Microglial CB₂ cannabinoid receptors are neuroprotective in Huntington’s disease excitotoxicity

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Cannabinoid-derived drugs are promising agents for the development of novel neuroprotective strategies. Activation of neuronal CB₁ cannabinoid receptors attenuates excitotoxic glutamatergic neurotransmission, triggers prosurvival signalling pathways and palliates motor symptoms in animal models of neurodegenerative disorders. However, in Huntington’s disease there is a very early downregulation of CB₁ receptors in striatal neurons that, together with the undesirable psychoactive effects triggered by CB₁ receptor activation, foster the search for alternative pharmacological treatments. Here, we show that CB₂ cannabinoid receptor expression increases in striatal microglia of Huntington’s disease transgenic mouse models and patients. Genetic ablation of CB₂ receptors in R6/2 mice, that express human mutant huntingtin exon 1, enhanced microglial activation, aggravated disease symptomatology and reduced mice lifespan. Likewise, induction of striatal excitotoxicity in CB₂ receptor-deficient mice by quinolinic acid administration exacerbated brain oedema, microglial activation, proinflammatory-mediator state and medium-sized spiny neuron degeneration. Moreover, administration of CB₂ receptor-selective agonists to wild-type mice subjected to excitotoxicity reduced neuroinflammation, brain oedema, striatal neuronal loss and motor symptoms. Studies on ganciclovir-induced depletion of astroglial proliferation in transgenic mice expressing thymidine kinase under the control of the glial fibrillary acidic protein promoter excluded the participation of proliferating astroglia in CB₂ receptor-mediated actions.
These findings support a pivotal role for CB2 receptors in attenuating microglial activation and preventing neurodegeneration that may pave the way to new therapeutic strategies for neuroprotection in Huntington’s disease as well as in other neurodegenerative disorders with a significant excitotoxic component.

**Keywords:** cannabinoid; microglia; Huntington’s disease; excitotoxicity; neurodegeneration

**Abbreviations:** BrdU = 5-bromo-2′-deoxyuridine; DARPP32 = dopamine- and cyclic AMP-regulated phosphoprotein; ECB = endocannabinoid; GFAP = glial fibrillary acidic protein; iNOS = inducible nitric oxide synthase; MRI = magnetic resonance imaging; PBS = phosphate buffered saline; WT = wild-type

### Introduction

Alterations in the endocannabinoid (ECB) system may be involved in the aetiopathology of different neurodegenerative disorders (Maccarrone et al., 2007; Katona and Freund, 2008). In particular, the potential therapeutic use of drugs targeting cannabinoid receptors in motor disorders, including multiple sclerosis, Parkinson’s and Huntington’s disease, is a subject of intense study (Centonze et al., 2007; Kreitzer and Malenka, 2007; Maccarrone et al., 2007; Maresz et al., 2007). In Huntington’s disease, neurodegeneration of stratal medium-sized spiny neurons and unbalanced neurotransmission are largely attributed to the interference of mutant huntingtin with transcriptional regulation (Cattaneo et al., 2005; Gil and Rego, 2008). One of the earliest neurochemical changes in Huntington’s disease patients is a strong depletion of neuronal CB1 cannabinoid receptors in the basal ganglia (Richfield and Herkenham, 1994; Glass et al., 2000). Likewise, striatal CB1 receptors are downregulated very early in transgenic mouse models of Huntington’s disease such as the R6/2 and HD94 models (Lastres-Becker et al., 2002; McCaw et al., 2004). These observations suggest that loss of neuronal CB1 receptors may be involved in the striatal dysfunction of the disease. The severe pre-symptomatic decline of CB1 receptors, together with the undesirable psychoactive effects triggered by CB1 receptor activation, prompts the search for alternative cannabinoid-based pharmacotherapies for Huntington’s disease.

Non-cell autonomous neurodegeneration may play a significant role in the progression of Huntington’s disease and other degenerative disorders. Overall, a dual role for microglial and astroglial cells has been proposed (Block et al., 2007; Lobsiger and Cleveland, 2007). An early activation of innate immune-response cells, including monocytes, macrophages and microglia, has been identified before the clinical onset of Huntington’s disease patients and models of the disease (Sapp et al., 2001; Simmons et al., 2007; Tai et al., 2007; Bjorkqvist et al., 2008). Activation of microglial cells during Huntington’s disease can exert a beneficial effect in neurodegenerative lesions (e.g. trophic and clearance activity), or instead increase damage severity by secreting pro-inflammatory mediators (Block et al., 2007; Lobsiger and Cleveland, 2007). In addition, astrocytes can contribute to Huntington’s disease neurotoxicity by impaired glutamate reuptake (Shin et al., 2005) or chemokine and cytokine homeostasis (Chou et al., 2008). The ECB system exerts an important role in the regulation of pivotal glial cell responses such as microglial reactivity (Eljaschewitsch et al., 2006) and astroglial neurotransmitter homeostasis (Navarrete and Araque, 2008). A protective effect of CB2 receptor activation in microglial cells upon inflammatory-induced CNS damage has been demonstrated in mouse models of multiple sclerosis (Maresz et al., 2007; Palazuelos et al., 2008). However, the precise role of microglial CB2 receptors in neurodegeneration remains uncertain (Miller and Stella, 2008). This study was therefore undertaken to address the role of microglial CB2 cannabinoid receptors in non-cell autonomous neurodegeneration during Huntington’s disease progression. We show that genetic ablation of CB2 receptors exacerbates Huntington’s disease-like behavioural symptoms and neurochemical alterations in R6/2 mice and, likewise, increases striatal injury after excitotoxicity. In addition, administration of CB2 receptor-selective agonists reduces striatal neurodegeneration by regulating microglial activation. Taken together, our results demonstrate that CB2 receptor activation is neuroprotective in Huntington’s disease models by controlling deleterious microglial activity.

### Materials and Methods

The following materials were kindly donated: CB2 receptor knockout mice by Nancy Buckley (National Institute of Health, Bethesda, MD, USA), and anti-iba-1 antibodies by Keiko Ohsawa (National Institute Neuroscience, Tokyo, Japan), respectively. HU-308 and other CB2 receptor-selective ligands by Raphael Mechoulam (Hebrew University, Israel) and Pharmos (Rehovot, Israel). Other antibodies used were: mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (Clon GA5, Sigma, St Louis, MO, USA), anti-NeuN (Chemicon International, Temecula, CA, USA) and anti-human CD68 (Dako, Glostrup, Denmark); rat monoclonal anti-5-bromo-2’-deoxyuridine (BrdU; Abcam, Cambridge, UK) and anti-mouse CD11b (clone M1/70; Becton Dickinson PharMingen, San Diego, CA, USA); rabbit polyclonal anti-CB2 receptor (PA1-746; Affinity Bioreagents, Golden, CO, USA), anti-dopamine- and cyclic AMP-regulated phosphoprotein (DARPP32, Chemicon), anti-synaptophysin (Synaptic Systems, Göttingen, Germany), anti-inducible nitric oxide synthase (Becton Dickinson) and anti-lysosomal-associated membrane protein 2 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA).

### Animal procedures and excitotoxicity experiments

Hemizygous male mice transgenic for exon 1 of the human huntingtin gene with 140–160 CAG repeats [R6/2 mice, code B6CBA-Tg(HDxen1)62Gpb/J] or expressing thymidine kinase under the control of the GFAP promoter [code B6.Cg-Tg(GFAP-Tk)7.1Msvs/J], as well as their corresponding wild-type (WT) littermates, were purchased from Jackson Laboratory (Ban Harbor, ME, USA). R6/1
transgenic Huntington’s disease mice with a CBA/BL6 background, expressing exon 1 of the mutant huntingtin gene with ~110–120 CAG repeats, were maintained and handled as described (Canals et al., 2004). Mice were housed under standard conditions (12 h light/dark cycle) in groups of mixed genotypes with access to food and water ad libitum. Both the housing and the experimental use of animals were approved by the Complutense University Animal Research Committee according to the European Union guidelines (86/609/EU) for the use of laboratory animals. Procedures were designed to minimize the number of animals used and their suffering. To generate double-mutant mice that express mutant huntingtin and are deficient in CB2 cannabinoid receptors, we cross-mated WT CBA female mice (Harlan, Barcelona, Spain) and CB2−/− (C57BL/6) male mice (Buckley et al., 2000) as described in Supplementary material online. For immunofluorescence experiments, mice were transcardially perfused at Week 12 (78–84 days) with phosphate buffered saline (PBS) followed by fixation with formalin and processed as described (Aguado et al., 2006). For experiments aimed to perform neurochemical determinations [γ-aminobutyric acid (GABA), nitrous oxide (NO), mRNA levels] mice were euthanized by decapitation and the indicated brain structures were carefully dissected and snap-frozen. GABA and NO quantification were performed as described (Supplementary material). Motor function was assessed by RotaRod analysis conducted with acceleration from 4 to 40 r.p.m. over a period of 570 s in a LE8200 device (Harvard Apparatus, Barcelona, Spain; Supplementary material). Experiments of depletion of astroglial cell proliferation were performed in GFAP-TK mice to which ganciclovir (100 mg/kg, Roche Farma, Madrid, Spain) or vehicle was administered by intraperitoneal (i.p.) injection daily starting 1 day prior to excitotoxicity. Excitotoxicity was induced by intrastriatal quinolinic acid (15 nmol), administration at the following coordinates: +0.6 posterior to bregma, +1.85 medio-lateral and –2.7 dorso-ventral to dura. Increased concentrations revealed dose-dependent striatal neurodegeneration (data not shown). Neurodegeneration and glial activation were investigated at different time points (4, 7 and 30 days). In pharmacological experiments, HU-308 (5 mg/kg) and additional CB2 receptor-selective agonists (cannabinor, PRS-639 and PRS-486) were administered i.p. after excitotoxicity and thereafter daily until sacrifice. Control animals received the corresponding vehicle injections (100 µl PBS supplemented with 0.5 mg/ml defatted bovine serum albumin and 4% dimethylsulphoxide). The number of mice with clonic–tonic seizures 2 h after excitotoxicity was determined in four independent experiments in CB2 receptor-deficient mice and WT littermates. BrdU injections (100 mg/kg, i.p.) were performed daily from 1 h after excitotoxicity and the following three consecutive days. Anti-inflammatory treatment was performed with minocycline administration (50 mg/kg, i.p.) 1 day prior to excitotoxicity and daily thereafter until sacrifice.

**Magnetic resonance imaging**

Magnetic resonance imaging (MRI) was performed in excitotoxicity experiments the day before sacrifice at the Nuclear Magnetic Resonance Centre of Complutense University (Madrid, Spain) using a Biospec 47/40 (Bruker, Ettlingen, Germany) operating at 4.7 T, equipped with a 12 cm gradient set and using a 4 cm radio frequency surface coil. The 3D T2-weighted spin-echo images were acquired using a fast spin-echo sequence. Diffusion water images delineated the area of neuroinflammation evidenced as hyperintense signals which are shown as pseudocoloured images. Acquisition information and further details can be found in Supplementary material.

**Immunofluorescence and confocal microscopy**

Free floating coronal brain slices (30 µm) were processed as described previously (Palazuelos et al., 2006). In brief, brain sections after blocking with 5% goat serum were incubated with the indicated primary antibodies (overnight incubation at 4°C) followed by secondary antibody incubation (1 h at room temperature). The appropriate mouse, rat and rabbit highly cross-adsorbed AlexaFluor 488, AlexaFluor 594 and AlexaFluor 647 secondary antibodies (Invitrogen, Carlsbad, CA, USA) were used. For double immunofluorescence with anti-iba-1 and anti-CB2 antibodies, the Fab fragment blocking system (Jackson Immunoresearch, Newmarket, UK) was employed following manufacturer’s instructions and the appropriate controls. All immunofluorescence data were obtained in a blinded manner by independent observers in a minimum of six correlative slices from 1-in-10 series located between –0.4 and +1.6 mm to bregma. Neurodegeneration and glial activation were determined by dopamine- and cyclic AMP-regulated phosphoprotein (DARPP32), CD11b, Iba-1 and GFAP immunostaining, and quantified with Image-J software. In transgenic mice and proliferation studies Iba-1, inducible nitric oxide synthase (iNOS) and BrdU-positive cells were quantified by cell counting using newCAST optical fractionator (Visiopharm, Hørsholm, Denmark) and data were given as mean cell number per square millimetre. Confocal fluorescence images were acquired by using Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with two passes by Kalman filter and a 1024 × 1024 collection box. In addition, fluorodecay staining was also performed (according to manufacturer’s instructions) and quantified as described in Supplementary material.

**Real-time quantitative PCR**

RNA was isolated using Trizol Reagent (Invitrogen). cDNA was obtained with Transcripter (Roche). Real-time quantitative PCR assays were performed using the FastStart Master Mix with Rox (Roche) and probes were obtained from the Universal Probe Library Set (Roche; primer sequences can be found in Supplementary Table S1). Amplifications were run in a 7900 HT-Fast Real-Time PCR System (Applied Biosystems). Each value was adjusted by using 18S RNA and β-actin levels as reference.

**Human brain tissue samples**

Post-mortem brain tissues from Huntington’s disease (n=6) and control donors (n=4) were provided by the ‘Banco de Tejidos para Investigación Neurologica’ (Madrid, Spain). Paraffin sections containing caudate putamen were cut into 4-µm thick sections (Benito et al., 2007) for immunohistochemical study, and non-fixed tissue was processed by Western blotting as described in Supplementary material.

**Statistical analyses**

Results shown represent the mean ± SEM, and the number of experiments is indicated in every case. Statistical analysis was performed by one- or two-way analysis of variance (ANOVA), as appropriate. A post hoc analysis was made by the Student Neuman–Keuls test. The occurrence of seizures in Table 1 was analysed by χ²-test.
Results

Increased CB2 cannabinoid receptor expression and microglial activation in the striatum of Huntington’s disease mouse models and patients

To address the potential involvement of CB2 cannabinoid receptors in Huntington’s disease pathogenesis, we used transgenic R6/2 mice that express exon 1 of human mutant huntingtin (Mangiarini et al., 1996). Real-time PCR analysis of striatal extracts revealed increased CB2 receptor transcript levels at pre-symptomatic (Week 4) and symptomatic stages (Week 8–12) of R6/2 mice (Fig. 1A). Similarly, in R6/1 mice, a more delayed Huntington’s disease murine model that expresses exon 1 of human huntingtin with fewer CAG repeats, CB2 transcripts were increased at Week 30 (Fig. 1B). Immunofluorescence characterization of R6/2 striatal sections at a symptomatic stage (Week 12) revealed increased microgliosis (Iba-1 + cells) and astrogliosis (GFAP + cells) (Fig. 1C). Microglial cells extended short processes and showed increased body size, indicating an activated rather than a resting state. Further confocal analysis was performed to elucidate the nature of the CB2 receptor-expressing cells. The glial response of R6/2 mice was accompanied by an increased number of CB2 receptor-positive cells. Microglial Iba-1-positive cells co-expressed CB2 receptors (Fig. 1D), whereas astrocytes and medium-sized spiny neurons (GFAP + and DARPP32 + cells, respectively) were negative for CB2 expression (Fig. 1E and data not shown).

To further validate the findings from the transgenic mouse models, we analysed the expression of CB2 receptors in brain samples of Huntington’s disease and control patients. Huntington’s disease patient samples revealed increased levels of CB2 receptors when compared with controls (Fig. 2A), and this occurred in parallel with increased levels of the microglial/macrophage markers CD11b and CD68. The cellular localization of CB2 receptors in the caudate putamen of Huntington’s disease patients was assessed by immunohistochemistry, which revealed that CB2 expression occurred in cells with the characteristic morphology of microglia (Fig. 2B). We next confirmed that cells

Table 1 Seizure score and survival of CB2 receptor-deficient mice after striatal excitotoxicity

<table>
<thead>
<tr>
<th>Mice group</th>
<th>Treatment</th>
<th>Seizure occurrence (%)</th>
<th>Survival (%)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>Vehicle</td>
<td>57</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Minocycline</td>
<td>29</td>
<td>100</td>
</tr>
<tr>
<td>CB2−/−</td>
<td>Vehicle</td>
<td>100a</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Minocycline</td>
<td>43*</td>
<td>100</td>
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The fraction of mice with seizures 2 h after quinolinic acid administration and the fraction of animal survival 24 h after excitotoxicity are indicated (n=7, each group). Mice were administered with minocycline or vehicle daily starting 1 day prior to striatal excitotoxicity.

*P<0.05 versus vehicle-treated CB2−/− mice.

#P<0.05 versus WT mice.

Figure 1 CB2 receptors are upregulated in microglial cells at symptomatic stages of Huntington’s disease animal models. (A and B) CB2 mRNA levels were determined by real-time quantitative PCR in striatal extracts of R6/2 and R6/1 mice at the indicated ages (black bars, n=6 each group). Transcript levels were normalized to β-actin RNA expression, referred to WT littermate levels (white bars, n=6) and given in arbitrary units (a.u.). *P<0.05, **P<0.01 versus the WT group at the corresponding ages. (C) Representative confocal microscopy analyses of striatal sections in WT and R6/2 mice at Week 12 were performed with anti-Iba-1 (green) and anti-GFAP (red) antibodies. (D) Expression of CB2 receptors (red) in Iba-1 (green) microglial cells was analysed in the same mice groups. Lower panels show orthogonal images of an example of CB2 receptor expression in Iba-1 + cell. (E) Expression of CB2 receptors (red) does not co-localize with GFAP (green) astroglial cells. Scale bars: 225 μm (C), 55 μm (D and E) and 15 μm (D, lower panels). CC = corpus callosum; Str. = striatum.
expressing CB2 receptors in Huntington’s disease correspond to CD68-positive microglia, but not to GFAP-positive astroglia (Fig. 2C and D).

**CB2 cannabinoid receptor deficiency exacerbates disease progression in R6/2 mice**

To investigate the role of CB2 cannabinoid receptor upregulation in R6/2 mice, genetic ablation experiments were achieved by crossing R6/2 mice with CB2 receptor-deficient mice (Buckley et al., 2000). R6/2 mice develop a rapid course of degeneration, with motor symptoms starting at Weeks 4–6 and mortality ensuing at Weeks 14–15. In the absence of CB2 receptors, R6/2 mice showed an aggravation of motor symptoms as determined by RotaRod analyses. Thus, a worse motor performance was observed in R6/2:CB2<sup>-/-</sup> mice than in R6/2:CB2<sup>+/+</sup> mice at Weeks 6 and 8 (Fig. 3A). Moreover, ablation of CB2 receptors reduced R6/2 mice lifespan (Fig. 3B).

We then analysed in detail striatal sections from double mutant R6/2:CB2<sup>-/-</sup> mice in comparison with R6/2:CB2<sup>+/+</sup> littermates. Iba-1-positive cells were quantified at Week 12 in R6/2:CB2<sup>-/-</sup> mice, revealing increased microglial activation when compared with CB2 receptor-expressing R6/2 littermates (Fig. 4A). The lack of CB2 receptors was also accompanied by increased astrogliosis (Fig. 4B). Importantly, the exacerbated phenotype of microglial and astroglial activation that occurred in R6/2:CB2<sup>-/-</sup> mice was associated with a higher expression of iNOS (Fig. 4C) and loss of the presynaptic marker synaptophysin (Cepeda et al., 2003) (Fig. 4D). Thus, during disease progression of transgenic R6/2 mice, microglial cell activation occurs in parallel with a concomitant upregulation of CB2 cannabinoid receptor expression, which appears to play a protective role against striatal dysfunction.

**CB2 cannabinoid receptor-deficient mice are more sensitive to striatal neurodegeneration after excitotoxicity and have an exacerbated glial activation**

In Huntington’s disease, loss of medium-sized spiny neurons is accompanied by excitotoxicity, that contributes to neurodegeneration (Gil and Rego, 2008; Kalia et al., 2008). To provide further support to the findings from CB2 receptor genetic deletion in mutant huntingtin-expressing mice, we sought to analyse the role of CB2 receptors in an excitotoxicity model of intrastriatal administration of the natural N-methyl-D-aspartic acid (NMDA) receptor agonist quinolinic acid. CB2 receptor-deficient mice showed higher occurrence of clonic–tonic seizures after quinolinic acid administration (Table 1). This higher sensitivity to excitotoxicity was reflected in an increased brain oedema volume (Fig. 5A), higher immunoreactivity of the microglial markers CD11b and Iba-1, as well as increased GFAP<sup>+</sup> reactive astroglial cells in the lesion area when compared with their WT littermates (Fig. 5B and C; Supplementary Fig. 1). A more detailed evaluation by gene expression analysis in the damaged striatum confirmed the enhanced activation of glial cells in CB2 receptor-deficient mice, which exhibit increased transcript levels of Iba-1, GFAP and the inflammatory markers iNOS, interleukin-1β, interleukin-6 and tumor necrosis factor-α. In addition, NO levels were also higher in CB2<sup>-/-</sup> mice than in their WT littermates (Supplementary Fig. 1). As in Huntington’s disease transgenic models, CB2 receptor transcripts were upregulated after striatal excitotoxicity and expressed by microglial but not astroglial cells (Supplementary Fig. 2A–C). Confocal microscopy analysis indicated that the
increase in microglial cells induced by excitotoxicity corresponds to actively phagocytizing cells as shown by the presence of neuronal debris in Iba-1 positive cells and the lysosomal-associated membrane protein 2 marker (Supplementary Fig. 2D and E).

Quantification of medium-sized spiny neuron degeneration by DARPP32 immunofluorescence evidenced an extended neuronal loss in CB2 receptor knockout mice 7 and 30 days after injury (Fig. 5D and Supplementary Fig. 1). The observed increase in microglial and astroglial response to excitotoxicity and the severe neurodegeneration of CB2 receptor-deficient mice appeared in concert with an exacerbated motor function impairment as revealed by RotaRod performance (Fig. 5E). Further analysis of the lesion by BrdU labelling after striatal excitotoxicity revealed that cell proliferation was enhanced in the absence of CB2 receptors (Supplementary Fig. 3). Immunofluorescence co-localization studies showed that the majority of the proliferative cells were astroglial and microglial cells (Supplementary Fig. 3).

Selective stimulation of CB2 cannabinoid receptors attenuates glial activation and protects from striatal neuronal damage

As loss-of-function studies indicated that CB2 receptor expression prevents neurodegeneration in Huntington’s disease models, we next turned to investigate the effect of direct CB2 receptor stimulation by using the non-psychotropic CB2-selective agonist HU-308 (Hanus et al., 1999). Mice were injected with quinolinic acid, and HU-308 or vehicle was administered daily after excitotoxicity. Analysis of neurological damage by MRI evidenced that brain oedema was significantly reduced in HU-308-treated mice after excitotoxicity (Fig. 6A). CB2 receptor engagement decreased microglial activation and astroglial reactivity in the striatum after excitotoxicity (Fig. 6B and C; Supplementary Fig. 4). This was further confirmed by real-time PCR analyses of different markers of glial activation and inflammation, as well as by the determination of NO levels (Supplementary Fig. 4). The relevance of CB2 receptor activation in HU-308-treated mice was evidenced by reduced loss of medium-sized spiny neurons as revealed by fluorojade staining and DARPP32 immunofluorescence (Fig. 6C and D). Furthermore, cannabinoid administration attenuated the loss of striatal GABA levels (Fig. 6E) and improved motor performance after excitotoxicity (Fig. 6F). In addition to HU-308, other CB2-selective agonists were effective in reducing striatal neurodegeneration, GABA deficits and glial activation (Supplementary Fig. 5).

Regulation of microglial activation is responsible for CB2 cannabinoid receptor-mediated neuroprotection

To address the role of exacerbated glial activation in striatal neurodegeneration of CB2 receptor-deficient mice after excitotoxicity, we employed the anti-inflammatory drug minocycline (Chen et al., 2000). Minocycline treatment reduced the occurrence of excitotoxicity-induced clonic–tonic seizures in CB2 receptor-deficient mice (Table 1), and this protective effect was associated to better RotaRod function (Fig. 7A). Anti-inflammatory treatment reduced the exacerbated glial activation (Fig. 7B–D) and medium-sized spiny neuronal loss (Fig. 7B and E) of CB2 receptor knockout mice. The protective action of the anti-inflammatory treatment against striatal excitotoxicity, together with the role of CB2 receptors in the control of microglial neuroinflammatory processes (Miller and Stella, 2008), supports that the beneficial effects of HU-308 rely specifically on the regulation of microglial activation by CB2 receptors. To validate the cellular target of cannabinoid protective actions after excitotoxicity, inducible transgenic mice that allow the depletion of proliferating astroglial cells were used. Thymidine kinase activity driven under the control of the GFAP promoter (GFAP-TK) was initiated by ganciclovir administration prior to striatal excitotoxicity. Excitotoxicity affected
Figure 4 CB₂ receptor deficiency in R6/2 mice exacerbates striatal glial activation and lowers synaptophysin expression. (A) Microglial cells were quantified by immunofluorescence with the anti-Iba-1 antibody in striatal sections of the indicated mice genotypes at Week 12 (n=5 each group). (B) Reactive astrogliosis was determined by GFAP immunofluorescence and quantification of immunoreactivity in the indicated mice groups. (C) iNOS immunofluorescence was determined as above. (D) Synaptophysin immunoreactivity was analysed as above. Representative images of each genotype are shown in the lower panels. Scale bars: 190 μm (A), 60 μm (B–D). **p < 0.01 versus WT mice group. ##p<0.01 versus R6/2:CB₂+/+ mice group.
motor coordination to the same extent in WT and GFAP-TK mice (Fig. 8A), and in the absence of newly generated astroglia the striatal damage induced by excitotoxicity was equivalent (Fig. 8B). In addition, pharmacological protection by HU-308 was equally effective in the presence or absence of proliferating astrocytes, with motor performance in the RotaRod analysis reaching similar levels in WT and transgenic mice (Fig. 8A). Confocal microscopy analysis revealed that ganciclovir-treated GFAP-TK mice showed equivalent HU-308-mediated effects of glial activation (Fig. 8C and D), and, likewise, prevention of medium-sized spiny neuron degeneration by HU-308 was not affected by ablation of astroglial proliferation (Fig. 8E).

Discussion

During Huntington’s disease progression, the ECB system is significantly affected and, specifically, CB1 receptors are known to be downregulated at early stages (Glass et al., 2000; McCaw et al., 2004). The consequent loss of CB1 receptor function may therefore contribute to unbalanced GABAergic neurotransmission (Centonze et al., 2005), deterioration of striatal brain functions as motor coordination (Kreitzer and Malenka, 2007) and impairment of CB1-mediated neuroprotection (Pryce et al., 2003; Pintor et al., 2006). Here we show that, in contrast to neuronal CB1 receptors, microglial CB2 receptors are induced in Huntington’s disease animal models and patients, and demonstrate for the first time in a transgenic model of a neurodegenerative disease that CB2 cannabinoid receptor ablation exacerbates microglial activation and accelerates the appearance of symptoms. Moreover, pharmacological activation of CB2 receptors in vivo, that does not induce undesirable psychotropic actions (Mackie, 2006), exerts a neuroprotective effect in Huntington’s disease models that relies on the control of deleterious microglial activity. CB2 receptors had been shown previously to confer neuroprotection in different animal models of acute brain toxicity and neuroinflammatory damage. Thus, CB2 receptor-deficient mice have enhanced susceptibility to neurodegeneration in experimental autoimmune
Figure 6 Pharmacological CB₂ receptor activation decreases striatal neurodegeneration after excitotoxicity. (A) Representative MRI images 2 days after intrastriatal injection of quinolinic acid or vehicle (Sham, dashed bars). HU-308 (5 mg/kg, black bars) or vehicle (white bars) were administered daily after excitotoxicity. Coloured areas correspond to the low water diffusion signal. The volume of striatal oedema was quantified 2 and 4 days after excitotoxicity and represented (n=5 each group). (B) CD11b and GFAP immunoreactivity was quantified in the total striatal area. (C) Characterization of neuronal damage by confocal microscopy was performed with antibodies against DARPP32 (green), CD11b (red) and GFAP (blue) in vehicle and HU-308-treated mice. (D) Fluorochrome staining and DARPP32 immunoreactivity were quantified and the number of positive cells as well as the area loss are represented, respectively. Representative images of fluorochrome-positive cells are shown. (E) GABA levels were determined in striatal extracts from treated mice and referred to total protein levels. (F) RotaRod analysis at the indicated days after striatal excitotoxicity. Scale bars: 2 mm (A), 170 μm (C), 25 μm (D). Determinations in B–E were performed 4 days after excitotoxicity. *P < 0.05, **P < 0.01 versus vehicle-treated mice.
encephalomyelitis (Maresz et al., 2007; Palazuelos et al., 2008) and upon intrastriatal malonate injection (Sagredo et al., 2008). Likewise, administration of CB2 receptor-selective agonists is neuroprotective in models of hypoxia or excitotoxicity (Palazuelos et al., 2006), experimental autoimmune encephalomyelitis (Docagne et al., 2007; Palazuelos et al., 2008), β-amyloid peptide inoculation (Ramirez et al., 2005) and lateral amyotrophic sclerosis (Shoemaker et al., 2007). These observations suggest that CB2 receptor activation may be a potential therapeutic strategy for neurodegenerative disorders (Mackie, 2006; Fernandez-Ruiz et al., 2007). The protective role of CB2 receptor activation is mostly attributed to its ability to reduce the deleterious microglial activation (Maresz et al., 2005; Ramirez et al., 2005; Eljaschewitsch et al., 2006; Palazuelos et al., 2008), although other studies support that CB2-mediated regulation of astroglial reactivity also contributes to cannabinoid neuroprotection (Docagne et al., 2007; Garcia-Ovejero et al., 2009). In this study, the use of GFAP-TK transgenic mice cogently shows that regulation of microglial rather than astroglial activation is responsible for CB2 receptor neuroprotective effect in Huntington’s disease models.

Regulation of non-cell autonomous neurodegeneration has been proposed as an important event in the progression of different neuroinflammatory disorders (Block et al., 2007; Lobsiger and Cleveland, 2007) and, in particular, the beneficial or detrimental influence of reactive microglia and astroglia remains a matter of intense debate. Ablation of reactive astrocytes increases the severity of the damage in traumatic brain injury (Myer et al., 2006) and, likewise, reactive astroglia may be beneficial in spinal cord injury models (Okada et al., 2006). However, conflicting findings on the role of astroglial cells have also been reported in, for example, models of motor neuron degeneration (Lepore et al., 2008; Yamanaka et al., 2008). Regarding Huntington’s disease, the role of non-cell autonomous neuronal death in the progression

**Figure 7** Minocycline treatment inhibits microglial activation and striatal neurodegeneration after excitotoxicity in CB2 receptor-deficient mice. (A) RotaRod performance after striatal excitotoxicity. The effect of daily injection of minocycline (i.p.) after quinolinic acid administration was compared in CB2−/− mice and their WT littermates (n=6 each group). (B) Confocal immunofluorescence images with antibodies for CD11b (red), GFAP (blue) and DARPP32 (green) in the indicated mice groups. Scale bar: 150μm. (C–E). Quantification of microglial and astroglial activation determined by CD11b and GFAP immunoreactivity analysis, and the area loss of striatal medium-sized spiny neurons determined by DARPP32 immunoreactivity. All determinations were performed 7 days after excitotoxicity. *P<0.05, **P<0.01 versus their respective WT mice group; #P<0.05, ##P<0.01 versus their respective vehicle-treated mice.
of the disease has been recently assessed. Both brain and peripheral inflammation are observed prior to Huntington’s disease onset and contribute to disease progression (Bjorkqvist et al., 2008). Likewise, anti-inflammatory treatment with minocycline exerts a neuroprotective effect and delays mortality of R6/2 mice (Chen et al., 2000; Wang et al., 2003), indicating a deleterious action of activated microglia on neuronal cells. In addition, astroglial cells can contribute to mutant huntingtin-induced neurodegeneration, as intranuclear aggregates impair GLT-1 and GLAST glutamate transporter expression (Shin et al., 2005). Thus, although the contribution of microglial cells to Huntington’s disease progression is highlighted from this and previous studies, the contribution of astrocytes cannot be completely ruled out (e.g. as microglia-activated secondary sources of neurotoxic mediators). Indeed, there is a clear parallelism in microglial and astroglial responses to brain injury, which makes a very difficult task to fully elucidate the precise participation of each glial cell type in non-cell autonomous neurodegeneration, even using selective cellular depletion strategies.

Our finding of increased CB₂ receptor expression in active striatal microglia of Huntington’s disease patients is in agreement with the recent indications of an innate inflammatory response occurring from early stages of the disease (Bjorkqvist et al., 2008). In addition, in Huntington’s disease pre-symptomatic gene carriers, positron emission tomography studies have evidenced that microglial activation is an early pathogenic step associated to disease progression (Tai et al., 2007). Likewise, CB₂ receptors are upregulated in reactive microglial cells in human neurodegenerative disorders such as multiple sclerosis (Benito et al., 2007; Palazuelos et al., 2008), Alzheimer’s disease (Ramirez et al., 2005) and others (Fernandez-Ruiz et al., 2007). The ECB-degrading enzyme fatty acid amide hydrolase constitutes...
an additional marker of neuroinflammation that labels reactive astroglia in multiple sclerosis (Benito et al., 2007). Thus, in addition to altered CB$_2$ receptor levels during neuroinflammation, changes in the levels of different ECB species have been reported in the striatum and cortex of R6/2 mice and Huntington’s disease patients (Maccarrone et al., 2007). Overall, the reduction of neuronal CB$_1$ receptors and the upregulation of microglial CB$_2$ receptors support a crucial role for the ECB system in the pathogenesis of Huntington’s disease. The use of drugs targeting the ECB system via CB$_1$ receptors aimed at restoring neurochemical alterations and palliating symptoms might constitute an interesting strategy for the management of Huntington’s disease and other neurodegenerative disorders with a significant excitotoxicity component (Katona and Freund, 2008). However, due to the early loss of CB$_1$ receptors during Huntington’s disease, the use of CB$_2$-selective agonists that avoid the undesirable psychotropic effects of CB$_1$ agonists may become an excellent alternative for disease control by taking advantage of the ability of these drugs to counteract neuron non-cell autonomous degeneration by active microglia.

**Acknowledgements**

We are grateful to J. Alberch, J. García de Yebenes and J. J. Lucas for fruitful discussions and sharing samples from Huntington’s disease models and patients and indebted to A. Chiarlone for excellent experimental assistance.

**Funding**


**Supplementary material**

Supplementary material is available at *Brain* online.

**References**


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