Excitotoxicity in a chronic model of multiple sclerosis: Neuroprotective effects of cannabinoids through CB1 and CB2 receptor activation

Fabian Docagne,† Vilma Muñetón, Diego Clemente, Carine Ali, Frida Loria, Fernando Correa, Miriam Hernangómez, Leyre Mestre, Denis Vivien, and Carmen Guaza

Grupo de Neuroinmunología - Instituto Ramón y Cajal, CSIC - Avda Dr Arce, 37 - 28002 Madrid, Spain
Neural Plasticity Group, Instituto Ramón y Cajal, Consejo Superior de Investigaciones Científicas, Madrid, Spain
INSERM Avenir “t-P4 in the working brain”, Centre Cycleron, BP 5229, 14074 Caen, France

Received 2 August 2006; revised 22 November 2006; accepted 10 December 2006
Available online 16 January 2007

Inflammation, autoimmune response, demyelination and axonal damage are thought to participate in the pathogenesis of multiple sclerosis (MS). Understanding whether axonal damage causes or originates from demyelination is a crucial issue. Excitotoxic processes may be responsible for white matter and axonal damage. Experimental and clinical studies indicate that cannabinoids could prove efficient in the treatment of MS. Using a chronic model of MS in mice, we show here that clinical signs and axonal damage in the spinal cord were reduced by the AMPA antagonist, NBQX. Amelioration of symptomatology by the synthetic cannabinoid HU210 was also accompanied by a reduction of axonal damage in this model. Moreover, HU210 reduced AMPA-induced excitotoxicity both in vivo and in vitro through the obligatory activation of both CB1 and CB2 cannabinoid receptors. Together, these data underline the implication of excitotoxic processes in demyelinating pathologies such as MS and the potential therapeutic properties of cannabinoids.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Multiple sclerosis; TMEV; Axonal damage; Excitotoxicity; Cannabinoids

Introduction

Multiple sclerosis (MS) is the most common chronic demyelinating disease of the central nervous system (CNS) in humans and leads to motor and sensory deficits, tremor and ataxia. While demyelination is considered as the main element in the pathology of MS, the deleterious processes leading to neurological decline include chronic inflammation, infiltration of T cells and macrophages, axonal damage, and autoimmune response against myelin. However, the way these different processes articulate with each other remains controversial. As demyelination does not necessarily correlate with the severity of neurological decline, an important issue to understand is the timing of axonal damage in this pathology, to characterize it as a cause or as a consequence of demyelination. In the Théller’s murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD)-model of MS, Tsuoda et al. (2003) suggested that demyelination could be secondary to axonal damage. Moreover, pioneer studies using experimental allergic encephalomyelitis (EAE) as a model of MS suggested that excitotoxic processes could induce axonal damage and may represent a key element in the pathogenesis of MS (Smith et al., 2000; Pitt et al., 2000). Finally, antagonising α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamatergic receptors led to lesser axonal damage and to better neurological score in EAE (Smith et al., 2000; Pitt et al., 2000).

Cannabinoids form a class of molecules that include active components of marijuana, endogenous ligands (endocannabinoids), and a variety of synthetic compounds. They induce their effects through activation of two G-protein-coupled receptors termed CB1 and CB2, and several yet-to-be-cloned receptors (Howlett et al., 2002) which might mediate some of the effects of cannabinoids. Therapeutic properties have been attributed to cannabinoids in various SNC pathologies including Parkinson’s disease (Lastres-Becker et al., 2005), Alzheimer’s disease (Ramírez et al., 2005), head trauma (Panikashvili et al., 2001) and MS. (Pryce et al., 2003) Arévalo-Martín and collaborators showed in 2003 that cannabinoids could exert therapeutic actions in the TMEV-IDD model of MS, reducing motor impairment and inflammation, and eventually promoting remyelination. However, the precise mechanisms promoting these beneficial effects of cannabinoids remain to be more clearly elucidated.

Several studies described neuroprotective actions of cannabinoids in different in vitro and in vivo models (Sarne and...
Mechoulam, 2005), including cellular models of excitotoxicity (Molina-Holgado et al., 2005; Kim et al., 2006). This neuroprotective effects is thought to be related to the capacity of cannabinoids to inhibit glutamatergic transmission (Domenici et al., 2006). Glial cells, and particularly astrocytes, have been implicated in the mediation of the effects of several neuroprotective agents, including cytokines such as TGF-β (Docagne et al., 2002). Astrocytes might participate in the neuroprotective action of cannabinoids as they may express both CB1 and CB2 receptors (Sheng et al., 2005). A related issue is the relative participation of CB1 and CB2 in cannabinoid-induced neuroprotective effects. These two last questions are addressed in the present study.

The present work aims at determining as to whether excitotoxic processes are implicated in the progression of TMEV-IDD. The relationship between excitotoxicity and axonal damage has also been studied in this chronic model of MS. Finally, we studied the therapeutic potential of cannabinoids in this context, for their protective activity against excitotoxicity.

Results

Blocking excitotoxic processes in the TMEV-IDD ameliorates clinical signs and reduces axonal damage

Excessive stimulation of AMPA/kainate glutamatergic receptors has been proposed as one of the principal toxic processes targeting axons in acute and chronic brain pathologies (see Stys, 2005 for review). We sought to study the possible implication of AMPA/kainate-induced excitotoxicity in the TMEV-IDD chronic viral model of MS, and investigate its potential relationship with axonal damage.

If AMPA/kainate-induced excitotoxic processes participate in the pathology of the TMEV-IDD, then the blockade of AMPA/kainate receptors should be beneficial for infected animals. To study this possibility, we treated animals with a twice daily i.p. injection of 450 μg NBQX, a typical AMPA/kainate antagonist. Similar doses have been used in previous studies (Pitt et al., 2000; Smith et al., 2000) and are considered sufficient to block AMPA/kainate neurotransmission in an efficient and specific manner. We started this treatment on day 39, when global motor activity started to decrease in vehicle-injected TMEV animals (Fig. 1A), and we maintained the exposure to NBQX until day 47. NBQX treatment induced a nearly complete reversion of TMEV-induced loss of global activity (Fig. 1A) that reached significance at day 44 (6 days of NBQX treatment, \( P < 0.05 \)), and remained significant until the end of the treatment period. The effect of the blockade of AMPA/kainate receptors on TMEV-IDD pathology was also evaluated using the rotarod test, as an index of motor coordination, which is known to be affected in the TMEV-IDD. Sham-operated animals treated with NBQX did not display any difference in the rotarod test when compared to vehicle-treated animals (Fig. 1B). Animals subjected to TMEV-IDD, when treated with NBQX, reached a
significantly higher score in this test as compared to TMEV-infected animals treated with vehicle (Fig. 1B).

Axonal damage is an important feature of MS lesions in humans and has been implicated in the two principal animal models of MS (Tsunoda et al., 2003; Wang et al., 2005). Axonal damage can be assessed through detection of abnormal dephosphorylation of the heavy chain neurofilament H (NF-H) (Trapp et al., 1998). Considering the reported presence of ionotropic glutamate receptors on myelinated axons (Rodriguez-Moreno et al., 1997), we hypothesized that the amelioration of the pathological state of the animals treated by NBQX could be related to a reduction in axonal damage. To test this possibility, we performed Western blot using the SMI32 antibody to detect the presence of the abnormally dephosphorylated NF-H in protein extracts of spinal cords of animals infected with TMEV and treated with NBQX, or corresponding vehicle. Axonal damage was dramatically reduced by the treatment with NBQX (Fig. 1C). These results indicate that axonal damage is detectable at early stages of symptomatology in the TMEV-IDD model of MS (50 days) and that blocking excessive glutamatergic transmission results in a reduction of axonal damage.

Taken together, these data indicate an amelioration of TMEV-IDD when the AMPA/kainate neurotransmission was blocked, suggesting an implication of excitotoxic processes in this MS model. Moreover, we suggest that excitotoxicity could lead to axonal damage in the spinal cord of TMEV-IDD animals, and that the better clinical state induced by the treatment with NBQX could be explained by a reduction of excitotoxicity-induced axonal damage.

**HU210 protects against AMPA-induced excitotoxic lesions in the spinal cord and displays therapeutic properties in the TMEV-IDD through reduction of axonal damage**

Our next step was to evaluate the potential therapeutic properties of the cannabinoid HU210 in the TMEV-IDD, given its reported anti-excitotoxic properties in other models of CNS pathology (Nagayama et al., 1999; Kim et al., 2006). First, in order to assess the relevance of such a therapeutic strategy, we studied the effects of this cannabinoid on excitotoxic processes in the spinal cord. AMPA (5 nmol in 2 μL) was microinjected directly into the dorsal horn of the spinal cord of rats in combination with vehicle or HU210 (1 μmol). Animals were pre-treated with an intraperitoneal injection of MK801, 1 h before the induction of the spinal cord damage, in order to avoid secondary NMDA receptor-dependent excitotoxic processes. Injection of AMPA together with vehicle in the grey matter of the dorsal horn induced an excitotoxic lesion with clear loss of cellular bodies and the induction, 48 h after the injection, of a glial scar in the injured area (Fig. 2A, panel ii). When co-injected with AMPA, HU210 reduced the severity of the lesion, with a minor loss of cell bodies and no appearance of glial scar, leading to a lesion characterized by a reduction of cell density (Fig. 2A, panel iii) as compared to control dorsal horn (Fig. 2A, panel i) rather than the severe necrotic lesion observed when AMPA was microinjected together with vehicle (Fig. 2A, panel ii). We then measured the extent of the lesion along the injected spinal cords, and observed that HU210 strongly reduced the size of the lesioned area induced by AMPA (Fig. 2B). These results indicate that HU210 is efficient in reducing AMPA/kainate induced excitotoxic lesions in the spinal cord.

To confirm that HU210 can influence the course of the disease in the TMEV-IDD, we submitted TMEV-infected mice to sub-chronic injection of this CB1/CB2 agonist (daily dose of 100 μg/kg i.p. between days 39 and 47 post-infection). TMEV-IDD induces a typical impairment in the motor coordination and activity as assessed by the rotarod test. Here, we evaluated the effects of HU210 treatment on TMEV-IDD-induced motor impairments. TMEV-infected animals, when injected with vehicle, showed a marked reduction in latency in the rotarod test (Fig. 3A: approximately 60 s spent on the wheel, as compared to 225 s for sham animals). However, 1 day after the end of HU210 treatment, they displayed a much higher latency (Fig. 3A: around 150 s), result which suggests a beneficial effect of this cannabinoid on motor activity. HU210 did not induce any significant changes in motor activity when administered to non-infected, sham animals (Fig. 3A), showing that the effect observed in TMEV animals is significantly higher score in this test as compared to TMEV-infected animals treated with vehicle (Fig. 1B).
likely due to a therapeutic action rather than to an effect of HU210 by itself on motor activity.

We observed by immunohistochemistry for abnormally dephosphorylated NF-H (SMI32 monoclonal antibody) that sham-operated animals treated with vehicle or HU210 displayed few SMI32-positive axons (Fig. 3B, panels i and ii). In contrast, mice subjected to TMEV-IDD suffered axonal damage in the spinal cord at 50 days post-infection (Fig. 3B, panel iii). Damaged spinal cord were characterized by abundant, swollen SMI32-positive axons, with multiple swellings and irregular shapes suggestive of a disrupted cytoskeleton (Fig. 3B, panel iii and insert), and with some swellings terminating in ovoids (axonal transections) (Trapp et al., 1998) (Fig. 3B, insert). Treatment with HU210 induced a global reduction in the density of SMI32-positive axons, together with a decrease in the fraction of swollen, irregularly shaped axons (Fig 3B, panel iv).
These observations were confirmed by Western blotting using the SMI32 antibody, from protein extracts of spinal cord of animals infected with the TMEV and treated with HU210 or the corresponding vehicle. Indeed, the immunoreactive band for abnormally dephosphorylated neurofilament H was largely reduced by HU210 treatment (Fig. 3C). Taken together, these data confirm the relevance of cannabinoid-based treatments in animal models of MS and show that the beneficial effects of the cannabinoid, HU210, are accompanied by a reduction of damage to axons, likely through its anti-excitotoxic properties.

**HU210 exerts a neuroprotective activity against AMPA-induced excitotoxicity through the activation of CB1 and CB2 receptor**

We showed that treatment with the cannabinoid, HU210, or with the AMPA/kainate antagonist, NBQX, resulted in an amelioration of disease in mice subjected to TMEV-IDD, and that these beneficial effects were accompanied, in both cases, by a reduction of axonal damage. These results suggest that HU210 could exert its beneficial effect, at least in part, through a protection against excitotoxic processes occurring in the TMEV-IDD model of MS.

As oligodendrocytes have been suggested to be a target of AMPA-mediated excitotoxic cell death in demyelinating diseases (Matute et al., 2001; Sánchez-Gómez et al., 2003), we first assessed the effect of HU210 on AMPA-induced oligodendrocyte toxicity in vitro. HU210 (250–1000 nM) failed to induce any protection against AMPA-induced oligodendrocyte cell death (data not shown). The other synthetic cannabinoid, (–)-Win-55521-2 (25 nM), did not induce any effect either in this paradigm of oligodendrocytes cell death (data not shown) at a dose sufficient to protect these cells from death induced by withdrawal of trophic factors (Molina-Holgado et al., 2002).

Then, to assess the putative neuroprotective effect of HU210 on neurons, we used an in vitro model of AMPA-induced excitotoxicity. The exposure of mixed cortical neuron-astrocytes cultures or pure cortical neuronal cultures (DIV 14) to AMPA (10 μM) produced acute swelling of neuronal bodies, followed 24 h later by widespread neuronal degeneration (Fig. 4A) while the glia remained intact. Pre-treatment (1 μM for 2 h, Fig. 4E) as well as co-treatment with increasing concentrations of HU210 (100–1000 nM, Figs. 4A, B) reduced neuronal death only when neurons were cultured in the presence of astrocytes, as HU210 did not induce any effect in cortical neurons cultures (Fig. 4C). Same experiments performed in mixed cortical neuron–astrocytes cultures where neurons were obtained from spinal cord also showed a neuroprotective effect of HU210 (1 μM) (Fig. 4D). HU210 (1 μM) failed to induce any significant neuroprotective effect in near pure neuronal cultures (containing less than 5% astrocytes), result that would indicate that the effects of HU210 could be mediated by astrocytes.

In order to study the respective implication of CB1 and CB2 in the neuroprotective effect displayed by HU210, HU210 (1 μM) was co-incubated with the CB1 antagonist, SR 141716 (1 μM), or with the CB2 antagonist, SR144528 (1 μM). Both CB1 and CB2 antagonist reversed HU210-induced neuroprotective activity (Fig. 5A). The treatment with the specific CB1 agonist ACEA (25 nM) (Figs. 5B, C), or with the specific CB2 agonist JWH133 at 300 nM (Fig. 5B) or at 5 μM (Fig. 5C) did not display any effect on AMPA-induced excitotoxic necrosis. However, the co-incubation of both ACEA (25 nM) and JWH133 (300 nM or 5 μM) induced a neuroprotection comparable to that observed with HU210 (Figs. 5B, C). These data indicate that the cannabinoid, HU210, induces neuroprotection against AMPA-induced excitotoxicity through a mechanism involving both CB1 and CB2 receptors.

The above result shows that neuroprotection by HU210 requires the presence of astrocytes, and also requires both CB1 and CB2 activation. This suggests that neuroprotection could be mediated through the activation of one subtype of CB receptor in astrocytes and the other subtype of receptor in neurons. We further investigated this issue with a strategy based on the use of mixed cultures of neurons and astrocytes obtained from CB1 knock-out or wild type neurons. In mixed cultures containing wild type neurons and CB1 receptor knock-out astrocytes, HU210 (1 μM) still induced a neuroprotective effect, similarly as what was observed in wild type cultures (Fig. 5D). However, when CB1 receptor knock-out neurons where cultured with wild type astrocytes, HU210 (1 μM) failed to induce any neuroprotection against AMPA-induced excitotoxicity (Fig. 5D). Given the importance of astrocytes in the protective effects of HU210 and the necessary activation of both CB1 and CB2 receptors, these results suggest that HU210 could induce its neuroprotective effect through the concomitant activation of CB1 in neurons and CB2 in astrocytes.

**Discussion**

Therapeutic strategies for the treatment of MS mainly include anti-inflammatory and immune-based therapies as well as neutralization of adhesion molecules. More recently, the possibility of a significant neurodegenerative component associated, with likely excitotoxic processes, raised new therapeutic opportunities based on neuroprotection. However, little is known about the actual implication of excitotoxicity in the progression of MS, with very few studies in animal models of this disease. In the present study, we show the beneficial effect of the administration of a classical AMPA/kainate receptor antagonist, NBQX, in the TMEV-induced model of MS, without effect when applied in healthy, uninfected animals. This absence of effect of the drug per se strengthens the idea that the therapeutic action of NBQX observed here is due to a blockade of excessive, excitotoxic AMPA transmission. These data reinforce previous observations of an excitotoxic component in the acute EAE model of demyelination (Pitt et al., 2000), and in the chronic relapsing EAE model of MS, considered to approximate the chronic relapsing remitting form of MS in human (Smith et al., 2000). Our study is the first indication of an excitotoxic component in an animal model which reproduces features of the primary progressive form of MS in human. Thus, excitotoxic processes, through activation of AMPA/kainate receptors, appear as a pathological mechanism common to different animal models of MS. New animal models are however required to study the secondary progressive form of MS, which currently lacks effective therapeutic strategies.

The cellular targets of excitotoxicity in chronic disease, and particularly in MS, are still unclear. White matter seems to be a particularly susceptible target for this pathological mechanism, as several of its cellular components express ionotropic glutamate receptors. Oligodendrocytes express functional AMPA/kainate receptors, of which their activation induces cell death (Sánchez-Gómez et al., 2003). Myelin sheath itself also expresses functional Ca2+-permeable glutamate receptors (Li and Sty, 2000). The presence of AMPA/kainate receptors on axons is still controver-
Harmal, but could explain the excitotoxic-dependent mechanisms implicated in the pathogenesis of MS. Oligodendrocyte death, myelin insult and/or excitotoxic damage directly targeting neurons are all likely to participate in the mechanisms responsible for axonal damage occurring in MS. A previous study, using the TMEV-IDD model of MS, describes the appearance of axonal damage in early phases of the disease, much before the first evidence of demyelination, in areas known to undergo myelin loss.
in later phases of the pathology (Tsunoda et al., 2003). Such studies indicate that axonal damage could be a cause, rather than a consequence, of myelin loss. Other reports on TMEV-IDD indicate that axonal loss can follow primary immune-mediated demyelination and that the severity of axonal loss correlates with the degree of spinal cord atrophy (McGavern et al., 2000). The timing with which axonal damage and demyelination occur in MS human pathology also remains poorly understood. Here, we show that the blockade of AMPA/kainate-dependent excitotoxicity reduces the appearance of axonal damage in relatively early stages of the TMEV-IDD symptomatology. Similar observations have been reported from the acute EAE model of MS (Pitt et al., 2000), yet to our knowledge, the present study is the first report of a relationship between excitotoxicity and axonal damage in a chronic model of MS.

Among the different therapeutic strategies designed to interfere with the deleterious processes involved in the pathology of MS, the use of cannabinoids compounds is currently one of the most promising ones. Growing literature describes a beneficial role of cannabinoids (Baker et al., 2000; Arévalo-Martín et al., 2003; Croxford and Miller, 2003; Cabranes et al., 2005) or endocannabinoid-reuptake inhibitors (Mestre et al., 2005; Ortega-Gutierrez et al., 2005; Cabranes et al., 2005) in animal models of MS. The mechanisms by which these molecules could promote their therapeutic effects are thought to include anti-inflammatory properties, effects on microglial activation, survival of oligodendrocytes

Fig. 5. Activation of both CB1 and CB2 receptors is required for the neuroprotective effect of HU210. (A) Mixed cultures of cortical neurons and astrocytes were exposed to 10 μM AMPA for 24 h and co-treated with HU210 (1 μM) and CB1 or CB2 antagonist (respectively SR1 and SR2, both at the dose of 1 μM) and neuronal death (%) was estimated by measuring the LDH release in the media (mean±SEM, n=12). *P<0.05 as compared to AMPA alone, one-way ANOVA followed by Bonferonni–Dunn’s test. (B) Mixed cultures of cortical neurons and astrocytes were exposed to 10 μM AMPA for 24 h and co-treated with HU210 and the selective CB1 or CB2 agonists (ACEA and JWH133, respectively, at the dose of 25 nM and 300 nM) and neuronal death (%) was estimated by measuring the LDH release in the media (mean±SEM, n=12). *P<0.05 as compared to AMPA alone, one-way ANOVA followed by Bonferonni–Dunn’s test. (C) Mixed cultures of cortical neurons and astrocytes were exposed to 10 μM AMPA for 24 h and co-treated with HU210 and the selective CB1 or CB2 agonists (ACEA and JWH133, respectively, at the dose of 25 nM and 5 μM) and neuronal death (%) was estimated by measuring the LDH release in the media (mean±SEM, n=12). *P<0.05 as compared to AMPA alone, one-way ANOVA followed by Bonferonni–Dunn’s test. (D) Mixed cultures of cortical wild type neurons and CB1 receptor knock-out astrocytes or CB1 receptor knock-out neurons and wild type astrocytes were exposed to 10 μM AMPA for 24 h and co-treated with HU210 and neuronal death (%) was estimated by measuring the LDH release in the media (mean±SEM, n=12). *P<0.05 as compared to AMPA alone, t-test.
progenitors, promotion of remyelination and neuroprotection. This last property of cannabinoids has been studied using a variety of in vitro models such as oxygen and glucose deprivation (Nagayama et al., 1999) as well as paradigms of NMDA-induced excitotoxicity (Kim et al., 2006). However, very few in vitro studies have focused on pharmacologically defined paradigms of excitotoxicity, more closely related to white matter damage, such as AMPA/kainate-induced necrotic neuronal death. Molina-Holgado and collaborators (2005) described a protective effect of the synthetic cannabinoid HU210 in this paradigm, and reported that this effect could be reversed by both CB1 and CB2 antagonists. Our study confirms these data, and comply them by the observation that the combination of CB1 and CB2 agonists induces a neuroprotective effect comparable to which observed with the mixed agonist HU210, indicating that both CB1 and CB2 receptors are required for cannabinoids to exert their beneficial effects. We also show that HU210 is effective as a neuroprotective drug only in the presence of astrocytes, and that knocking-out CB1 gene in astrocytes does not reverse the neuroprotection by HU210. However, we observed that HU210 requires the expression of CB1 in neurons to play its neuroprotective effect. Taken together, these results allow us to propose an original mechanism of neuroprotection by HU210 that would require the concomitant activation of CB1 in neurons and CB2 in astrocytes. However, we cannot discard the possible implication of microglia in the effects of HU210. For instance, the activation of cannabinoid receptors has been shown to modulate the activation (Ramirez et al., 2005) and migration (Walter et al., 2003) of microglial cells and to inhibit the production of inflammatory cytokines by this cell type (Puffenbarger et al., 2000). Even if a very small number of microglial cells are contained in the mixed cultures (≤1%) we cannot completely exclude their possible participation in the effects of HU210.

In addition, using an acute model of excitotoxic lesion to the rat spinal cord, we report that HU210, when co-injected with the excitotoxin AMPA, reduced the extent of damaged tissue, showing that cannabinoids are able to induce neuroprotection against excitotoxic processes that may occur in the spinal cord. We also show that the same cannabinoid HU210, administrated in a sub-chronic manner, induced a therapeutic effect in the TMEV-IDD model, as previously observed with other cannabinoids in the same model as well as in others. Interestingly, we show here that this beneficial action is accompanied by a reduction in axonal damage induced by the disease. This result must be considered along with the previously published observation that mice knock-out for CB1 gene are susceptible to axonal injury in the chronic relapsing EAE model (Jackson et al., 2005) of MS. Thus, exogenously administered as well as endogenous cannabinoids could exert their therapeutic actions in animal models of MS through a reduction in axonal damage.

Overall, our study suggests that interfering with excitotoxic processes in MS could induce therapeutic effects. The observation that excitotoxicity-induced axonal damage could appear at relatively early stages of symptomatology indicates that the window of therapeutic opportunities using anti-excitotoxic drugs could offer possibilities of early treatment of demyelinating disease. Efficient anti-excitotoxic strategies may be obtained through the modulation of the endogenous cannabinoid system, as well as with the exogenous application of cannabinoid-related molecules and could thus represent a promising strategy for the treatment of chronic neurodegenerative diseases such as MS.

Experimental methods

Material

Theiler’s virus (DA strain) was a kind gift from Dr M. Rodriguez at Foundation Mayo Clinic (Rochester, MN, USA). SMI32 monoclonal antibody was purchased from Stenberger Monoclonals Inc. (Lutherville, MA, USA). Mouse anti-mouse MAP2, was obtained from Calbiochem (Spain). LDH Cytotoxicity Detection Kit was from Roche diagnostics GmbH (Germany). Cannabinoid agonists are available in Tocris Cookson Ltd. (UK). Cannabinoid antagonists SR141716 and SR144528 were obtained from Sanofi Research (Montpellier, France). Unless stated elsewhere in the text, all other chemicals are available at Sigma (Spain).

Animals and Theiler’s virus inoculation

We used female SJL/J mice, susceptible to TMEV-IDD, from our in-house colony ( Cajal Institute, Madrid, Spain), maintained on food and water ad libitum in a 12-h light/dark cycle. Four-week-old mice were inoculated intracerebrally in the right cerebral hemisphere with 106 pfu of DA TMEV strain in 30 μL of DMEM supplemented with 10% of FCS as previously described (Lledo et al., 1999). Congenic ABH CB1 gene (Cnr1)-deficient mice (CB1 knock-out mice) were obtained as a kind gift from Dr David Baker, Institute of Neurology, U.C. London and were bred in our animal house. Handling of animals was performed in compliance with the guidelines of animal care set by the European Union (86/609/EEC).

Animal sub-chronic treatments

Sham or TMEV-IDD mice were injected intraperitoneally with vehicle, HU210 (100 μg/kg, once daily) or NBQX (450 μg/animal, twice daily) between days 39 and 47 post-infection. The duration of treatment was chosen based on two previous studies undertaken in the EAE model of MS (Smith et al., 2000; Pitt et al., 2000). While the MS models used in these studies are different from the TMEV-IDD, we adapted the treatment so that the timing of the initiation would correspond to a comparable clinical stage in the disease. In both cases the initiation of the treatment occurred at the time of the first clear evidence of motor impairment. Animals were killed by the injection of an overdose of anaesthetic at day 50 post-infection and processed for tissue collection.

Behaviour tests

Evaluation of motor coordination

To evaluate motor neurological deficits of mice, we used the rotarod test, which measures balance, coordination, and motor control. The rotarod apparatus (Ugo Basile, Comerio, Italy) consists of a suspended rod able to run at constant or at accelerating speed. All mice were exposed to a 5-min training period, at constant speed (4.5 rpm), immediately before the test, to familiarize them with the apparatus. The test was performed at gradually accelerating rod speed. Data were collected from mouse rotarod performance 1 day after the end of the period of sub-chronic HU210 or NBQX treatment, which corresponds to 49 days after TMEV infection. The trial was terminated when mice fell from the apparatus or after a maximum of 5 min.

Spontaneous motor activity

The screening for locomotor activity was performed using an activity cage (Activity Monitor System Omnitech Electronics, Inc., Columbus, OH, USA) coupled to a Digiscan Analyser. This test was performed 6 h after NBQX or HU210 treatments to eliminate any direct effect of these compounds on locomotor activity. Total distance was measured for each animal during the last 5 min of a session of 10 min.
Proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) prior to immobilization onto poly-(vinylidene difluoride) membranes. Blots were exposed, for 1 h at room temperature, to the primary antibody (anti-SMI32, 1:1000) in a blocking reagent (Tris buffer saline [TBS]: Tris 10 mM, NaCl 200 mM, pH 7.4, containing 0.1% Tween-20, 0.65% dry milk). The membranes were then washed and exposed for 1 h to the appropriate secondary peroxidase-conjugated antibody (1:8000) prior to revelation using a chemiluminescence kit.

**Cell cultures**

**Astrocyte cultures**

Astrocyte cultures were prepared from postnatal mice (1–3 days after birth), as previously described (Rose et al., 1993). Dissociated cortical cells were grown in multiwell dishes coated with poly-L-lysine (PDL) or on PDL-coated coverslips, using a media stock (MeS, Eagle’s minimal essential medium containing 25 mM glucose) supplemented with 5% horse serum, 5% fetal bovine serum, and 2 mM glutamine. Cultures were kept at 37 °C in a humidified 5% CO2-containing atmosphere until they reached confluency (7–10 days in vitro). Confluent cultures were used as a support for mixed cultures of neurons and astrocytes.

**Mixed cortical cultures**

Mixed cortical cultures containing neurons and astrocytes were prepared from mice at 14–16 days gestation, as described by Rose et al. (1993). Briefly, dissociated cortical or spinal cord cells were plated in 24 wells on a previously established layer of confluent astrocytes, using MeS supplemented with 5% horse serum, 5% fetal bovine serum, and 2 mM glutamine. After 3 days in vitro (DIV), non-neuronal cell division was halted by exposure to 10 μM cytosine-D-arabinofuranoside (Ara C). Subsequently, partial medium replacement was performed twice a week and, after 12 DIV, cultures were shifted to a maintenance medium identical to the plating medium but lacking serum. The amount of cells others that neurons and astrocytes (mainly microglia and more rarely oligodendrocytes) was quantified using Mac1 and P0FGRX immunostaining, and was estimated to a total of less than 2% of the numbers of neuronal cells. The same method was used to prepare mixed spinal cord cultures, in which neurons were obtained from spinal cords of mice at 14–16 days of gestation.

**Near pure neuronal cell cultures**

These cultures containing less than 5% astrocytes were prepared as detailed previously (Rose et al., 1993). Dissociated cortical cells in MeS supplemented with 5% fetal bovine, 5% horse serum, and 2 mM glutamine were plated in multiwell vessels that had been coated previously with PDL and laminin. After 3 DIV, non-neuronal cell division was halted by an exposure to 10 μM Ara-C. There was no further exchange of the media. After 12 DIV, cultures did not need the presence of serum to survive. They were shifted to a maintenance medium identical to plating medium but lacking serum. Cultures were used after 7 DIV for serum deprivation (SD) and after 13–14 DIV for excitotoxic injury.

**Excitotoxicity**

Slowly triggered excitotoxicity was induced at 37 °C by a 24-h exposure to 10 μM AMPA in MeS (Choi, 1992) in the presence of 10 μM MK801 to block secondary N-methyl-D-aspartate (NMDA)-dependent neuronal death. Neuronal death was estimated by microtubule-associated protein 2 (MAP-2) immunostaining and examination of the cultures under bright-field microscopy, and quantified by measurement of lactate dehydrogenase (LDH) release from damaged cells into the bathing medium, 1 day following the onset of excitotoxic exposure (Koh and Choi, 1987). The LDH level corresponding to complete neuronal death (100% without glial cell death) was determined in sister cultures exposed to 100 μM/L NMDA for 24 h. Background LDH levels were determined in sister cultures subjected to sham wash and subtracted from experimental values to yield the signal specific for experimentally induced injury. Percentage of cell death in experimental conditions was calculated using the formula: [% of cell death = (experimental value − BK value)/(FK − BK)], where BK stands for “blank” (sham wash) and FK stands for full kill (complete neuronal death).

**Immunocytochemistry in cultured cells**

For immunostaining of mixed neuron–astrocyte cultures, cells plated onto PDL-coated coverslips were fixed in 4% paraformaldehyde for 30 min before incubation for 2 h at room temperature in PBS containing 5% fetal goat serum and 0.1% Triton X-100 with primary antibodies for MAP-2 (1:250). After being rinsed with PBS, the cells were incubated for 1 h at room temperature with secondary Alexa-conjugated anti-mouse IgG. Then the coverslips were washed with phosphate buffer saline (PBS) and mounted on slides. Non-specific interactions of secondary antibodies were verified by omitting the primary antibodies.

**Immunohistology**

Animal tissue was processed as previously described (Docagne et al., 2005). Briefly, animals were perfuse-fixed with periodicate-lysine paraformaldehyde containing 0.25% glutaraldehyde (PLP) after saline perfusion and then the spinal cords were removed, postfixed for 1 h in PLP, cryoprotected for 12 h in 30% sucrose solution followed by 6 h in 7% sucrose solution, dried for 15 min at room temperature and frozen in Tissue Tek.

Immunostaining was performed as previously described (Clemente et al., 2004) in frozen, 20-μm-thick longitudinal sections of mouse spinal cords, using the monoclonal mouse SMI32 antibody (1:2500) and a horse biotinylated anti-mouse secondary antibody (1:200). The mouse-on-mouse kit (M.O.M™, Vector Laboratories) was used to reduce possible background due to the use of monoclonal anti-mouse antibody on mouse tissue. Non-specific interactions of secondary antibodies were verified by omitting the primary antibodies.

**Surgery and tissue analysis**

The experimental protocols adhered to the recommendations of the European Council and Spanish Department of Health for Laboratory Animals. Rats of every experimental group: (1) Normal (non-lesioned), (2) Vehicle (with laminectomy and PBS injection), (3) Lesioned (with laminectomy and AMPA injection) and (4) Treated (with laminectomy, AMPA +HU210 injection), were used in two different assays. Animals of groups 2, 3 and 4 were injected i.p. with MK801 (1 mg/kg), in order to avoid secondary lesions produced by NMDA receptor stimulation. Briefly, adult male Wistar rats (10 weeks old) were anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories: 50 mg/kg i.p.; and Rompín, Bayer: 3 mg/kg i.p.). Cervical spinal cord was identified and a hemi-laminectomy between C7 and T1 was performed under aseptic conditions, opening the dura mater with fine iridectomy scissors. A solution of AMPA–EtOH (5 mmol suspended in 2.0 mL of PBS) or HU210+–AMPA–EtOH (1 mmol suspended in 2.0 mL of PBS) or PBS alone for vehicle, were injected into C8 dorsal horn, using a borosilicate glass capillary and a depth of 300–500 μm. The spinal cord was then covered with Gelfoam (Upjohn Co.) soaked in sterile saline, and the muscle and skin layers were put together with chromic gut suture and surgical clips. The animals were maintained in a warm environment until full recovery from anaesthesia.

Spinal cords (C5–T1) were processed as described in the “Immunohistology” part of this section. Analysis was performed in frozen, 20-μm-thick transversal sections, after toluidine-blue staining using a standard protocol. In order to determine the lesion extent, two investigators, blind to the experiment, analysed the sections of spinal cord in levels C6, C7 and C8. One of every five sections (distance between two sections: 100 μm) was analysed for cell body loss in the grey matter. Every section analysed was then classified as undamaged or damaged for the presence or not of area of cell loss. The number of damaged sections in each
animal was determined as the mean value from the two investigators. The extent of the lesion for each animal was then calculated as the number of damaged sections multiplied by 100 μm.

Statistical analysis
All results are expressed as mean±SEM. For in vitro experiments, when n = 12 is indicated, this value corresponds to 12 different well pools derived from three independent dissections. As stated in the figure legend, statistical analysis consisted of Student’s t-test or one-way ANOVA, followed by Bonferroni–Dunn’s test. For the in vivo injections of AMPA, statistical analysis consisted in non-parametric Mann–Whitney U-test.

Acknowledgments
We would like to thank Elisa Baides, Joaquín Sancho and Rufino Fernández-Senso for their excellent technical support. FD is an I3P postdoctoral fellow (CSIC, Spain). This work was supported by the CICYT (Grant #002004/416, Ministerio de Educación y Ciencia, Spain).

References


