synaptic NMDA receptors in the post-synaptic neuron (31, 32).

In the precise case of HD, it has been long suggested that the early and massive down-regulation of CB_1 receptors located on MSNs plays a pathogenic role in promoting disease onset and

progression (12, 33, 34). Thus, as the CB_1 receptor couples to several cell-autonomous neuroprotective pathways (6, 35), one might suppose that its down-regulation in MSNs would render these cells more susceptible to damage. However, as it is well established that CB_1 receptors located on MSNs inhibit GABA release (4, 5), it would also be conceivable that their notable loss enhanced extracellular GABA availability, thereby constituting an adaptive mechanism aimed at attenuating excitatory transmission and, in turn, excitotoxicity of MSNs. These possibilities notwithstanding, here, by using various pharmacological and genetic approaches, we were unable to detect any overt effect of the MSN-CB_1 receptor pool on striatal damage. In contrast, impairing the function of the corticostriatal-terminal CB_1 receptor pool produced remarkable deleterious effects in the striatum. This strongly supports that (i) the detrimental effects elicited by the complete genetic elimination of CB_1 receptors in HD mouse models (18, 19) are due to the inactivation of CB_1 receptors located on corticostriatal projections rather than on MSNs, and (ii) the beneficial effects exerted by pharmacological administration of THC on HD-like progression in symptomatic R6/2 mice (18) reflects the engagement of CB_1 receptors located on corticostriatal projections rather than those on MSNs. Hence, from a translational point of view, it is tempting to speculate that the glutamatergic-neuron CB_1 receptor pool may constitute a therapeutic target to attenuate neurodegeneration in HD patients. THC and other cannabinoids have a favorable drug safety profile and are already used in the clinic as anti-emetic, anti-cachectic, anti-spastic and analgesic compounds (36). Although exhaustive clinical studies are indeed necessary to assess whether cannabinoid-based medicines could be used for the management of neurodegenerative diseases, the findings reported here, by providing a specific neurobiological substrate for cannabinoid-evoked neuroprotection in preclinically-relevant models, may contribute to improving the development of therapeutic approaches aimed at targeting the glutamatergic-neuron CB_1 receptor population.

Materials and Methods

Animals. We used conditional mutant mice, generated by the Cre-lox technology, in which the CB_1 receptor gene is primarily absent from cortical glutamatergic neurons of the dorsal telencephalon ($CB_1^{floxed/floxed;Nex\text{-}Cre/+}$ mice; herein referred to as Glu-$CB_1^{-/-}$ mice) or from forebrain GABAergic neurons ($CB_1^{floxed/floxed;Dlx5/6\text{-}Cre/+}$ mice; herein referred to as GABA-$CB_1^{-/-}$ mice)(26, 27). Hemizygous mice transgenic for exon 1 of the human huntingtin gene with an expanded CAG tract (~160 CAG repeats; R6/2 mice) (37) were purchased from The Jackson Laboratory [Bar Harbor, ME; code B6CBA-Tg(HDexon1)62Gpb/1J]. Double-mutant R6/2:$CB_1^{-/-}$ mice were generated by crossing R6/2 mice with $CB_1^{-/-}$ mice as described (18). In some experiments we used another HD-like mouse line, designated as R6/2L, which expresses a longer mutant tract (~250 CAG repeats) and has a longer survival (~30 weeks) than the aforementioned "normal" R6/2 line. This new line was obtained by exploiting the fact that transmission of the CAG tract in R6/2 mice is unstable, with a tendency to expand through the male line. We crossed R6/2L mice with $CB_1^{floxed/floxed}$ mice to generate the double-mutant R6/2L:$CB_1^{floxed/floxed}$ line as follows: R6/2L males were first cross-mated with $CB_1^{floxed/floxed}$ females. The R6/2L:$CB_1^{floxed/+}$ F1 males were back-crossed with the aforementioned $CB_1^{floxed/floxed}$ females to obtain the R6/2L:$CB_1^{floxed/floxed}$ double-mutants and the respective $CB_1^{floxed/floxed}$ controls. The colony was maintained by back-crossing R6/2L:$CB_1^{floxed/floxed}$ males with $CB_1^{floxed/floxed}$ females. In all the experiments, mutant mice were compared with their corresponding littermates. Animal housing, handling and assignment to the different experimental groups were conducted as described (18). All the experimental procedures were performed in accordance with the guidelines and with the approval of the Animal Welfare Committee of Madrid Complutense University (DC 86/609/EU).

Drug treatments. THC (The Health Concept) was stored in DMSO. Just before the experiments, solutions of vehicle [1% (v/v) DMSO in Tween-20/saline (1:18, v/v)] and THC (2 mg/kg body weight/day) were prepared for i.p. injections. CNO (Santa Cruz) was prepared fresh in saline just before the experiments and administered i.p. at 10 mg/kg body weight/day. Stock solutions of MK-801 (Sigma) and picrotoxin (Sigma) were prepared in ethanol and, just before the experiments, diluted into sterile distilled H_2O (final ethanol concentration: 2%). Animals received i.p. injections of vehicle, MK-801 (0.03 mg/kg body weight/day) or picrotoxin (0.3 mg/kg body weight/day). These doses of MK-801 and picrotoxin were selected from our previous studies on feeding behavior (27) and memory (38), as well as from preliminary experiments on motor activity. Specifically, the drug doses used here were the highest ones that had no significant effect *per se* but were able to block CB_1 receptor-evoked effects on those parameters.

Quinolinic acid-induced excitotoxicity. Conditional mutant mice lacking CB_1 receptors in glutamatergic or GABAergic neurons, as well as WT [$CB_1^{floxed/floxed}$ littermates or C57BL6/N mice (Harlan), depending on the experiment], were injected stereotactically (unilaterally) with vehicle (1 µL PBS) or quinolinic acid (from 30 to 150 nmol in 1 µL PBS, pH adjusted to 7.5) (39) at the following dorsolateral-striatum coordinates (to bregma): antero-posterior +0.6, lateral +2.0, dorso-ventral -3.0. RotaRod performance was evaluated along the 3 following days. Mice were sacrificed the day after by intracardial perfusion and their brains were excised for immunofluorescence analyses.

Viral vectors. G_q-coupled human M3 muscarinic DREADD (hM3Dq) fused to mCherry (10) (kindly provided by Dr. Brian L. Roth, University of North Carolina, Chapel Hill, NC) and HA-tagged Cre recombinase, or mCherry and EGFP as respective controls, were subcloned in a recombinant adeno-associated virus (rAAV) expression vector with a minimal CaMKIIα promoter (kindly provided by Dr. Karl Deisseroth, Stanford University, Stanford, CA) by using standard molecular cloning techniques. All vectors used were of an AAV1/AAV2 mixed serotype, and were generated by calcium phosphate transfection of HEK293T cells and subsequent purification as described (22).

DREADD-induced excitotoxicity. Eight week-old male C57BL6/N mice were injected stereotactically with CaMKIIα-hM3Dq-rAAV or CaMKIIα-mCherry-rAAV (in 1.5 µL PBS) aimed at targeting the motor cortex projecting onto the dorsolateral striatum. Each animal received 2 bilateral injections at coordinates (to bregma): antero-posterior +1.5, lateral ±1.2, dorso-ventral -1.7; and antero-posterior -0.5, lateral ±1.2, dorso-ventral -1.2. Six weeks after surgery mice were assigned to different experimental groups and injected i.p. with vehicle or CNO (10 mg/kg body weight/day) (10), together with vehicle, MK-801 (0.03 mg/kg body weight/day) or THC (2 mg/kg body weight/day), for 4 weeks. RotaRod performance was analyzed along the last 3 days of treatment. Mice were subsequently sacrificed by intracardial perfusion and their brains were excised for immunofluorescence analyses.

Cre recombinase-driven deletion of CB_1 receptors. Four week-old R6/2L:$CB_1^{floxed/floxed}$ mice and their $CB_1^{floxed/floxed}$ littermates were injected stereotactically with CaMKIIα-Cre-rAAV or CaMKIIα-EGFP-rAAV (in 1.5 µL PBS) either into the motor cortex projecting onto the dorsolateral striatum or into the dorsolateral striatum. In the case of the cortex, each animal received 2 bilateral injections at coordinates (to bregma): antero-posterior +1.5, lateral ±1.2, dorso-ventral -1.7; and antero-posterior -0.5, lateral ±1.2, dorso-ventral -1.2. In the case of the striatum, each animal received one bilateral injection at coordinates (to bregma): antero-posterior +0.6, lateral +2.0, dorso-ventral -3.0. At week 20 of age RotaRod performance was analyzed. Mice were subsequently sacrificed by intracardial perfusion and their brains were excised for immunofluorescence analyses and *in situ* hybridization.

Organotypic cultures. Corticostriatal slices (300 µm-thick) were obtained from adult WT ($CB_1^{floxed/floxed}$) and Glu-$CB_1^{-/-}$($CB_1^{floxed/floxed;Nex\text{-}Cre/+}$) littermates, and cultured under semidry conditions in neurobasal medium supplemented with B27 (1%), N2 (1%), glutamine (1%), penicillin/streptomycin (1%), fungizone (1%) and ciprofloxacine (5 µg/ml), as described (40). Slices were incubated for 24 hours with vehicle (PBS) or quinolinic acid (50 µM), alone or in combination with vehicle (0.1% DMSO), THC (1 µM) and/or rimonabant (5 µM). Slices were subsequently fixed with formalin and processed in 15-µm sections, which were analyzed at equivalent regions of the rostral to caudal axis. Counting of DARPP-32 inmmunoreactivity was conducted in the dorsolateral striatum in a 1-in-6 series per slice.

Synaptosomes. Synaptosomes were obtained from mouse striata as described (41) and used for immunomicroscopy analyses (see below). Glutamate release was assayed in synaptosomal preparations from the P2 fraction with glutamate dehydrogrenase, and the fluorescence of NADPH was followed by on-line fluorimetry (PerkinElmer LS-50 luminescence spectrometer) (41). Stock solutions of WIN-55,212-2 were prepared in DMSO (final concentration in the assay: 0.1%).

***In situ* hybridization.** Cryosections (14 µm-thick) were incubated with digoxigenin-labelled riboprobes against mouse CB_1 receptor and/or FITC-labelled riboprobes against mouse vGluT-1 or GAD-67 as described(27). For signal amplification we used the TSA Plus Cyanine 3 & Fluorescein System (PerkinElmer). Cell nuclei were visualized with DAPI. Preparations were analyzed in an Axioplan 2 microscope (Carl-Zeiss). Co-expression data were obtained with Image J software (NIH) by counting fluorescence in a 1-in-6 series per animal in the deep motor cortex (layers 5/6) and the dorsolateral striatum, ranging from bregma +1.5 to -0.5 coronal coordinates.

Immunomicroscopy (mouse samples). Coronal free-floating sections (30 µm-thick) were obtained from paraformaldehyde-perfused mouse brains. Synaptosomes were seeded onto polylysine-coated cover glasses. Samples were incubated with antibodies against DARPP-32 (1:1000; BD), CB_1 cannabinoid receptor (1:500; kindly provided by Dr. Ken Mackie, Indiana University, Bloomington, IN), vGluT-1 (1:500; Synaptic Systems), and/or Bassoon protein (1:500; Synaptic Systems), followed by staining with the corresponding Alexa Fluor 488, 594 or 647 antibodies (1:1000; Life Technologies) (18).

Nuclei were visualized with Hoechst 33342 or DAPI. Counting of DARPP-32 immunoreactivity in the dorsolateral striatum was conducted in a 1-in-10 series per animal (from bregma +1.5 to -0.5 coronal coordinates), and data were calculated as immunoreactive area per total cell nuclei, except for the quinolinic acid-induced *in vivo* excitotoxicity experiments (Fig. 1*A*), in which data of immunoreactive area were referred to total counted area. Confocal fluorescence images were acquired using TCS-SP2 software and a SP2 AOBS microscope (Leica). Pixel quantification and co-localization were analyzed with Image J software.

Immunomicroscopy (human samples). Paraffin-embedded *post-mortem* 4 µm-thick brain sections containing caudate-putamen were kindly provided by Dr. Jean-Paul Vonsattel (New York Brain Bank at Columbia University, NY), are were obtained and handled following the ethical guidelines of that institution. Samples (4 sections per individual) were obtained from HD donors [grades 3-4; n=7; age (year-old) and sex: 54♂, 56♂, 56♂, 58♀, 59♀, 61♀, 72♀] and control subjects with no background of neuropsychiatric disease [n=5; age (year-old) and sex: 49♂, 57♂, 57♂, 68♀, 74♂]. Immunohistochemical analysis (42) was performed with anti-CB_1 cannabinoid receptor antibody (1:100; Thermo Scientific). Sections were further incubated with biotinylated goat anti-rabbit antibody (1:200), avidin-biotin complex (Vector Laboratories) and a diaminobenzidine substrate-chromogen system (Dako) to give a visible reaction product. For immunofluorescence analysis (42) sections were sequentially incubated with anti-vGluT-1 (1:250; Synaptic Systems), Alexa Fluor 488 (Life Technologies), anti-CB_1 cannabinoid receptor (1:50; Thermo Scientific) and Alexa Fluor 546 (Life Technologies) antibodies. Sections were treated with 1% Sudan Black in 70% ethanol to quench autofluorescence.

Behavior. Motor coordination (RotaRod performance) was evaluated as described (18). All assays were conducted before drug injections.

MRI. Striatal volume was measured by MRI in a BIOSPEC BMT 47/40 (Bruker) operating at 4.7 T as described (18).

Statistics. Data are presented as mean ± SEM. Statistical comparisons were made by ANOVA with *post hoc* Student-Neuman-Keuls test or by unpaired Student's *t* test. A *P* value of less than 0.05 was considered significant.

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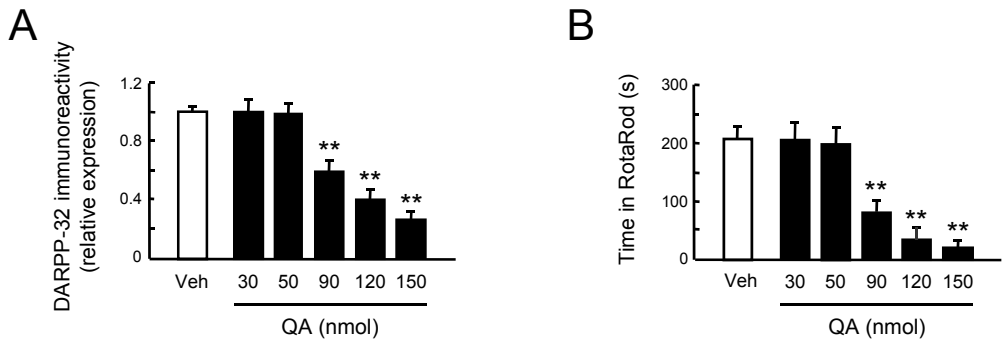

Fig. S1. Dose-dependent striatal damage induced by focal quinolinic acid injection. (*A*, *B*) WT (C57BL/6N) mice were injected intrastriatally with vehicle (Veh) or the indicated doses of quinolinic acid (QA; in 1 µL PBS, unilaterally) (n=6-8 animals per group). RotaRod performance was evaluated along the following 3 days (*B*), and the day after animals were sacrificed for determination of DARPP-32 immunoreactivity in the dorsolateral striatum (*A*; data expressed as relative values from the Veh-treated group). ***P*<0.01 from the Veh-treated group.

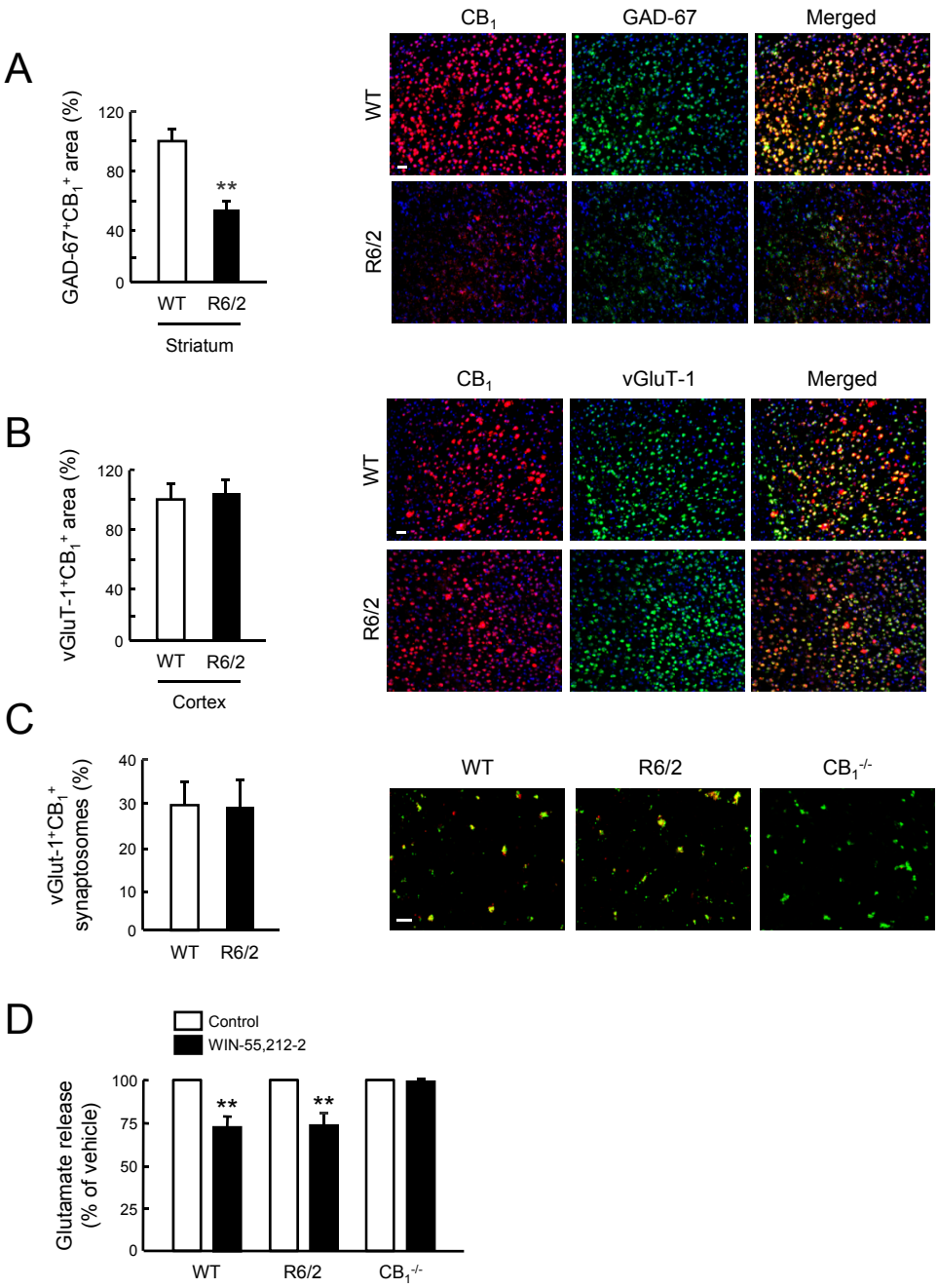

Fig. S2. CB_1 cannabinoid receptors are preserved on glutamatergic terminals in the striatum of symptomatic R6/2 mice. (*A*, *B*) Brain sections were obtained from 10 week-old R6/2 mice and WT littermates. The co-expression of CB_1 and glutamic acid decarboxylase of 67 kDa (GAD-67) mRNAs in the dorsolateral striatum (*A*) or CB_1 and vesicular glutamate transporter-1 (vGluT-1) mRNAs in the deep motor cortex (*B*) was quantified by *in situ* hybridization. Data are given as area of co-expression relative to total cells recorded (DAPI staining) (n=4 animals per group). Scale bars, 50 µm. ***P*<0.01 from the corresponding WT group. (*C*) Striatal synaptosomes were isolated from 10 week-old R6/2 mice and WT littermates. CB_1-expressing glutamatergic synaptosomes ($Bassoon^+vGluT\text{-}1^+CB_1^+$ structures) were counted and given as percentage of total synaptosomes ($Bassoon^+$ structures). Data correspond to 3 pools of R6/2 or WT mouse-derived synaptosomes, each of which was obtained by combining the striata of 4 R6/2 or WT mice. Representative images are shown (CB_1, red; vGluT-1, green; Bassoon, blue, omitted for clarity). Striatal synaptosomes from $CB_1^{-/-}$ mice were used as control of CB_1 staining. Scale bar, 20 µm. (*D*) CB_1 receptors preserved on glutamatergic terminals in the striatum of symptomatic R6/2 mice are functionally active. Effect of the cannabinoid receptor agonist WIN-55,212-2 (5 µM) on 30 mM KCl-evoked glutamate release in striatal synaptosomes from 10 week-old R6/2 mice and WT littermates. Striatal synaptosomes from $CB_1^{-/-}$ mice were used as control of WIN-55,212-2 action. ***P*<0.01 from the corresponding control group.

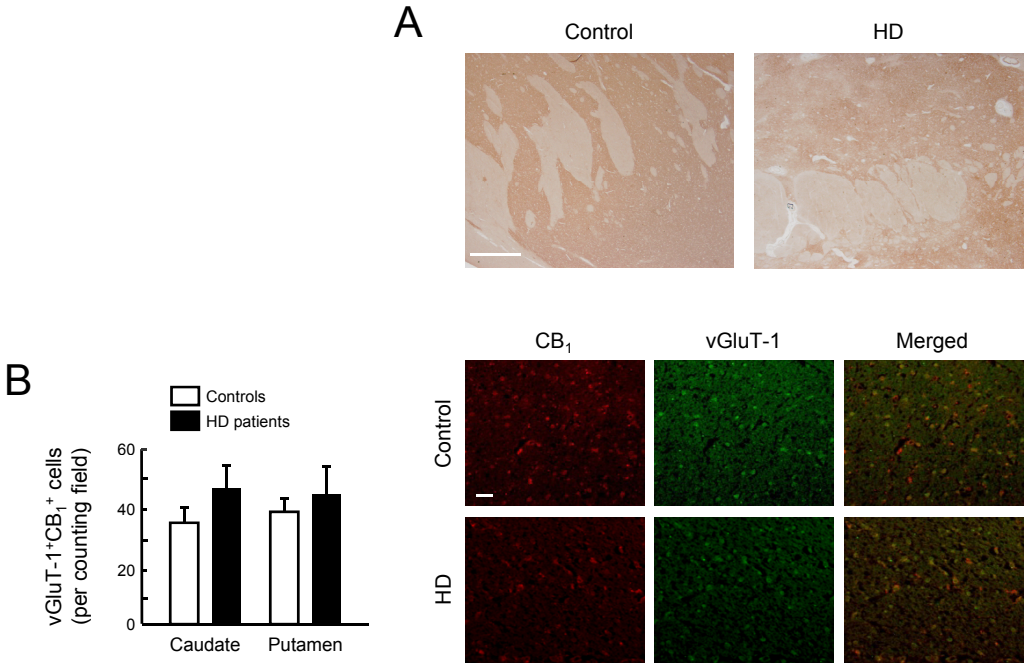

Fig. S3. CB_1 cannabinoid receptors are preserved on glutamatergic terminals in *post-mortem* caudate-putamen specimens of HD patients. (*A*) Representative low-magnification immunohistochemistry images of CB_1 receptor staining in a control subject and a HD patient. Scale bar, 800 µm. (*B*) Immunofluorescence analysis of CB_1/vGluT-1 co-expression in control subjects (n=5) and HD patients (grades 3-4; n=7). Representative images are shown (DAPI, blue, omitted for clarity). Scale bar, 50 µm.

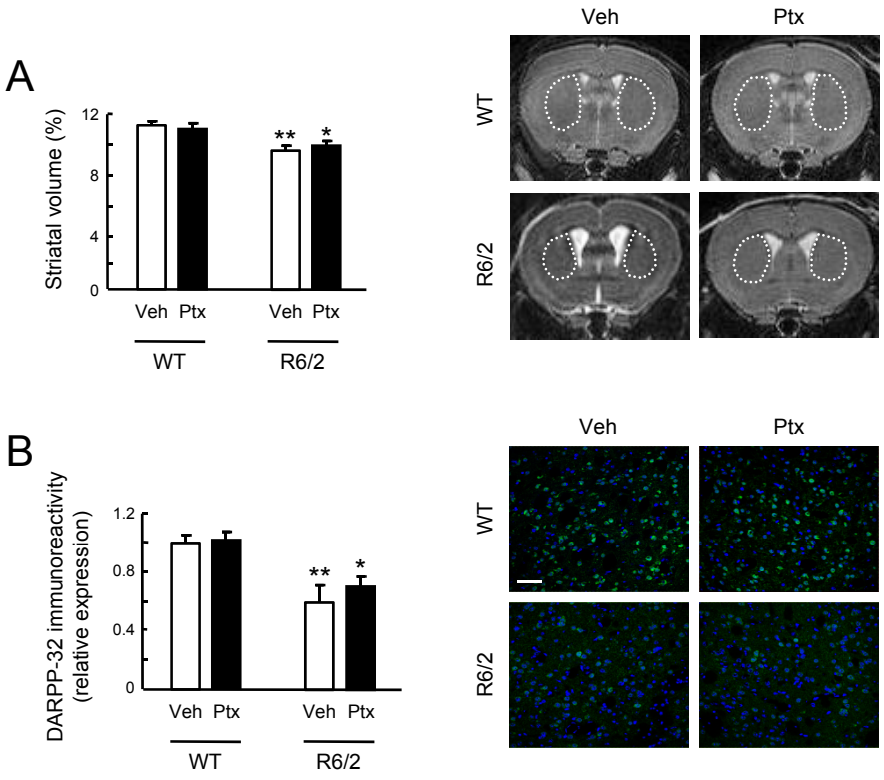

Fig. S4. Pharmacological blockade of $GABA_A$ receptors does not prevent HD-like striatal neurodegeneration in symptomatic R6/2 mice. (*A*, *B*) R6/2 mice and WT littermates were treated i.p. with vehicle (Veh) or picrotoxin (Ptx; 0.3 mg/kg body weight/day) from week 8 to week 12 of age. (*A*) Striatal volume (percentage of total brain volume). Representative MRI pictures are shown. The striata are outlined. (*B*) DARPP-32 immunoreactivity in the dorsolateral striatum (relative values from the Veh-treated WT group). Representative images are shown (DARPP-32, green; Hoechst 33342, blue). Scale bar, 50 µm. Data in *A* and *B* correspond to 12 week-old mice at the end of the treatments (n=6-8 animals per group). **P*<0.05, ***P*<0.01 from the corresponding WT group.

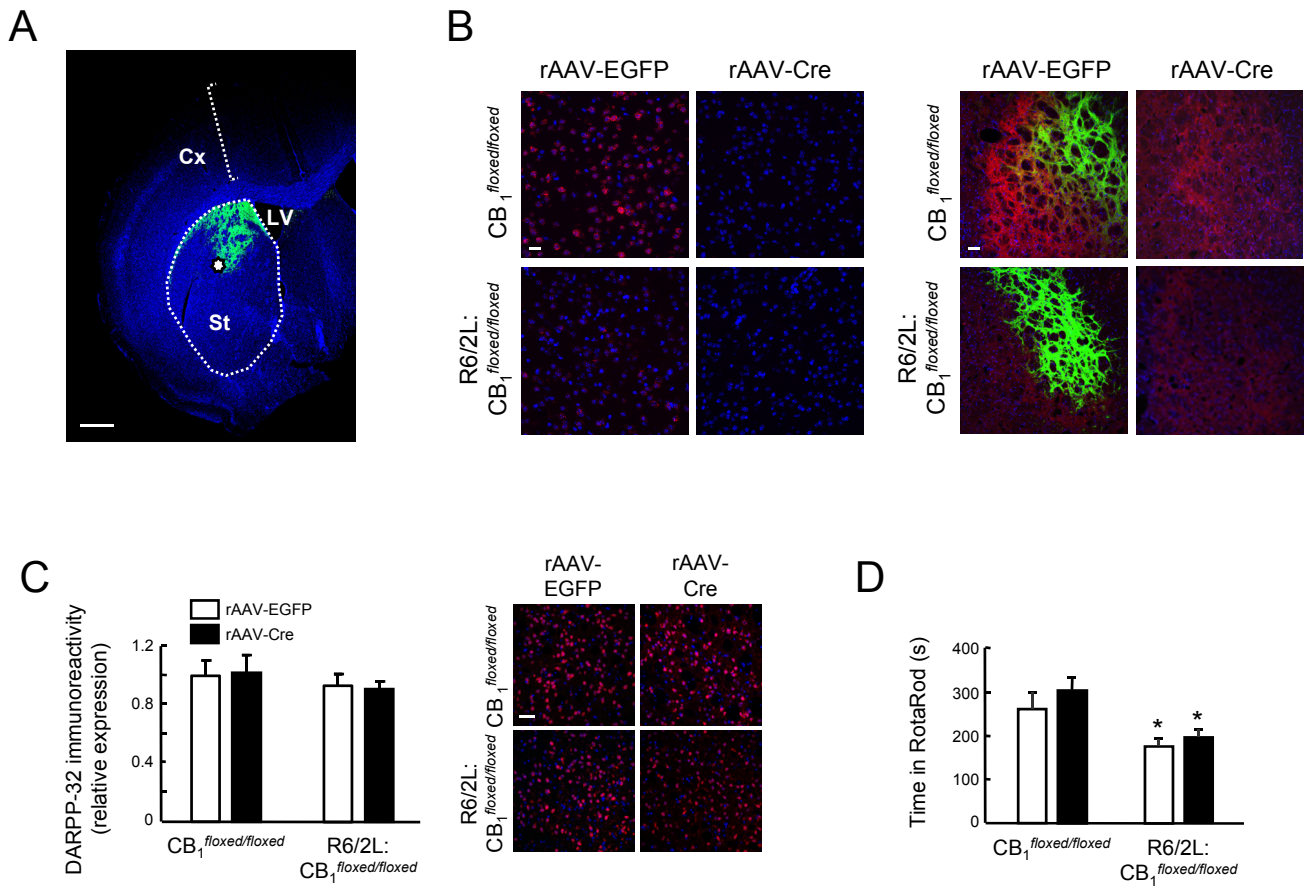

Fig. S5. Cre recombinase-driven deletion of CB_1 cannabinoid receptors in the dorsolateral striatum does not affect HD-like neurodegeneration. (*A-D*) Four week-old R6/2L:$CB_1^{floxed/floxed}$ mice and $CB_1^{floxed/floxed}$ littermates were injected stereotactically into the dorsolateral striatum with a recombinant adeno-associated virus (rAAV) encoding Cre recombinase (or EGFP) under the control of the CaMKIIα promoter (n=8-12 animals per group). At week 20 of age RotaRod performance was evaluated, and the day after animals were sacrificed for histological analyses. (*A*) Example of a brain hemisphere injected with rAAV-CaMKIIα promoter-EGFP (EGFP, green; DAPI, blue). Cx, cortex; St, striatum; LV, lateral ventricle; asterisk, approximate site of injection. Scale bar, 500 µm. (*B*) *Left*: Representative images of CB_1 receptor mRNA *in situ* hybridization in the dorsolateral striatum (CB_1, red; DAPI, blue). Note the Cre-mediated reduction of CB_1 mRNA expression. Scale bar, 50 µm. *Right*: Representative images of CB_1 receptor immunostaining in the globus pallidus (a major projecting area of MSNs; CB_1, red; EGFP, green; DAPI, blue). Note the Cre-mediated reduction of CB_1 protein expression. Scale bar, 50 µm. (*C*) DARPP-32 immunoreactivity in the dorsolateral striatum (relative values from the corresponding rAAV-EGFP-injected $CB_1^{floxed/floxed}$ group). Representative images of DARPP-32 staining are shown (DARPP-32, red; DAPI, blue). Scale bar, 50 µm. (*D*) RotaRod performance (time to fall). **P*<0.05 from the corresponding $CB_1^{floxed/floxed}$ group.

GENERAL DISCUSSION

Huntington's disease is a devastating neurodegenerative disorder caused by a single mutation that endows the huntingtin protein with new toxic functions, deleterious for brain cells. At the same time, the mutation also impairs the ability of the WT protein to exert molecular activities that are fundamental for the survival and functioning of the neurons that predominantly degenerate in the disease. Since the cloning of the HTT gene, the effort of several research groups contributed to the elucidation of several mechanisms responsible for the pathophysiology of the disease, although the reasons whereby the mutation in huntingtin causes the observed neuronal degeneration are still unclear. Among the molecular dysfunctions occurring in the disease, a decline in CB_1 receptor binding and expression in basal ganglia of HD patients and animal models has been widely reported (Denovan-Wright and Robertson, 2000; Glass et al., 2000; Lastres-Becker et al., 2002; McCaw et al., 2004), although its contribution to the pathogenesis and symptomatology of HD is still unclear.

Results from this Thesis contributed to describe the molecular mechanisms underlying the downregulation of the CB_1 receptor, and to define the potential contribution of the loss of the receptor to HD pathogenesis. These findings also demonstrated that the protective effect of cannabinoid stimulation is due to the CB_1 activation in a particular set of glutamatergic neurons projecting from the cortex to the striatum, revealing a novel putative therapeutic target.

Molecular mechanisms by which mHtt downregulates CB_1 receptor transcription

Huntingtin is a ubiquitous multi-domain protein involved in a plethora of cellular processes, including transcriptional regulation of gene expression (fully reviewed (Zuccato et al., 2010)). Wt HTT controls gene expression through the interaction with several transcription factors, and these interactions are usually disrupted in the presence of the mutant form of the protein (Rubinsztein and Carmichael, 2003). This induces alterations in the expression of a large number of genes, especially in striatum and motor cortex (Cha, 2007; Luthi-Carter et al., 2000).

The best established factor that participates in the Htt-mediated control of neuronal gene expression is the repressor element 1 silencing transcription factor (REST) (Cattaneo et al., 2005; Zuccato et al., 2003). REST is a transcriptional repressor that acts as a master regulator of neuronal genes in both neuronal (Palm et al., 1998; Wood et al., 2003) and non-neuronal cells (Belyaev et al., 2004). The interaction of WT Htt with REST has been vastly studied by Elena Cattaneo's group, in their quest to unravel the mechanisms responsible for the BDNF reduction in HD (Cattaneo et al., 2005; Zuccato et al., 2003). They found that wt Htt mantains REST inactive, retaining it in the cytoplasm, while the mutant form of the protein is unable to sequester the repressor, which translocates to the nucleus, binds the RE1 sequences on its target genes and inhibit their transcription (*Figure 13*).

Results from this Thesis (**Chatper 1**) demonstrated that the presymptomatic decline of

CB_1 receptor, observed both in patients and animal models of HD, is the result of an mHtt-mediated alteration of the CB_1 receptor gene (CNR1)

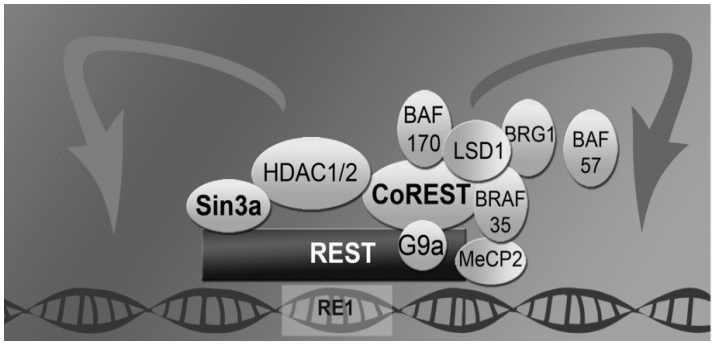

Figure 14: REST binds RE1 sites in the genome and exerts its repressive function by its interaction with a variety of co-regulator proteins. In order for REST to bind the RE1 sequence and repress the transcription of its target genes, other proteins are needed. These are: co-repressor Sin3a; CoREST; histone modifying enzymes such as HDACs, the histone methyltransferase G9a and the histone methylase LSD1; chromatin remodelling enzymes BAF170, BRG1, BAF57 and BRAF35; and the methyl CpG-binding protein, MeCP2.(Taken form Bithell et al Biochem Soc Trans. 2009 Dec;37(Pt 6):1270-5).

transcription through a REST-dependent mechanism, similar to that observed for BDNF. Even though REST-mediated repression of transcription is the best studied mechanism by which mHtt alters gene expression, the decrease in CB_1 mRNA and protein expression could be due to multiple mechanisms. Firstly, other than REST, Huntingtin can interact with other serveral specific transcription factors and co-factors, whose binding sites are also found on CNR1 promoter (Borner et al., 2008; Laprairie et al., 2013; McCaw et al., 2004; Miller and Devi, 2011). These include Sp1, CREB and CBP, $TAF_{II}130$, and NFKB (Buckley et al., 2010).

Apart from transcriptional activators and repressors, regulation of gene expression also relies on alteration of chromatine structure governed by histone post-translational modifications, such as acetylation, methylation, phosphorylation and ubiquitination (Strahl and Allis, 2000)). In general, acetylation of lysine residues on H3 or H4 corresponds to transcriptionally active chromatin and promotes transcription (Roth et al., 2001). In the context of HD, mutant huntingtin can impair histone acetylation, and in turn alter gene expression, through its interaction with histone acetyltransferase (Steffan et al., 2000). In a recent work of (Sadri-Vakili et al., 2007), the authors observed that genes normally downregulated in HD, being CNR1 one of those, are associated with hypoacetylated histones, thus are less transcripted.

Another post-transcriptional mechanism for the control of gene expression is represented by miRNAs. These small nucleotides (20-22 nucleotides) bind to the 3′ or 5′ untranslated region (UTR) of their target mRNA and promote the inhibition of its transcription and/or its degradation. miRNA are involved in a large variety of biological functions, including the regulation of neuronal functions (Kosik, 2006), and their dysregulation has been associated with several human disorders of the CNS (Abe and Bonini, 2013). In the context of HD, two recent reports have demonstrated that the neural miRNA system is indeed perturbed in HD probably via a REST-dependent mechanism (although other mechanisms cannot be excluded) (Johnson et al., 2008; Packer et al., 2008).

Interestingly, many of the neural miRNAs contain the conserved repressor element (RE1),

hence can be downregulated once REST is in the nucleus, contributing to the transcriptional dysregulation observed in the disease (Buckley and Johnson, 2011; Lee et al., 2011). Among these miRNAs, mir-124, whose function is to promote neuronal differentiation in the developing brain by suppressing non-neuronal genes, is downregulated.

Intriguingly, unpublished results of our group showed, by an *in silico* prediction, that mir-124 can putatively bind to the 3'UTR of the CB_1 receptor mRNA, being probably involved in the receptor's regulation of expression. We analyzed levels of mir-124 in the striatum of R6/2 mice and observed an reduction in the levels of the miRNA compared to the WT (as already described in the literature), whereas chronic treatment with THC was capable to restore the miRNA expression levels in these mice (*Figure 15A*). In parallel, we analyzed levels of mir-124 in mice lacking CB_1 receptors and observed a decline in mir-124 levels (*Figure 15B*). These results, although very preliminary, indicate that the stimulation of CB_1 receptor in HD context may be inducing mir-124 expression, in order to promote its own transcription: it has been shown that mir-124 antagonizes the REST/SCP1 pathway during embryonic development (Visvanathan et al., 2007) and prevent REST increase in the context of focal cerebral ischemia (Doeppner et al., 2013), hence CB_1 may be inducing mir-124, that, in turn, would repress REST (or another protein involved in the regulation of expression of the cannabinoid receptor).

In addition to transcriptional dysregulation of CNR1, mutant htt-mediated disruption of anterograde axonal transport (Zuccato et al., 2010) could participate in the cell-specific decrease of CB_1 receptor expression at axonal boutons (Horne et al., 2013).

Taken together, all these evidence point to the existance of several mechanisms that may be involved in the specific downregulation of CB_1 receptor in the striatum.

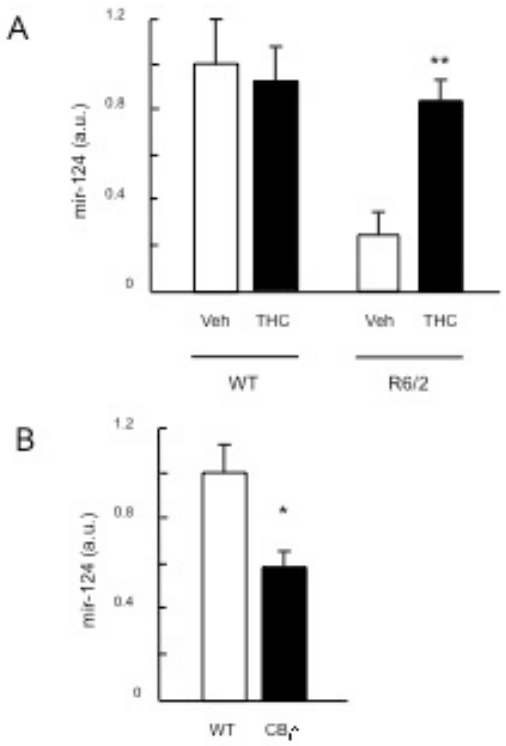

Figure 15: mir-124 levels in the striatum
A: Stritatal mir124 levels in R6/2 mice and their WT littermates, treated with either vehicle or THC (2 mg/kg/day; 4 weeks); B: striatal mir124 levels in CB1-/- mice and their WT littermates. *P<0.05; **P<0.01

Neuroprotective mechanisms mediated by CB_1

It is well established that CB_1 receptor engagement inhibits excitotoxic neurotransmission by blunting pre-synaptic glutamate release, and this has been put forward as a major event underlying CB_1 receptor-mediated neuroprotection (Galve-Roperh et al., 2008; Katona and Freund, 2008; Marsicano and Lutz, 1999; Shohami et al., 2011). However, it is plausible that additional processes contribute to the neuroprotective activity of the CB_1 receptor, since the receptor stimulation directly evokes signaling pathways associated with cell survival, such as those reliant on phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinases (MAPKs) and cAMP/protein kinase A (PKA) (Pertwee et al., 2010).

Neuronal survival depends on the local concentration gradients of growth factors, and neuronal viability may be enhanced by increasing the availability of neurotrophic factors. BDNF, one of the master neurotrophins in the mammalian forebrain (Nagahara and Tuszynski, 2011; Park and Poo, 2013), is crucial for sustaining neuronal survival and several studies have reported a close association between CB_1 receptor activity and the expression levels of BDNF (De March et al., 2008; Khaspekov et al., 2004; Marsicano et al., 2003). Anyway, despite the experimental evidence available, the molecular mechanism that connects BDNF and CB_1 in neuroprotection has not been elucidated yet.

In our group, we are currently trying to decipher the signaling pathways that underlie the neuroprotective effect evoked by CB_1 stimulation, and to assess the link between CB_1 receptor activation and BDNF expression. Preliminary results in a cellular model (STHdh mouse striatal neuroblasts) showed that the PI3K/Akt/mTORC1

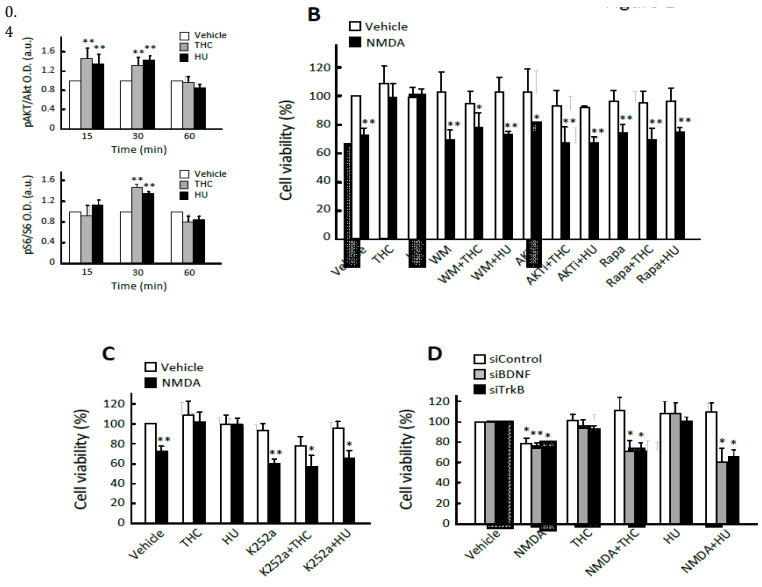

Figure 16: CB_1 receptor protects cultured striatal cells from excitotoxicity via PI3K/Akt/mTORC1/BDNF.
*A:*STHdh cells were incubated for the times indicated with vehicle, 0.5 µM THC or 10 nM HU-210. Cells were lysed and Western blot analyses were conducted. Quantification of mean optical density (O.D.) values relative to those of loading controls (respective total proteins*). B, C:* STHdh cells were preincubated for 5 h in Locke's solution with or without 1 mM NMDA together with vehicle, 0.5 µM THC, 10 nM HU-210, 0.2 µM wortmanin, 0.1 µM Akti-1/2, 30 nM rapamicin and/or 25 nM K252a, and subsequently incubated for 24 h in NMDA-free medium. Relative cell viability is shown (n=6-8 experiments). (**D**) STHdh cells were transfected with a non-targeted siRNA or with siRNAs directed against *BDNF* or *TrkB* and subsequently incubated for 5 h with or without NMDA, THC and/or HU-210 as in panel B. Relative cell viability is shown (n=4-6 experiments). **P*<0.05, ***P*<0.01 from the corresponding vehicle-treated cells.

pathway is activated upon cannabinoid stimulation (*Figure 15A*), and that its blockade with specific inhibitors abrogated the cannabinoid-evoked anti-excitotoxic action (*Figure 15B*). Furthermore, the pharmacological blockade of TrkB (the BDNF receptor; *Figure 15C*) and the silencing of both the neurotrophin and the receptor (*Figure 15D*) demonstrated that BDNF is involved in cannabinoid-induced neuroprotection. These results, despite being in the early stages, suggest that CB_1 evoke neuroprotection by activating the PI3K/Akt/mTORC1 axis.

Differential physiological relevance of CB_1 receptors subpopulations.

CB_1 receptors are expressed in different neuronal populations in the mammalian brain, and the physiological role of these various receptor populations has currently became a hot topic in the cannabinoid research. For example, a recent behavioral study demonstrated that the orexigenic effect of cannabinoids is mediated by CB_1 receptors residing on glutamatergic terminals, while the hypophagic effect of high doses of cannabinoids is modulated by the GABAergic pool of the receptor (Bellocchio et al., 2010). Likewise, the cannabinoid-mediated anxiolytic effect depends on the activation of glutamatergic CB_1, while the anxiogenic effect relies on the GABAergic pool of the receptor (Rey et al., 2012). Having described the pathophysiological relevance of CB_1 receptor in Huntington's disease (Chapter 1), we tried to define the precise pool of CB_1 receptors responsible for the neuroprotective effects of the endocannabinoid system modulation. In **Chapter 2** of this Thesis we observed that the CB_1 receptors located on glutamatergic corticostriatal terminals play a crucial role in protecting the brain from excitotoxic stimuli.

The biological reasons behind this differential action of the receptor are still uncertain, although several factors could influence cannabinoid signaling, thus granting differential proterties to the receptor, which may be specific of certain neuronal populations. Firstly, although CB_1 usually couples to $G_{o/i}$, it has been demonstrated that stimulation of CB_1 receptor results in the activation of various $G_{\alpha i}$ and $G_{\alpha o}$ subtypes in several brain regions (Prather et al., 2000), and that the efficacy and potency of cannabinoid agonists vary considerably for individual G protein subtypes, suggesting that different intracellular responses are produced by the CB_1 receptor depending on the preferential activation of different effectors by each G-protein. Moreover, in a recent report, Steindel et al. (2012)(Steindel et al., 2013), described that in glutamatergic cells of the hippocampus, the coupling between CB_1 and G protein is more efficient, being the ratio 1:3, compared to GABAergic neurons, where 2 receptor units bound to one G protein. In addition, the authors observed that glutamatergic pool of CB_1 has only a minimal tonic/constitutive activity (5-7%) (Roberto et al., 2010), compared to that located on GABAergic neurons (30-40%) (Slanina and Schweitzer, 2005). Therefore the tonic and phasic activities of the endocannabinoid system seem

unevenly distributed among CB_1 subpopulations. Thus, these findings could explain why CB_1 receptors located on glutamatergic neurons respond to low doses of cannabinoids and why they seem to be engaged in more physiological processes.

Besides the distinct coupling proterties of different CB_1 receptor populations, other factors, can contribute to the heterogeneous CB_1 functions in different neuronal circuits, for example, interacting partners. The fine-tuning of the basal activity of GPCRs relies on their interaction with ancillary proteins that affect receptors' signaling and trafficking. It has recently been shown that CB1 receptors interact with a novel protein, the cannabinoid receptor-interacting protein (CRIP1) (Niehaus et al., 2007). This protein exists in two isoforms, CRIP1a and CRIP1b, although, the best characterized is the former.

CRIP1a contains a C-terminus PDZ ligand, that may allow its interaction with other proteins and act as a scaffolding site to establish variations in signal transduction, enable the formation of homo/heterodimerization between CB_1 and/or other receptors and modulate CB_1 trafficking events such as localization, desensitization or internalization. Moreover, CRIP1a is differentially expressed in the mouse brain, co-localizing with CB_1 only in excitatory glutamatergic neurons, but not in inhibitory GABAergic interneurons (Ludanyi et al., 2008). A recent report demonstrated that the binding of CRIP1a to CB_1 reduces the receptor-mediated tonic inhibition of voltage-gated Ca^{2+} currents, probably in an agonist-independent manner (Niehaus et al., 2007). Subsequently, it has been shown that CRIP1a modulates the CB_1-mediated neuroprotection against glutamate excitotoxicity (Stauffer et al., 2011), and that CRIP1a overexpression disrupted the ability of cannabinoid receptor activation to protect cells against glutamate-induced excitotoxicity. Clearly, further research is needed to elucidate how CRIP1a influences CB_1 receptor activity; although these initial studies indicate that the presence or absence of CRIP1a may determine whether CB_1 activity is modulated in a specific neuronal population.

Regarding CB_1 trafficking, an elegant study has identified the G-protein-coupled receptor-associated sorting protein (GASP1) to be the main responsible for ligand-induced down regulation of CB_1 receptor (Martini et al., 2007). Since this process is highly variable among different brain areas (De Vry et al., 2004), different expression pattern of GASP-1 may underlie the differential CB_1 signaling.

Another mecahnism that could grant new biochemical characteristics to CB_1 receptors is the formation of homo- or heteromers that could grant the receptor with new biochemical characteristics. Like many other GPCRs, CB_1 physically and functionally interacts with itself to form homodimers and with other GPCRs or other receptor types as heterodimers or higher-order oligomers (Pertwee et al., 2010). The first evidence for CB_1 homodimerization came from a electron microscopy study in the amygdala, where CB_1 was proposed to dimerize in order to regulate GABAergic transmission in the

interneurons (Katona et al., 2001).

The most thoroughly studied CB_1 heteromer is the CB_1/D2 heteromer. It was initially demonstrated that the co-stimulation of CB_1 and D2 in striatal neurons led to an accumulation of cAMP, whereas stimulation of either receptor alone led to an inhibition of cAMP (Glass et al., 1997). This response was suggested to be the result of a D2-mediated switching of CB_1 from G_i to G_s signaling, and was eventually demonstrated the formation of this heteromer stabilizes a CB_1 active state with increased coupling to Gs (Kearn et al., 2005). To date, CB_1 has been found to form heteromers also with the μ-, κ-, and δ-opioid receptors, the orexin-1 receptor, the A2A adenosine receptor, the beta2 adrenergic receptor (β2AR), CB_2 and GPR55 (Callen et al., 2012; Carriba et al., 2007; Ellis et al., 2006; Henstridge et al., 2010; Wager-Miller et al., 2002).

Similar to the effect of the CB_1/D2 heterodimer on CB_1-G_s signalling, A2A adenosine receptor co-activation was required for effective CB_1-G_i signalling, as measured by inhibition of forskolin-mediated cAMP accumulation, in the context of a CB1/A2A heterodimer (Carriba et al., 2007). When CB_1 and A2A were co-expressed in heterologous systems, CB_1 only signalled through G_i when A2A was co-activated, (Carriba et al., 2007). These findings indicate that the physical interaction between CB_1 and A2A in the absence of an A2A agonist results in a conformation of CB_1 that does not allow for CB_1-G_i signalling to occur.

To further analyze the relevance of the heteromer formation in the differential activity of CB_1 receptor, our group conducted a preliminary

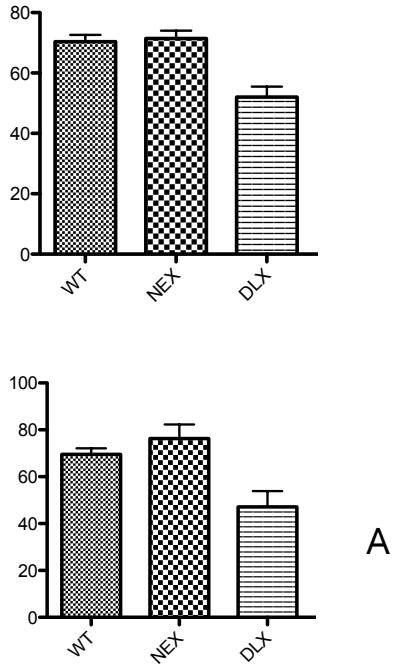

Figure 17: CB_1-A2A receptor heteromers differential expression in CB_1 knock-out contitional mutant mice (% dotted cells vs total nuclei **P<0.01.

screening in the brain of conditional mutant mice lacking CB_1 either in glutamatergic (Glu-$CB_1^{-/-}$ mice) or GABAergic neurons (GABA-$CB_1^{-/-}$ mice). We analyzed the formation of the following heteromers —CB_1-A2A, CB_1-A1, CB_1-CB_2, CB_1-D1 and CB_1-D2— using a newly developed technique, the so-called proximity ligation assay (PLA) (Gustafsdottir et al., 2005) that allows the easy detection of heteromers directly from tissues, in their physiological context. Among all the "couples" analyzed, we observed a significative reduction of the CB_1-A2A, both in the cortex and in the striatum of GABA-$CB_1^{-/-}$ mice (*Figure 16*). This preliminary result could explain the differential activity of CB_1 located on GABAergic

terminals, although additional experiment will be necessary to study whether this heteromer signals through different signaling pathways than each individual receptor.

As future perspective, a detailed neuroanatomical characterization is needed to explain the heterogeneicity of CB_1 receptor signaling. This can be done by further analyzing the signaling pathways underliyng the neruoprotective role of CB_1 in neuroprotection, by analyzing CB_1 receptor interaction with its accessory proteins and with other GPCRs in different neuronal populations.

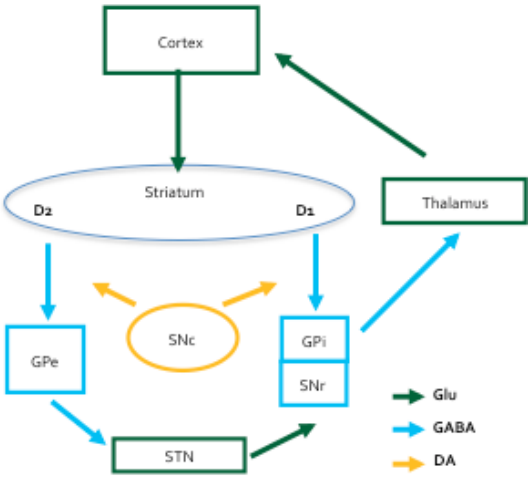

Figure 18: Cerebral circuits in which CB_1 is involved.

CONCLUSIONS

Despite the well-established role of the endocannabinoid system in neuroprotection, the mechanisms underlying this process remain unclear. In this Doctoral Thesis, we studied the physiological relevance and the therapeutic potential of the CB_1 receptor in neurodegeneration, especially in Huntington's disease.

The results obtained allow us to conclude that:

1-The down-regulation of CB_1 receptors by mutant huntingtin is dependent on a loss of WT huntingtin function, involves the control of the CB_1 receptor gene promoter by repressor element-1 silencing transcription factor.

2- CB_1 receptor is strongly involved in the pathophysiology of Huntington's disease. The loss of the receptor aggravates symptoms, neuropathology and molecular pathology of the disease, while the pharmacological stimulation of the receptor evokes beneficial effects.

3-The neuroprotective effects of the cannabinoid stimulation rely on the activation of a specific pool of CB_1 receptors, namely those located on the glutamatergic neurons that project from the cerebral cortex to the striatum.

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