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Microglial CB_2 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 CB_1 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 CB_1 receptors in striatal neurons that, together with the undesirable psychoactive effects triggered by CB_1 receptor activation, foster the search for alternative pharmacological treatments. Here, we show that CB_2 cannabinoid receptor expression increases in striatal microglia of Huntington's disease transgenic mouse models and patients. Genetic ablation of CB_2 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 CB_2 receptor-deficient mice by quinolinic acid administration exacerbated brain oedema, microglial activation, proinflammatory-mediator state and medium-sized spiny neuron degeneration. Moreover, administration of CB_2 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 CB_2 receptor-mediated actions.

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These findings support a pivotal role for CB_2 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 striatal 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 CB_1 cannabinoid receptors in the basal ganglia (Richfield and Herkenham, 1994; Glass *et al*., 2000). Likewise, striatal CB_1 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 CB_1 receptors may be involved in the striatal dysfunction of the disease. The severe pre-symptomatic decline of CB_1 receptors, together with the undesirable psychoactive effects triggered by CB_1 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 homoeostasis (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 homoeostasis (Navarrete and Araque, 2008). A protective effect of CB_2 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 CB_2 receptors in neurodegeneration remains uncertain (Miller and Stella, 2008). This study was therefore undertaken to address the role of microglial CB_2 cannabinoid receptors in non-cell autonomous neurodegeneration during Huntington's disease progression. We show that genetic ablation of CB_2 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 CB_2 receptor-selective agonists reduces striatal neurodegeneration by regulating microglial activation. Taken together, our results demonstrate that CB_2 receptor activation is neuroprotective in Huntington's disease models by controlling deleterious microglial activity.

Materials and methods

The following materials were kindly donated: CB_2 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 CB_2 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-CB_2 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

Hemizigous male mice transgenic for exon 1 of the human huntingtin gene with 140–160 CAG repeats [R6/2 mice, code B6CBA-Tg(HDexon1)62Gpb/1J] or expressing thymidine kinase under the control of the GFAP promoter [code B6.Cg-Tg(GFAP-Tk)7.1Mvs/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 CB_2 cannabinoid receptors, we cross-mated WT CBA female mice (Harlan, Barcelona, Spain) and $CB_2^{-/-}$ (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 neurodegeration (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 CB_2 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 CB_2 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 T_2-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-CB_2 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, fluorojade 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 Transcriptor (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 χ^2-test.

Table 1 Seizure score and survival of CB_2 receptor-deficient mice after striatal excitotoxicity

Mice group	Treatment	Seizure occurrence (%)	Survival (%)
WT	Vehicle	57	100
	Minocycline	29	100
$CB_2^{-/-}$	Vehicle	100#	71
	Minocycline	43*	100

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 $CB_2^{-/-}$ mice.
#$P < 0.05$ versus WT mice.

Results

Increased CB_2 cannabinoid receptor expression and microglial activation in the striatum of Huntington's disease mouse models and patients

To address the potential involvement of CB_2 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 CB_2 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, CB_2 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 CB_2 receptor-expressing cells. The glial response of R6/2 mice was accompanied by an increased number of CB_2 receptor-positive cells. Microglial Iba-1-positive cells co-expressed CB_2 receptors (Fig. 1D), whereas astrocytes and medium-sized spiny neurons (GFAP$^+$ and DARPP32$^+$ cells, respectively) were negative for CB_2 expression (Fig. 1E and data not shown).

To further validate the findings from the transgenic mouse models, we analysed the expression of CB_2 receptors in brain samples of Huntington's disease and control patients. Huntington's disease patient samples revealed increased levels of CB_2 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 CB_2 receptors in the caudate putamen of Huntington's disease patients was assessed by immunohistochemistry, which revealed that CB_2 expression occurred in cells with the characteristic morphology of microglia (Fig. 2B). We next confirmed that cells

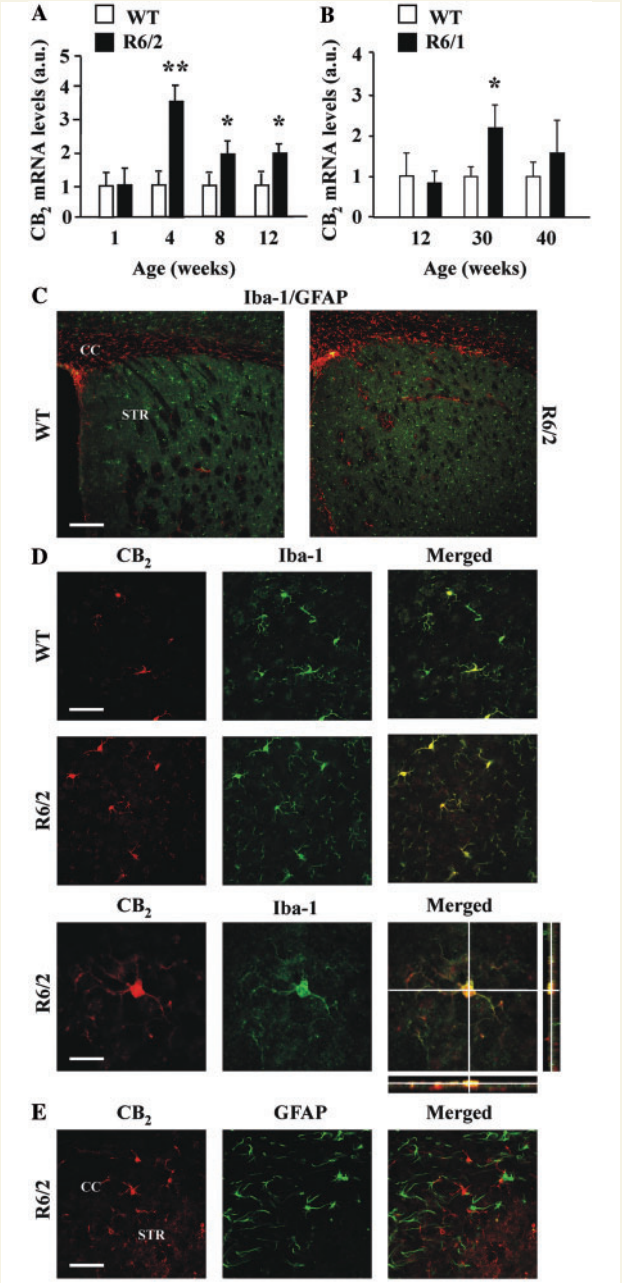

Figure 1 CB_2 receptors are upregulated in microglial cells at symptomatic stages of Huntington's disease animal models. (**A** and **B**) CB_2 mRNA levels were determined by real-time quantitative PCR in striatal extracts of R6/2 and R6/1 mice at the indicated ages (black bars, $n = 6$ each group). 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 CB_2 receptors (red) in Iba-1 (green) microglial cells was analysed in the same mice groups. Lower panels show orthogonal images of an example of CB_2 receptor expression in Iba-1$^+$ cell. (**E**) Expression of CB_2 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.

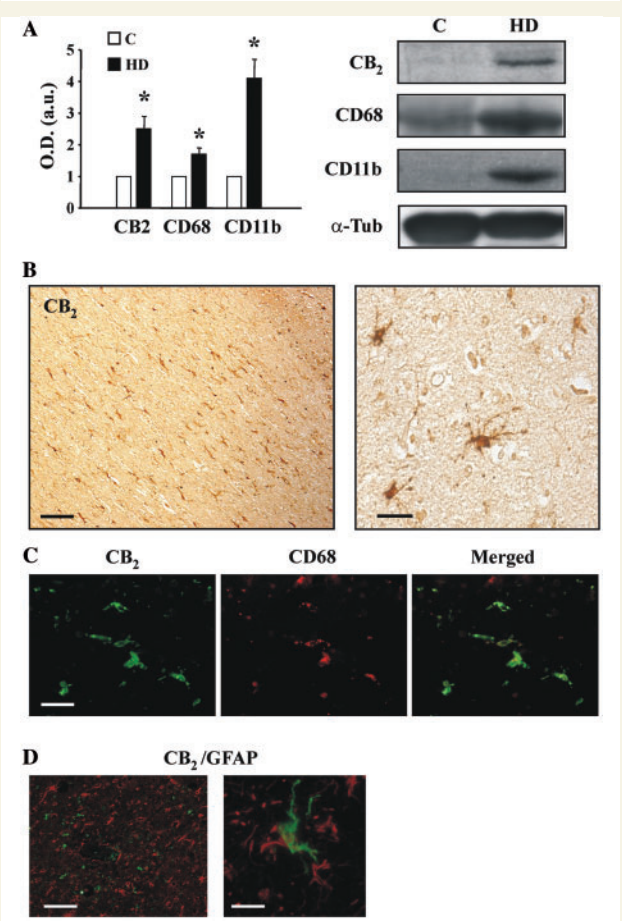

Figure 2 CB_2 receptors are expressed in caudate-putamen microglial cells of Huntington's disease patients. (**A**) Western blot analysis of caudate-putamen specimens from Huntington's disease patients and control subjects. A representative blot of a Huntington's disease patient and a control subject is shown (females of 68 and 77 years old, respectively). Optical density values relative to those of loading controls (α-tubulin) are given ($n = 4$ per group; $^*P < 0.05$ from control). (**B**) Representative immunohistochemistry images at low and high magnification of CB_2 receptor immunoreactivity in the caudate-putamen of a representative Huntington's disease patient. (**C**) CB_2 receptors (green) are expressed in microglial cells as revealed by their co-localization with CD68 (red). (**D**) CB_2 receptors are not expressed in astroglial cells as evidenced by the lack of co-localization of CB_2 (green) and GFAP (red) signals. Scale bars: 220 μm (**B**); 45 μm (**C**); 330 μm (**D**); 15 and 30 μm (high magnification images in **B** and **D**, respectively).

expressing CB_2 receptors in Huntington's disease correspond to CD68-positive microglia, but not to GFAP-positive astroglia (Fig. 2C and D).

CB_2 cannabinoid receptor deficiency exacerbates disease progression in R6/2 mice

To investigate the role of CB_2 cannabinoid receptor upregulation in R6/2 mice, genetic ablation experiments were achieved by crossing R6/2 mice with CB_2 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 CB_2 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:$CB_2^{-/-}$ mice than in R6/2:$CB_2^{+/+}$ mice at Weeks 6 and 8 (Fig. 3A). Moreover, ablation of CB_2 receptors reduced R6/2 mice lifespan (Fig. 3B).

We then analysed in detail striatal sections from double mutant R6/2:$CB_2^{-/-}$ mice in comparison with R6/2:$CB_2^{+/+}$ littermates. Iba-1-positive cells were quantified at Week 12 in R6/2:$CB_2^{-/-}$ mice, revealing increased microglial activation when compared with CB_2 receptor-expressing R6/2 littermates (Fig. 4A). The lack of CB_2 receptors was also accompanied by increased astrogliosis (Fig. 4B). Importantly, the exacerbated phenotype of microglial and astroglial activation that occurred in R6/2:$CB_2^{-/-}$ 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 CB_2 cannabinoid receptor expression, which appears to play a protective role against striatal dysfunction.

CB_2 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 CB_2 receptor genetic deletion in mutant huntingtin-expressing mice, we sought to analyse the role of CB_2 receptors in an excitotoxicity model of intrastriatal administration of the natural N-methyl-D-aspartic acid (NMDA) receptor agonist quinolinic acid. CB_2 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 $GFAP^+$ 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 CB_2 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 $CB_2^{-/-}$ mice than in their WT littermates (Supplementary Fig. 1). As in Huntington's disease transgenic models, CB_2 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

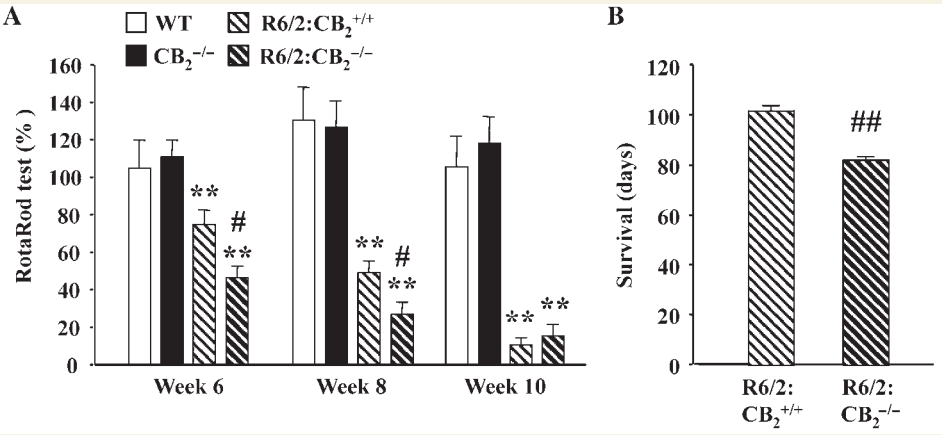

Figure 3 Evolution of motor symptoms and lifespan of CB_2 receptor-deficient R6/2 mice. (**A**) Motor coordination was determined by RotaRod analysis at the indicated ages in WT (white bars), $CB_2^{-/-}$ (black bars), R6/2:$CB_2^{+/+}$ (hatched white bars) and double mutant R6/2:$CB_2^{-/-}$ (hatched black bars) mice. Values correspond to the percentage of RotaRod time from basal determinations at Week 4. (**B**) Lifespan of R6/2:$CB_2^{+/+}$ (hatched white bars) and R6/2:$CB_2^{-/-}$ (hatched black bars) mice ($n=5$ each group). $^{**}P < 0.01$ versus RotaRod of WT mice; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus R6/2:$CB_2^{+/+}$ mice RotaRod (**A**) or survival (**B**).

increase in microglial cells induced by excitotoxicity corresponds to actively phagocyting 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 CB_2 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 CB_2 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 CB_2 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 CB_2 cannabinoid receptors attenuates glial activation and protects from striatal neuronal damage

As loss-of-function studies indicated that CB_2 receptor expression prevents neurodegeneration in Huntington's disease models, we next turned to investigate the effect of direct CB_2 receptor stimulation by using the non-psychotropic CB_2-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). CB_2 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 CB_2 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 CB_2-selective agonists were effective in reducing striatal neurodegeneration, GABA deficits and glial activation (Supplementary Fig. 5).

Regulation of microglial activation is responsible for CB_2 cannabinoid receptor-mediated neuroprotection

To address the role of exacerbated glial activation in striatal neurodegeneration of CB_2 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 CB_2 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 CB_2 receptor knockout mice. The protective action of the anti-inflammatory treatment against striatal excitotoxicity, together with the role of CB_2 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 CB_2 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

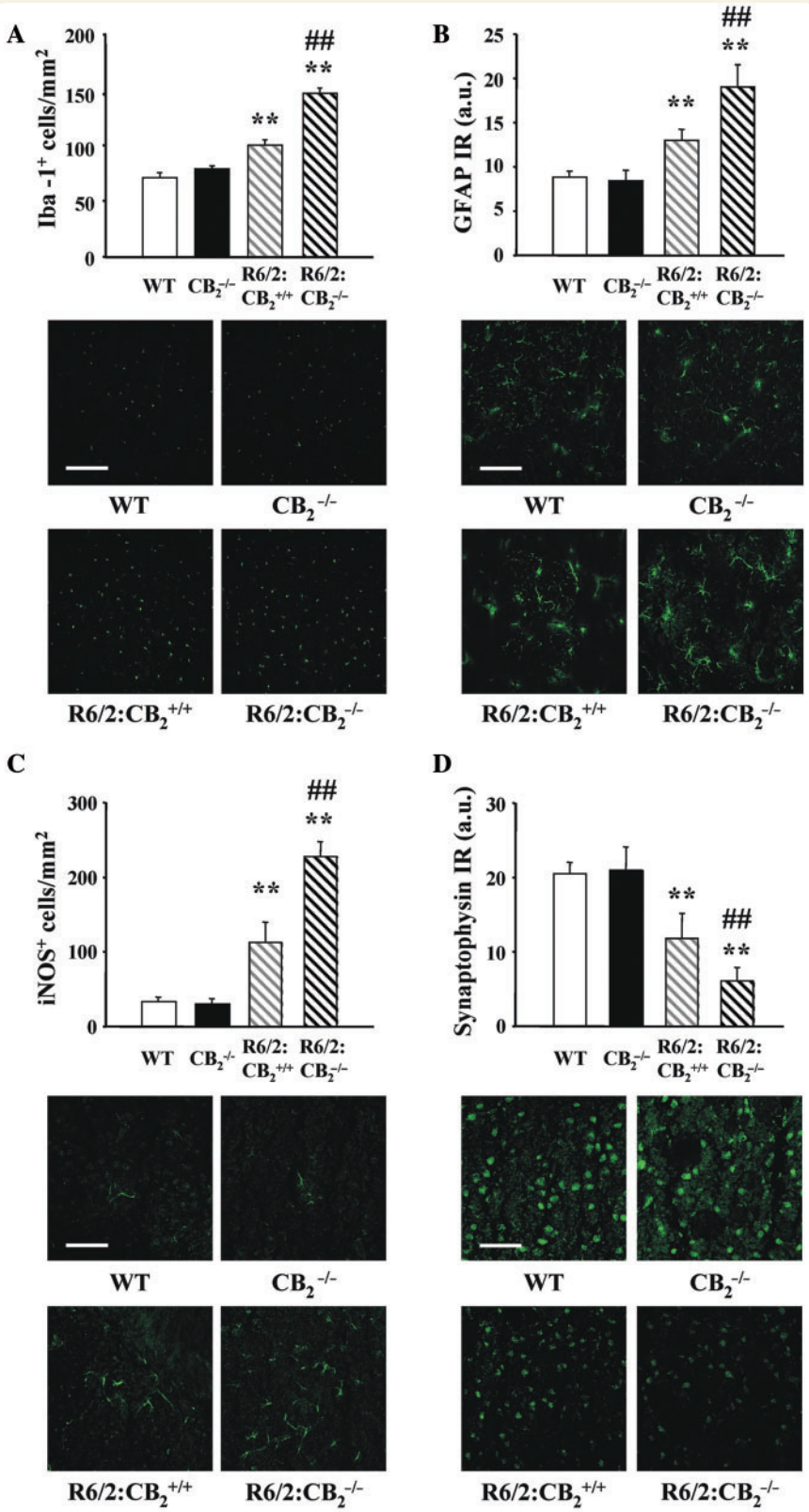

Figure 4 CB_2 receptor deficiency in R6/2 mice exacerbates striatal glial activation and lowers synaptophysin expression. (**A**) Microglial cells were quantified by immunofluorescence with the anti-Iba-1 antibody in striatal sections of the indicated mice genotypes at Week 12 ($n = 5$ each group). (**B**) 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:$CB_2^{+/+}$ mice group.

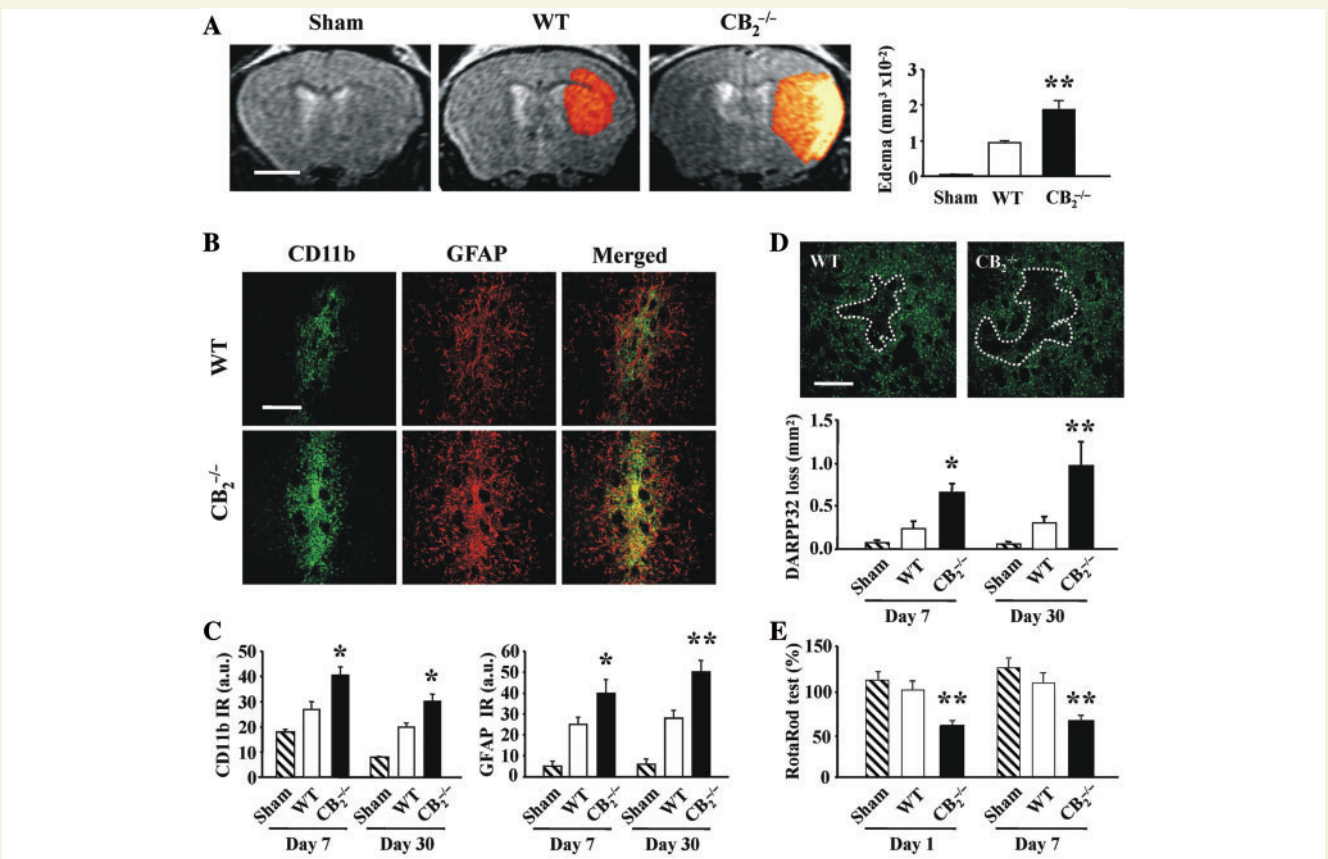

Figure 5 Increased striatal excitotoxicity in CB_2 receptor-deficient mice. (**A**) Representative MRI images of WT (white bar) and $CB_2^{-/-}$ (black bar) mice 5 days after intrastriatal injection of quinolinic acid or vehicle (Sham). Coloured areas correspond to the low water diffusion signal. The volume of striatal oedema was quantified and represented ($n = 5$ each group). (**B**) Representative images of striatal gliosis 30 days after excitotoxic injury obtained by confocal immunofluorescence with antibodies against CD11b (green) and GFAP (red). (**C**) Quantification of CD11b and GFAP immunoreactivity in the striata of the indicated mice 7 and 30 days after excitotoxicity. (**D**) Neurodegeneration was quantified by loss of DARPP32 immunoreactivity in the striata of treated WT and $CB_2^{-/-}$ mice. Representative immunofluorescence images at the end of the experiment are shown (upper panels). (**E**) RotaRod analysis was performed 24 h and 7 days after excitotoxic injury. Values correspond to the percentage of RotaRod time determinations prior to excitotoxicity. Scale bars: 2 mm (**A**), 95 μm (**B**), 185 μm (**D**). **C–E**, $n = 8$ each group. $^*P < 0.05$, $^{**}P < 0.01$ versus the WT group at the corresponding time points.

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, CB_1 receptors are known to be downregulated at early stages (Glass *et al*., 2000; McCaw *et al*., 2004). The consequent loss of CB_1 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 CB_1-mediated neuroprotection (Pryce *et al*., 2003; Pintor *et al*., 2006). Here we show that, in contrast to neuronal CB_1 receptors, microglial CB_2 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 CB_2 cannabinoid receptor ablation exacerbates microglial activation and accelerates the appearance of symptoms. Moreover, pharmacological activation of CB_2 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. CB_2 receptors had been shown previously to confer neuroprotection in different animal models of acute brain toxicity and neuroinflammatory damage. Thus, CB_2 receptor-deficient mice have enhanced susceptibility to neurodegeneration in experimental autoimmune

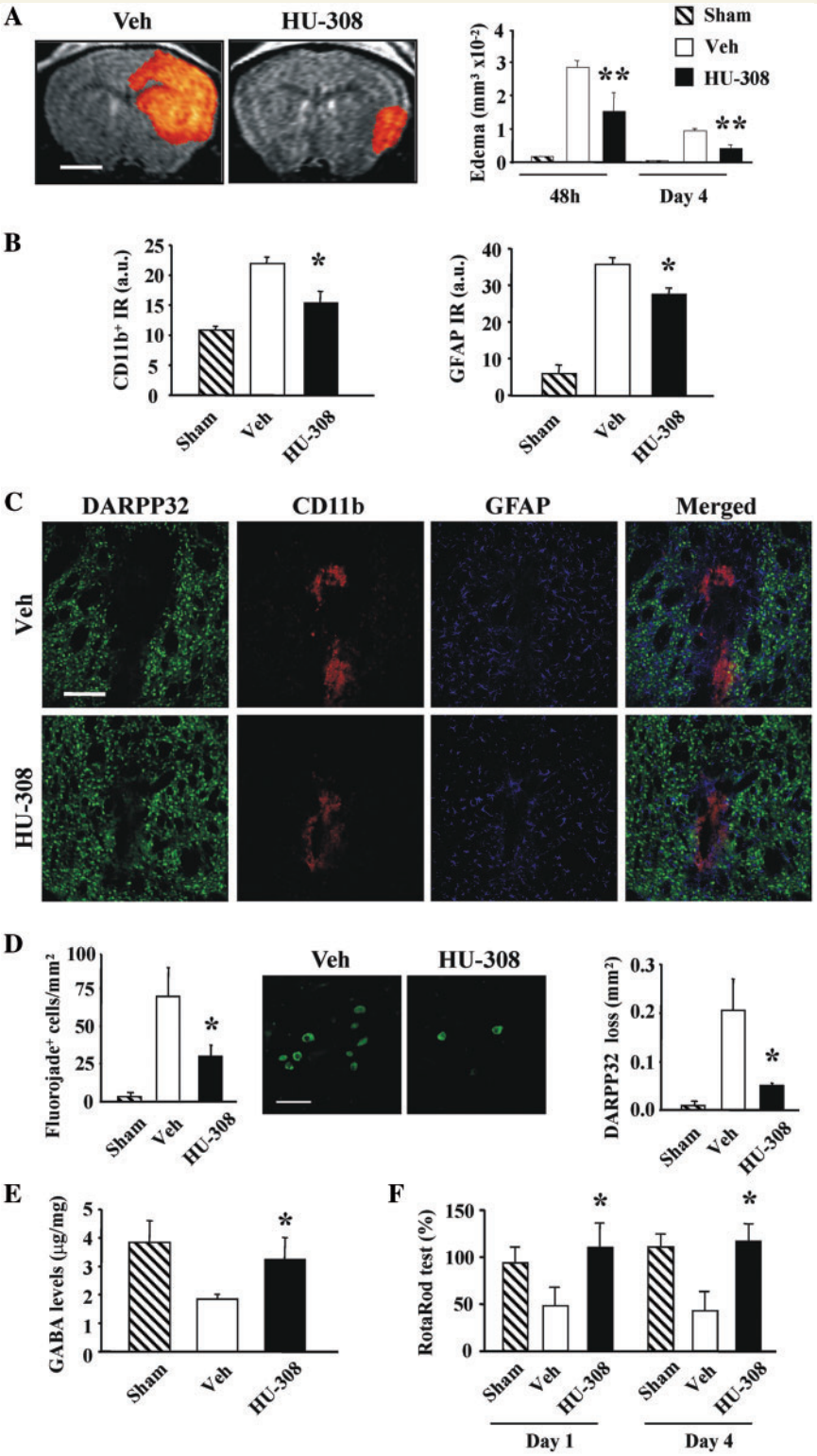

Figure 6 Pharmacological CB_2 receptor activation decreases striatal neurodegeneration after excitotoxicity. (**A**) Representative MRI images 2 days after intrastriatal injection of quinolinic acid or vehicle (Sham, dashed bars). HU-308 (5 mg/kg, black bars) or vehicle (white bars) were administered 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**) Fluorojade staining and DARPP32 immunoreactivity were quantified and the number of positive cells as well as the area loss are represented, respectively. Representative images of fluorojade-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.

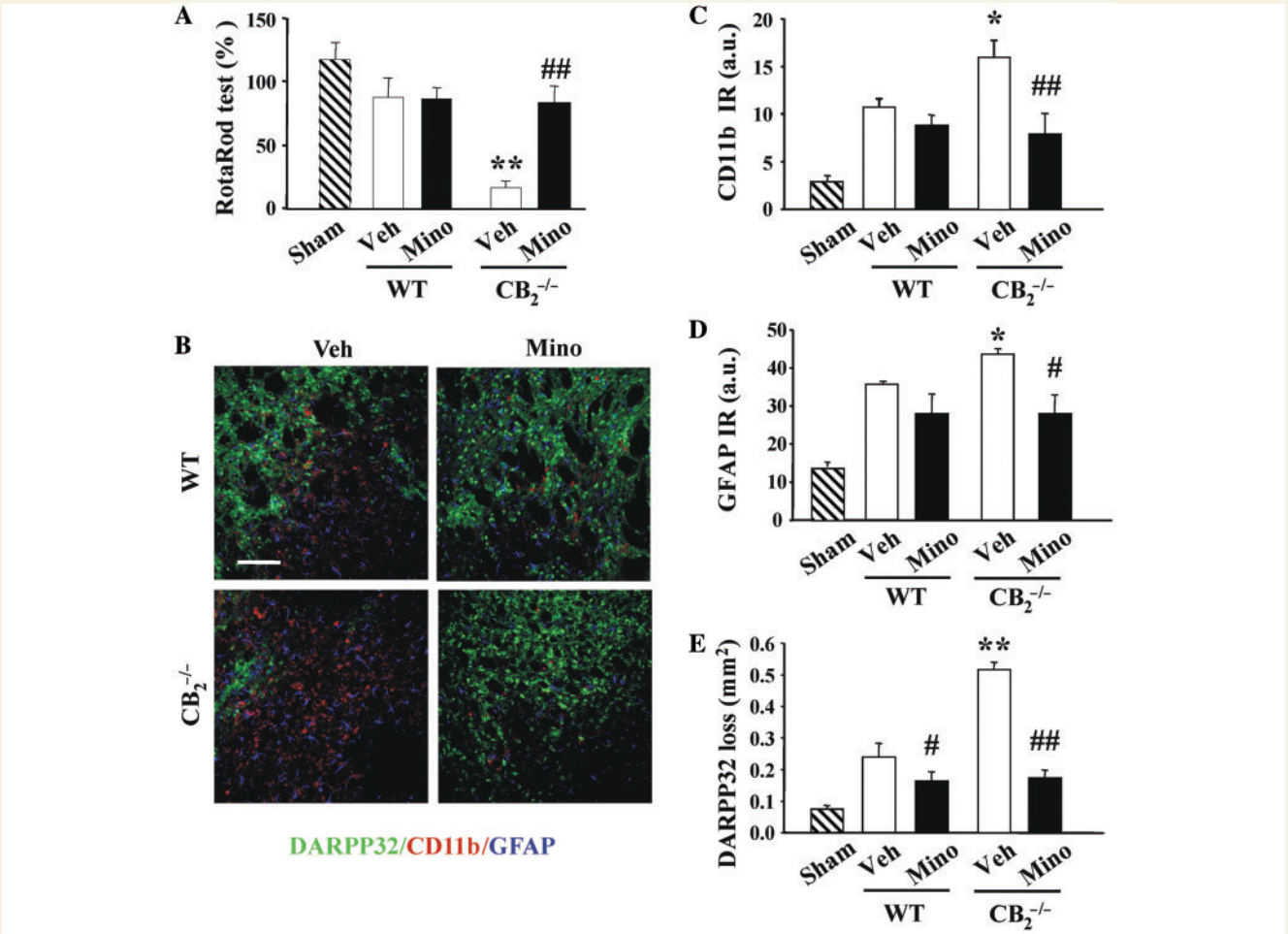

Figure 7 Minocycline treatment inhibits microglial activation and striatal neurodegeneration after excitotoxicity in CB_2 receptor-deficient mice. (**A**) RotaRod performance after striatal excitotoxicity. The effect of daily injection of minocycline (i.p.) after quinolinic acid administration was compared in $CB_2^{-/-}$ 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: 150 μm. (**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.

encephalomyelitis (Maresz *et al*., 2007; Palazuelos *et al*., 2008) and upon intrastriatal malonate injection (Sagredo *et al*., 2008). Likewise, administration of CB_2 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 CB_2 receptor activation may be a potential therapeutic strategy for neurodegenerative disorders (Mackie, 2006; Fernandez-Ruiz *et al*., 2007). The protective role of CB_2 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 CB_2-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 CB_2 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

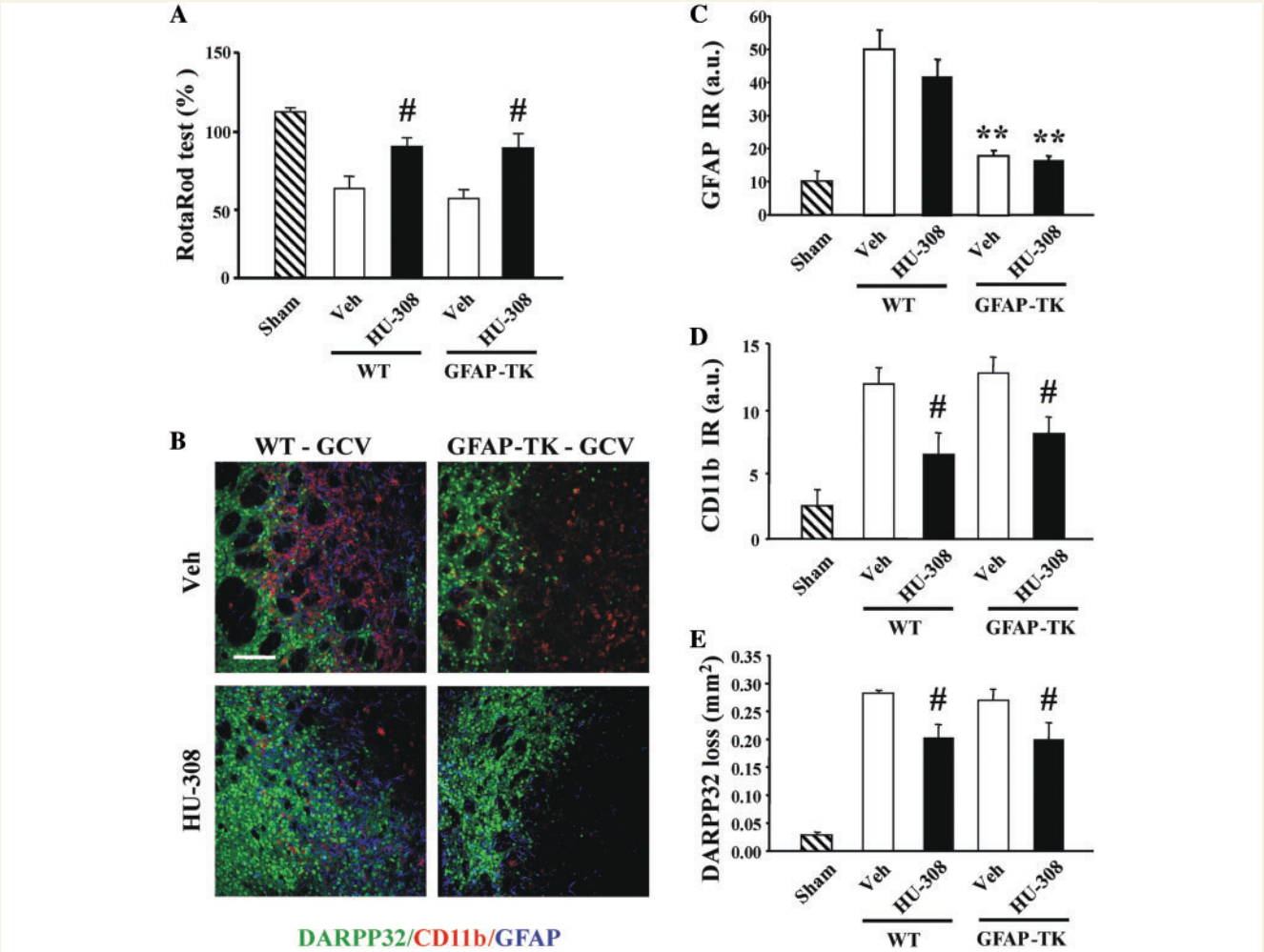

Figure 8 Ablation of astroglial proliferation does not affect CB_2 receptor-mediated neuroprotection. (**A**) RotaRod analysis 4 days after induction of striatal excitotoxicity in GFAP-TK mice and WT littermates. Treatment with HU-308 (5 mg/kg, black bars) or vehicle (white bars) was performed after excitotoxicity and then daily until sacrifice ($n = 5$ each group). (**B**) Confocal immunofluorescence images with antibodies against CD11b (red), GFAP (blue) and DARPP32 (green) was performed to assess glial cell activation and medium-sized spiny neuronal loss in the indicated mice groups. Scale bar: 150 μm. (**C–E**) Astroglial activation, microglial activation and striatal neurodegeneration were, respectively, determined by immunofluorescence quantification of GFAP, CD11b and DARPP32 immunoreactivity in the indicated mice groups. $^{**}P < 0.01$ versus their respective WT mice group; $^{\#}P < 0.05$ 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 completely be 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 CB_2 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, CB_2 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.

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Supplementary material

Supplementary material is available at *Brain* online.

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