

Cannabinoids inhibit neurodegeneration in models of multiple sclerosis

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Summary

Multiple sclerosis is increasingly being recognized as a neurodegenerative disease that is triggered by inflammatory attack of the CNS. As yet there is no satisfactory treatment. Using experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis, we demonstrate that the cannabinoid system is neuroprotective during EAE. Mice deficient in the cannabinoid receptor CB₁ tolerate inflammatory and excitotoxic insults poorly and develop substantial neurodegen-

eration following immune attack in EAE. In addition, exogenous CB₁ agonists can provide significant neuroprotection from the consequences of inflammatory CNS disease in an experimental allergic uveitis model. Therefore, in addition to symptom management, cannabis may also slow the neurodegenerative processes that ultimately lead to chronic disability in multiple sclerosis and probably other diseases.

Keywords: cannabinoids; excitotoxicity; experimental allergic encephalomyelitis; multiple sclerosis; neuroprotection

Abbreviations: 2-AG = 2-arachidonoyl glycerol; CB = cannabinoid receptor; CREAE = chronic relapsing experimental allergic encephalomyelitis; EAE = experimental allergic encephalomyelitis; EAU = experimental allergic uveitis; ELISA = enzyme-linked immunosorbent assay; i.p. = intraperitoneal; IRBP = interphotoreceptor retinoid binding protein; NMDA = N-methyl-D-aspartate; Δ^9 -THC = tetrahydrocannabinol

Introduction

Multiple sclerosis is a chronic disease of the CNS, where autoimmunity is thought to drive the development of inflammatory lesions that induce the primary demyelination, which results in the inhibition of normal neurotransmission (Compston and Coles, 2002). However, the observation that disability often continues to worsen despite immunotherapy, which reduces blood–brain barrier dysfunction and relapse rate (Coles *et al.*, 1999; SPECTRIMS Study Group, 2001; Wiendl and Hohlfeld, 2002) underscores that neurodegenerative changes are of major importance in disease progression (Barnes *et al.*, 1991; Ferguson *et al.*, 1997; Trapp *et al.*, 1998; Coles *et al.*, 1999). This correlates with gross atrophy of the CNS, axonal loss and the accumulation of permanent disability (Bjartmar *et al.*, 2000; Compston and Coles, 2002). Axonal pathology is an early feature of multiple sclerosis lesions and is initially associated with inflammation (De

Stefano *et al.*, 2001; Filippi *et al.*, 2003); likewise, axonal damage is a feature in experimental allergic encephalomyelitis (EAE), an autoimmune model of multiple sclerosis (Baker *et al.*, 1990; Wujek *et al.*, 2002). During multiple sclerosis and EAE, destruction of myelin results in the redistribution and aberrant expression of axonal ion channels, and demyelinated axons are particularly sensitive to the damaging effects of free-radicals and glutamate excitotoxicity, which may additionally contribute to chronic neurodegeneration in CNS autoimmune disease (Foster *et al.*, 1980; Black *et al.*, 2000; Pitt *et al.*, 2000; Smith *et al.*, 2000, 2001; Werner *et al.*, 2001; Lo *et al.*, 2002; Kapoor *et al.*, 2003). Therapeutic strategies in multiple sclerosis have concentrated on immunomodulation (Wiendl and Hohlfeld, 2002). There is an urgent need for agents that can inhibit progressive multiple sclerosis.

Table 1 *CB₁-deficient mice are susceptible to the development of EAE*

Strain	No. EAE/total	Clinical score (± SEM)	Day of onset (± SD)
ABH (wild type)	15/15	4.0 ± 0.1	14.7 ± 1.0
ABH. <i>Cnr1</i> ^{+/+} (wild type homozygous)	6/6	4.0 ± 0.0	16.3 ± 1.8
ABH. <i>Cnr1</i> ^{+/-} (heterozygous)	9/9	4.2 ± 0.2	15.4 ± 1.0
ABH. <i>Cnr1</i> ^{-/-} (knockout)	15/15	4.1 ± 0.1	16.3 ± 1.8

The results represent the number of animals within a group that developed clinical EAE, the mean maximal clinical score ± SEM that developed during acute phase EAE (up to day 24 post-inoculation) and the mean day of onset ± SD of clinical EAE (score >0.5) The disease incidence, onset and severity of congenic mice was comparable ($P > 0.05$) to that found in wild-type ABH mice, as assessed using the Mann–Whitney *U*-test.

Cannabis contains many compounds but it has been found that the major psychoactive ingredient is Δ^9 -tetrahydrocannabinol (Δ^9 -THC) (Mechoulam and Gaoni, 1967). Δ^9 -THC mediates the majority of its activities through stimulation of cannabinoid receptors (CB), notably CB₁, which are expressed throughout the CNS (Matsuda *et al.*, 1990; Howlett *et al.*, 2002). Following the discovery of the receptors, fatty acid endogenous ligands, such as anandamide and 2-arachidonoyl glycerol (2-AG), and a degradation system including a re-uptake mechanism and hydrolytic enzymes have been identified (Devane *et al.*, 1992; Deutsch and Chin, 1993; Mechoulam *et al.*, 1995; Dinh *et al.*, 2002). The cannabinoid system functions to regulate synaptic neurotransmission (Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001) and tonically controls clinical signs such as spasticity and tremor that develop in chronic EAE (Baker *et al.*, 2000, 2001). This provides objective evidence to support the claims of multiple sclerosis patients that cannabis may have a benefit in symptom management (Consroe *et al.*, 1997), a claim further supported by some recent clinical trials of medical cannabis extracts (Killestein *et al.*, 2002; Robson *et al.*, 2002; Vaney *et al.*, 2002). There is *in vitro* evidence that cannabinoids can also regulate glutamate release, oxidant free radicals and calcium influxes (Twitchell *et al.*, 1997; Hampson *et al.*, 1998; Kreitzer and Regehr, 2001; Howlett *et al.*, 2002), which, in excess, can cause neuronal death in neuroinflammatory disease (Pitt *et al.*, 2000; Smith *et al.*, 2000; Kapoor *et al.*, 2003). The lack of specificity of all available cannabinoid reagents (Howlett *et al.*, 2002) and the potential presence of additional CB-like receptors (Di Marzo *et al.*, 2000; Breivogel *et al.*, 2001; Monory *et al.*, 2002) means that gene-deleted transgenic mice (Ledent *et al.*, 1999; Zimmer *et al.*, 1999) provide powerful tools to definitively investigate the potential role of the cannabinoid system in neuroprotection.

Material and methods

Animals

Biozzi ABH and CB1 gene (*Cnr1*)-deficient mice were from stock bred at the Institute of Neurology. They were fed

RM-1(E) diet and water *ad libitum*. Congenic (N5) ABH.*Cnr1*^{-/-} ^{-/+} and ^{+/+} were generated from CD1.*Cnr1*^{-/-} knockout mice (Ledent *et al.*, 1999) and screened as described previously (Brooks *et al.*, 2002). B10.RIII mice were purchased from Harlan Olac, Oxford, UK. All experiments were ethically performed according to the UK Animals (Scientific Procedures) Act (1984), under the control of the UK Government, Home Office.

Chemicals

The cannabinoid receptor agonists *R*(+)-WIN55,212 and CP55,940 were purchased from Tocris (Bristol, UK). *N*-methyl-D-aspartate (NMDA) and the NMDA receptor antagonist MK-801 were obtained from Sigma (Poole, UK). The CB₁ selective antagonist rimonabant (SR141617A; Rinaldi-Carmona *et al.*, 1994) and Δ^9 -THC were from the National Institute for Drugs and Abuse (NIDA) drug supply program. These were dissolved in ethanol : cremophor : PBS (1 : 1 : 18) and 0.1–0.2 ml was injected intraperitoneally (i.p.) daily.

Induction of chronic relapsing EAE (CREAE)

Mice were injected subcutaneously in the flank on day 0 and 7 with 1 mg mouse spinal cord homogenate in complete Freund's adjuvant [60 µg *Mycobacterium tuberculosis* H37Ra, *Mycobacterium butyricum* (4 : 1) per injection] on day 0 and 7 (Baker *et al.*, 1990). Clinical disease was assessed daily and scored: 0 = normal, 1 = limp tail, 2 = impaired righting reflex, 3 = paresis of hindlimbs, 4 = complete paralysis of hindlimbs and 5 = moribund/death (O'Neill *et al.*, 1992). The activity of animals was monitored over 5 min in a 27 × 27 cm open-field activity chamber (Brooks *et al.*, 2002). Tissues were either snap-frozen or formaldehyde-fixed for immunohistology (Baker *et al.*, 1990; Ahmed *et al.*, 2002), western blotting for caspase activity (Ahmed *et al.*, 2002) and enzyme-linked immunosorbent assay (ELISA) detection of CNS proteins.

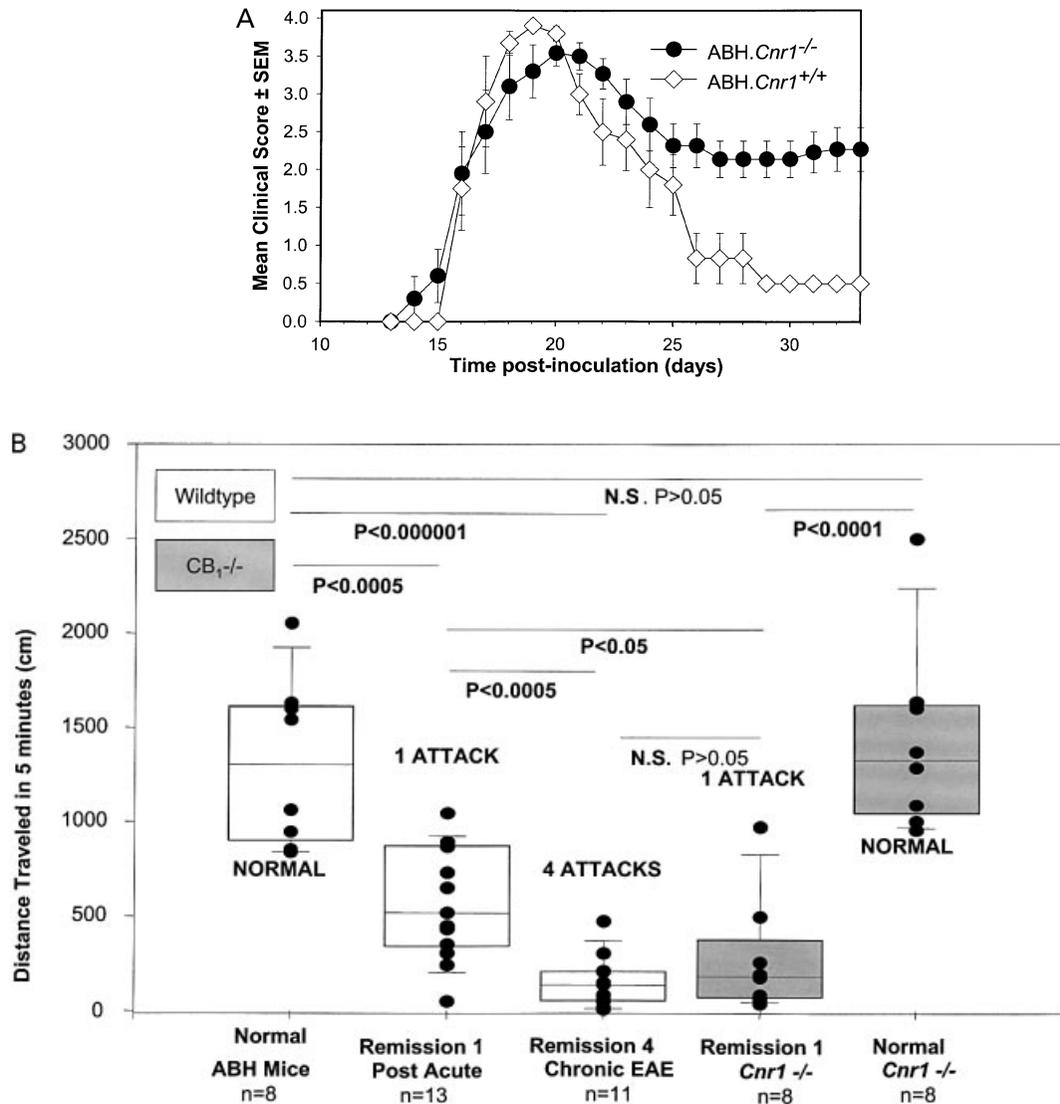


Fig. 1 Cannabinoids limit accumulation of disability in EAE. CREAE was actively induced in wild-type ABH or CB₁ gene (*Cnr1*)-deficient, congenic ABH mice with mouse spinal cord homogenate in complete Freund's adjuvant on day 0 and 7. (A) The mean ± SEM daily clinical scores (post-induction) demonstrate that CB₁ knockout mice (filled circles) show poor recovery from paralysis compared with wild-type mice (open diamonds). (B) Movement activity of normal and animals with EAE in remission after one or four paralytic disease episodes were measured in an activity chamber over 5 min. The results represent the individual data points (solid circles) and box plot (25–75% percentile) with 5–95% percentile range of activity wild-type (open box) and CB₁ knockout (shaded box) mice.

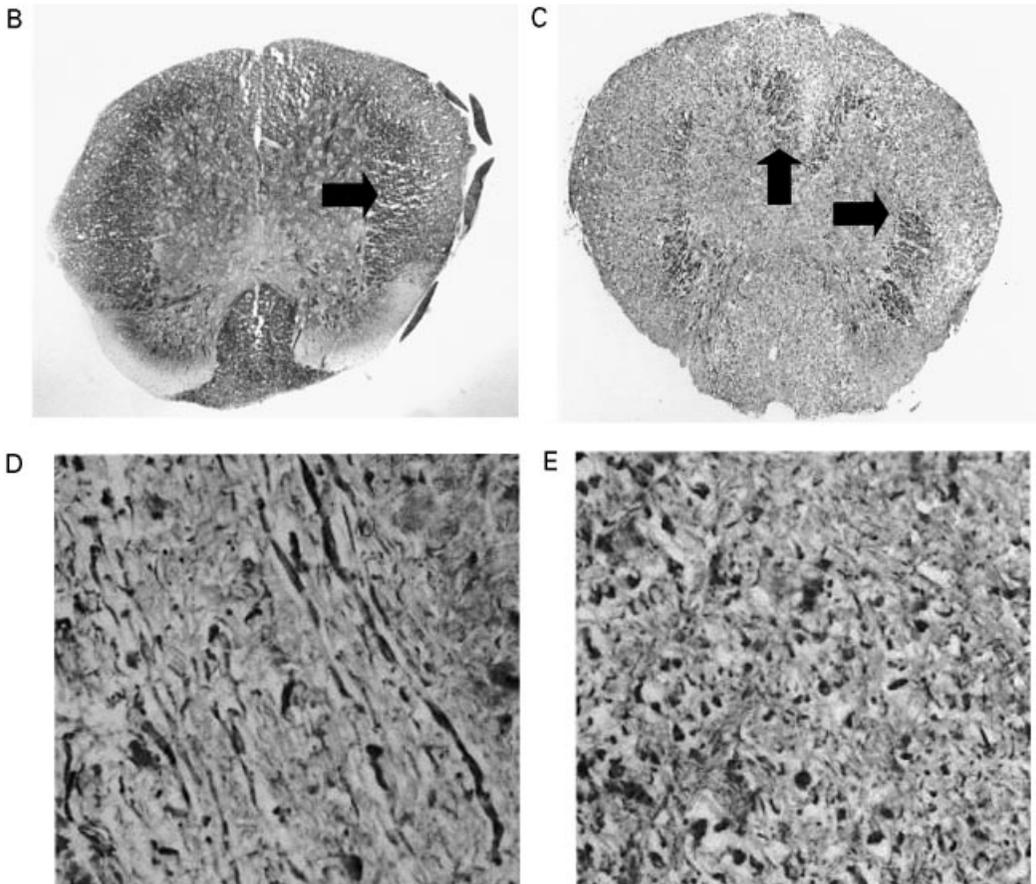
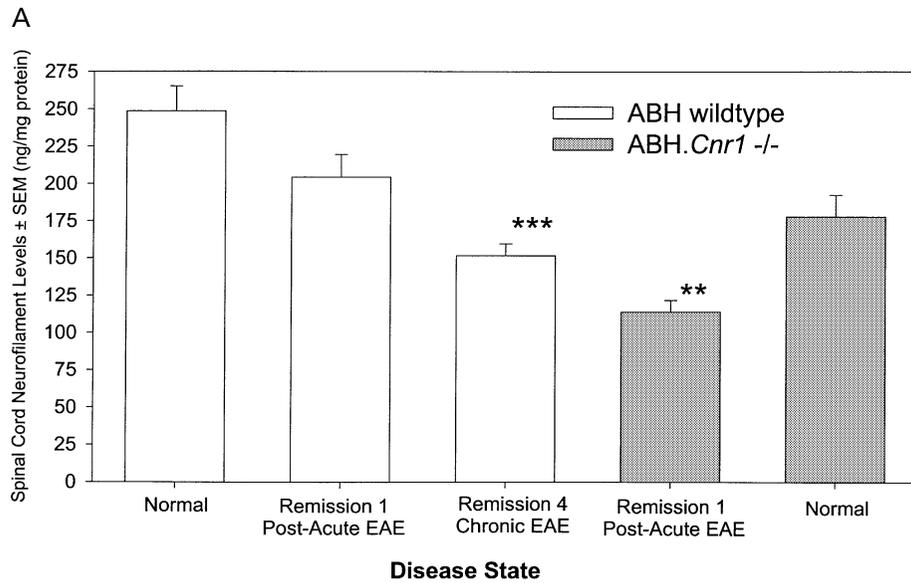
Neurofilament ELISA

Whole spinal cords were homogenized on ice by trituration and sonication in 500 µl of barbitor buffer [11 mM barbital, 63 mM sodium barbital, 1.2 mM EDTA (Sigma)] containing a protease inhibitor cocktail and 4 mM EGTA. Lipids were extracted from the sample by adding diisopropyl-ether (Sigma) at 1 : 5000 and centrifuging for 5 min at 20 000 g. The supernatant was frozen and stored in aliquots at -70°C, and the total protein was measured using the standard Lowry method. Ninety-six-well microtitre plates (Maxisorp; Nunc, Rochester, NY, USA) were coated overnight at 4°C with the SMI35 coat monoclonal antibody (SMI35; Sternberger Monoclonals Inc., Lutherville, MD,

USA) against neurofilament heavy chain diluted in 0.05 M sodium carbonate (pH 9.6). This was followed by a wash step with barbitor buffer containing 5 mM EDTA, 1% bovine serum albumin and 0.05% Tween-20 (Sigma). Non-specific protein binding was blocked by incubation with 1% bovine serum albumin in barbitor buffer for 1 h at room temperature, followed by a wash with wash buffer as above. Spinal cord homogenates were serially diluted to 1 : 10 000 in barbitor buffer containing 5 mM EDTA, and incubated at room temperature for 2 h. After washing, a rabbit polyclonal anti-neurofilament H antibody (N-4142; Sigma), diluted 1 : 1000, was incubated at room temperature for 1 h. Following another wash, horseradish

peroxidase-conjugated anti-rabbit immunoglobulin diluted 1 : 1000 was incubated for 1 h at room temperature. The tetramethylbenzidine chromogenic reagent (R & D Systems Europe, Minneapolis, MN, USA) was used, signal devel-

opment stopped using 1 M phosphoric acid, and the plate read at 450 nm, with a reference reading at 620 nm. The antigen concentration for each sample was calculated from an internal standard curve ranging from 0 to 250 ng/ml



(high-performance liquid chromatography-purified bovine neurofilament H; Affiniti Bioreagents, Golden, Colorado, USA). All samples were analysed in duplicate.

Induction of experimental allergic uveitis (EAU)

B10.RIII mice were injected subcutaneously with 25 µg interphotoreceptor retinoid binding protein (IRBP^{151–181}) peptide in complete Freund's adjuvant (as used in EAE experiments) on day 0 and 7, disease was assessed histologically, by haematoxylin and eosin-stained 5 µm paraffin wax sections, for the level of infiltration (score 0–6) and structural damage (score 0–5), as described previously (Hankey *et al.*, 2001).

Glutamate excitotoxicity

NMDA-induced Ca²⁺ influx

Cerebellar neurons obtained from 6-day-old wild-type ABH.*Cnr1*^{+/+} and CB₁ knockout mice were cultured for 9 days in poly-D-lysine coverslips as described previously (Evans and Pocock, 1999). At 36 h and 6 days, 10 µM cytosine arabinoside was added to inhibit non-neuronal proliferation (Evans and Pocock, 1999). Cells were loaded with 5 µM of the fluorescent Ca²⁺ indicator dye fura-2 acetoxymethyl ester (Calbiochem, Nottingham, UK), prior to ionotropic glutamate receptor stimulation with 100 µM NMDA and subsequent inhibition with the NMDA receptor

antagonist, 10 µM MK-801. This concentration of MK-801 is required to give maximal block of imaged NMDA-induced Ca²⁺ influxes in cerebellar neurons (Pocock and Nicholls, 1998). The CB₁ receptor agonist CP55,940 (0.01–5 µM) was added 5 min before imaging commenced and was present throughout the experiment. A 12-bit digital camera acquired images and the output visualized with a Life Science Resources Merlin Imaging system, version 1.8630 (Perkin Elmer Life Science, Cambridge, UK). Data were analysed by calculating the 340/380 nm fluorescence ratios with time.

Kainic acid induced lesion

Mice were deeply anaesthetized with halothane and stereotactically injected unilaterally (bregma 2.5 mm, medial-lateral 1.7 mm and dorsoventral 1.6 mm) with 1.5 nmol of kainic acid in 30 µl of 0.9% saline over 30 s; the injection needle was retained for 1 min to prevent reflux of fluid (Chen and Strickland, 1997).

Statistical analysis

Non-parametric data were assessed using the Mann–Whitney *U*-test with Minitab software (Coventry, UK), parametric data were assessed using *t*-tests with Sigmasat software. The group score represents the maximal clinical grade developed by all animals within the group.

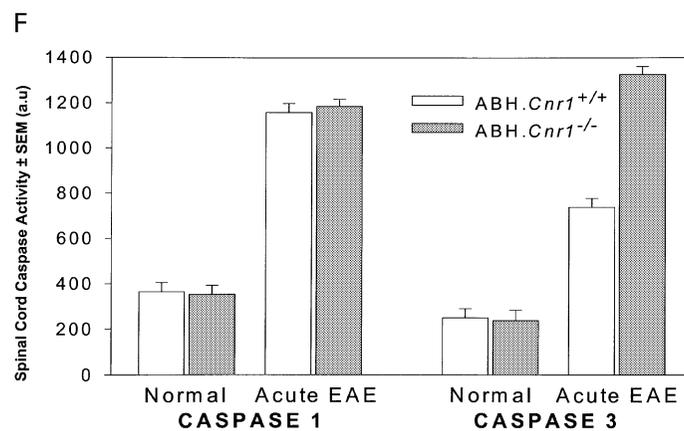


Fig. 2 Cannabinoids mediate neuroprotection in experimental allergic encephalomyelitis. CREAE was actively induced in wild-type ABH or CB₁ gene (*Cnr1*)-deficient, congenic ABH mice with mouse spinal cord homogenate in complete Freund's adjuvant on day 0 and 7, and disease progression in wild-type mice is associated with axonal damage and loss. (A) Spinal cord neurofilament levels from tissue homogenates from wild-type (open boxes) and CB₁ knockout (shaded boxes) mice were measured by ELISA from normal and animals with EAE in remission after one or four paralytic disease episodes during EAE. The results represent the mean ± SEM neurofilament levels ($n = 6–8$ per group). ** $P < 0.01$, *** $P < 0.001$ compared with respective normal controls. (B–E) Axonal damage was reflected histologically. Bielschowsky silver stain of paraffin 5 µm wax sections of lumbar spinal cord in (B) normal and (C) chronic EAE after four attacks, demonstrating few surviving axons (arrows) and note the loss of axons in the dorsal horn. Neurofilament-specific immunocytochemistry of the spinal cord from a CB₁ knockout mouse (D) before and (E) after a single paralytic episode of EAE. Note the many transactions of the white matter axons. (F) Caspase-1 and -3 levels were assessed using western analysis and caspase-3 levels are significantly ($P < 0.001$) elevated in CB₁ knockout mice (shaded boxes) during acute EAE compared with wild type (open boxes) (Student's *t*-test; $n = 6$ per group).

Results

Development of chronic paresis in EAE is associated with accumulation of axonal loss

Following actively, spinal cord homogenate-induced CREAE, ABH mice develop a relapsing–remitting disease progression of distinct paralytic disease episodes followed by remission with an increasing residual deficit (Baker *et al.*, 1990, 2000). Although histological axonal damage occurs in the initial acute phase of CREAE in ABH mice, this becomes much more evident, particularly in the spinal cord, following the development of relapsing disease (Baker *et al.*, 1990; Ahmed *et al.*, 2002). Whilst EAE has classically been assessed using a subjectively scored scale of paralysis (Table 1; Fig. 1A) (Baker *et al.*, 1990; Smith *et al.*, 2000; Lo *et al.*, 2002), accumulating residual deficit could be quantitatively demonstrated through assessment of mobility of remission animals in an open-field activity chamber (Fig. 1B). Whilst ABH mice remitted to exhibit more immobility after one episode of paralysis ($P < 0.001$; clinical score 0.5), movement activity was further reduced ($P < 0.001$) after three to four episodes (clinical grade 2.5–3), where animals had chronically developed residual hind limb paresis upon recovery from relapsing paralytic episodes (Fig. 1B). This immobility was associated with accumulating axonal loss, which could be quantitatively assessed using a neurofilament ELISA (Fig. 2A), and demonstrated histologically (Fig. 2B and C).

The cannabinoid system regulates EAE-induced neurodegeneration

Wild-type (ABH) and congenic wild-type homozygous (ABH.*Cnr1*^{+/+}), heterozygotes (ABH.*Cnr1*^{+/-}) and CB₁-deficient (ABH.*Cnr1*^{-/-}) mice developed EAE with comparable incidence, severity and onset (Table 1). There was, however, an increased frequency (>5%) of EAE-induced mortality in CB₁-deficient mice and also in heterozygotes, which have reduced CB₁ expression (Selley *et al.*, 2001). However, most strikingly CB₁-deficient mice exhibited significantly ($P < 0.05$) more immobility and residual paresis (Fig. 1) and axonal pathology (Fig. 2A and D–F) than wild-type mice following recovery after the first paralytic episode. These animals relapse and accumulate more deficits that rapidly reach an acceptable severity limit, including the development of permanent hindlimb paralysis. Consistent with the enhanced neurodegeneration after a single attack, spasticity (Baker *et al.*, 2000) developed early in ABH.*Cnr1*^{-/-} mice, which in wild-type ABH mice usually only occurs after three to four attacks (Baker *et al.*, 2000). Injection of wild-type mice with the CB₁ antagonist (twice daily with 5 mg/kg rimonabant *i.p.* from onset; $n = 8$) induced greater mortality than usually occurs (<5%). However, using clinical signs of remission, the level of neurodegeneration was not as consistent as that found in CB₁-deficient animals (data not shown). Although multiple pathways contribute to

axonal damage in EAE, the final effector mechanism in neuronal death is probably toxic ion influxes (Ca²⁺) and caspase-3-mediated apoptosis (Ahmed *et al.*, 2002), and consistent with this, ABH.*Cnr1*^{-/-} mice exhibited significantly ($P < 0.001$) elevated levels of active caspase-3 during acute-phase EAE compared with wild-type ABH.*Cnr1*^{-/-} mice, although levels of caspase-1 activity were comparable (Fig. 2F). Caspase-3 could be detected immunocytochemically in dying axons and these axons demonstrated many transections, which is a feature of multiple sclerosis (Trapp *et al.*, 1998) (Fig. 2D and E). Therefore, neurodegeneration is clearly elevated in CB₁-deficient mice following inflammatory insults, suggesting that CB₁ agonism should have neuroprotective potential in CB₁-wild-type animals, in addition to controlling neurological symptoms such as tremor and spasticity (Baker *et al.*, 2000).

Exogenous CB₁ agonism is neuroprotective in inflammatory CNS disease

In CREAE in ABH mice, the neurological deficit accumulates slowly over a number of months and multi-focal lesions can occur anywhere along the neuroaxis (Baker *et al.*, 1990), complicating assessment and treatment of neurodegeneration, especially as limited quantities of Δ^9 -THC were available for study. In contrast, neurodegeneration is restricted to a focal site and develops rapidly in EAU. Following sensitization of B10.RIII mice with IRBP^{153–180} peptide, the neuroretina is almost completely destroyed within 14–16 days (Hankey *et al.*, 2001) (Fig. 3A–E), again associated with caspase-3-induced neuronal pathology (not shown). CB₁ receptor agonism with either *R*(+)-WIN-55,212-2 (Figs 3C and 4A), at doses that demonstrated no immunosuppressive effect in EAE (vehicle: $n = 9/9$, clinical score 3.3 ± 0.4 , day of onset 17.6 ± 1.2 ; compared with 5 mg/kg *i.p.* *R*(+)-WIN 55,212-2 from day 10–22, $n = 7/8$, clinical score 3.4 ± 0.5 , day of onset 17.3 ± 1.8), and Δ^9 -THC significantly inhibited photoreceptor damage, without any apparent inhibition of inflammatory infiltrate (Figs 3E and 4B). Therefore, CB₁ agonism can mediate neuroprotection during inflammatory insults.

Cannabinoids regulate/inhibit glutamate excitotoxicity

Although immunosuppression, shown by a reduction in the degree of infiltrate, was not evident following treatment with *R*(+)-WIN-55, 212–2 or Δ^9 -THC (Fig. 3) shortly before expression of disease, Δ^9 -THC has been reported to have immunosuppressive effects (Lyman *et al.*, 1989; Wirguin *et al.*, 1994), which could influence neurodegenerative potential. As glutamate excitotoxicity has been implicated in neuronal damage in this and other EAE models (Achiron *et al.*, 2000; Pitt *et al.*, 2000; Smith *et al.*, 2000), glutamate-induced excitotoxicity was examined *in vitro* and following CNS injection of kainic acid *in vivo* to examine neuro-

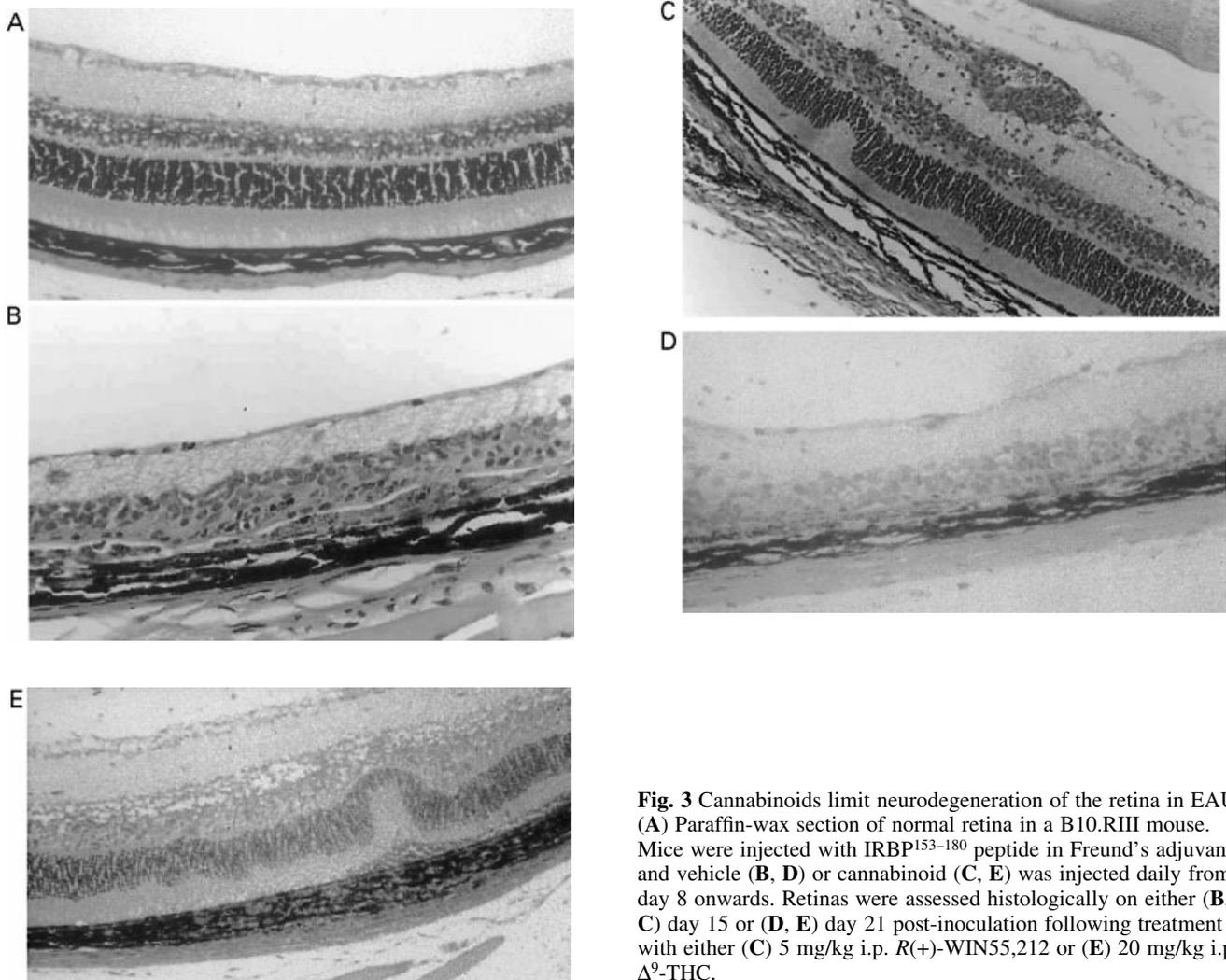


Fig. 3 Cannabinoids limit neurodegeneration of the retina in EAU. (A) Paraffin-wax section of normal retina in a B10.RIII mouse. Mice were injected with IRBP^{153–180} peptide in Freund's adjuvant and vehicle (B, D) or cannabinoid (C, E) was injected daily from day 8 onwards. Retinas were assessed histologically on either (B, C) day 15 or (D, E) day 21 post-inoculation following treatment with either (C) 5 mg/kg i.p. *R*(+)-WIN55,212 or (E) 20 mg/kg i.p. Δ^9 -THC.

protective potential in the absence of a compounding immunomodulation. Following *in vitro* stimulation of NMDA receptors there was a marked (cerebellar) neuronal Ca^{2+} influx, which was more pronounced in CB_1 -deficient mice than in controls, suggesting that the cannabinoid system exhibits tonic control of this response (Fig. 5A). In addition, the NMDA receptor antagonist (MK-801) was slower at reducing Ca^{2+} to basal levels in CB_1 -deficient mice compared with ABH.*Cnr1*^{+/+} wild type, suggesting Ca^{2+} dysregulation in the absence of CB_1 receptors had occurred. Exogenous CB_1 agonism by CP55,940 inhibited this NMDA-induced cytosolic Ca^{2+} influx in wild-type animals, maximally at 1 μM using these culture conditions, but was relatively ineffective in CB_1 -knockout mice, suggesting that post-synaptic control of NMDA-receptor activation is lost in CB_1 -deficient mice (Fig. 5A). The injection of kainic acid (>0.15 nM) into CB_1 -deficient mice induced seizures and caused mortality, usually within 10 min post-injection, which did not occur in wild-type ABH and ABH congenic wild-type (Fig. 5B) mice ($P < 0.01$), despite using 50-fold higher

doses of kainic acid. This elevated mortality was also evident in *CD1.Cnr1*^{-/-} mice (Fig. 5B), and therefore CB_1 receptors are also clearly regulating ionotropic glutamate receptor activity (Fig. 5), which has been implicated in neural excitotoxicity.

Discussion

Neurological disability in multiple sclerosis correlates with spinal cord axonal loss (~50–70% in paralysed multiple sclerosis patients) and reduced *N*-acetyl aspartate (NAA) levels in chronic multiple sclerosis patients (Bjartmar *et al.*, 2000). This study provides the first and definitive evidence that the cannabinoid system controls the development of neurodegeneration, which occurs as a result of inflammatory insult of the CNS. This provides a novel avenue for neuroprotection in multiple sclerosis and other neurodegenerative diseases.

In diseases such as EAE and multiple sclerosis, it is unlikely that there is a single route to neurodegenerative events, and these may change during the disease course. The

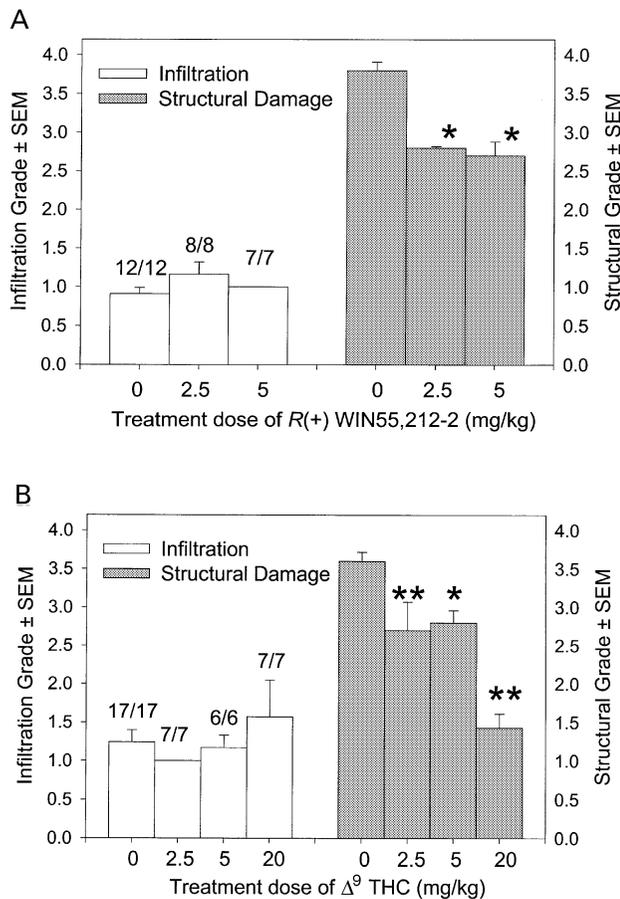


Fig. 4 Cannabinoids mediate neuroprotection in EAU. Following active induction of EAU in B10.RIII mice with IRBP¹⁵³⁻¹⁸⁰ peptide in complete Freund's adjuvant, eyes were processed for routine wax histology and the level of infiltration (scale 0–5) and structural damage (scale 0–6) assessed. Animals received daily injections of (A) 5 mg/kg i.p. R(+)-WIN55,212 or (B) 20 mg/kg i.p. Δ⁹-THC, dissolved in ethanol : cremophor : PBS (1 : 1 : 18), from day 8 onwards. The data represent the results on days 15 and 21 post-inoculation, respectively. The number of animals with EAU per group is indicated. * $P < 0.05$, ** $P < 0.01$ compared with respective control group.

clinical outcome will be determined by the rate that these accumulate and how the genetic background of the individual enables them to adapt to the insult. Whilst axonal loss occurs very early in the course of multiple sclerosis (Filippi *et al.*, 2003), it can remain clinically silent for some time, and irreversible neurological disability appears to develop when a threshold (15–30% in mice) of axonal loss is reached and compensatory CNS resources are exhausted (Confavreux *et al.*, 2000; Wujek *et al.*, 2002). In this model, marginal, statistically non-significant axonal loss occurred after the initial neurological attack, assessed here using a novel, relatively rapid neurofilament ELISA and by changes in NAA levels (Preece *et al.*, 1994). Once chronic paresis was evident there was significant axonal loss (~40%) as shown here by ELISA and also by magnetic resonance spectroscopy of

spinal cord NAA levels (R. A. Page, H. G. Parkes, D. Baker, G. Giovannoni and C. A. Davie, unpublished observations). However, most interestingly, CB₁-deficient mice accumulated significant axonal loss (~36%) even after a single acute episode, indicating that the presence of CB₁ was mediating a degree of neuroprotection during autoimmune attack. Surprisingly, analysis of spinal cord axonal content indicated that apparently normal, CB₁-deficient ABH mice have fewer spinal nerves than wild-type animals ($P < 0.001$), which may be reflective of CB₁ involvement in neural plasticity during development (Kim and Thayer, 2001), or there could be inherent neurodegeneration in these animals. This requires further study.

In EAE and, at least initially, in multiple sclerosis axonal damage occurs at least concordantly with inflammation (Ferguson *et al.*, 1997; Trapp *et al.*, 1998), which produces many potentially damaging elements such as cytokines and oxidative stress (Koprowski *et al.*, 1993; Werner *et al.*, 2001; Lock *et al.*, 2002). Ionotropic glutamate receptor systems can also signal damaging mechanisms, at the blood–brain barrier and within the neural microenvironment, in EAE and multiple sclerosis (Bolton and Paul, 1997; Achiron *et al.*, 2000; Pitt *et al.*, 2000; Smith *et al.*, 2000; Kalkers *et al.*, 2002). As shown here, cannabinoids can tonically regulate NMDA glutamate receptor activity *in vitro* and support the *in vivo* observation that CB₁ regulates NMDA-induced and ischaemic excitotoxicity (Nagayama *et al.*, 1999; Parmentier-Batteur *et al.*, 2002). We also show definitively that CB₁ receptor activity regulates kainate glutamate receptor activity *in vivo*. Cannabinoids also have anti-oxidant properties that could further limit damaging events during inflammation (Hampson *et al.*, 1998; Howlett *et al.*, 2002). In addition, cellular changes such as neural and oligodendrocyte death and gliosis will change the CNS microenvironment, for example through redistribution of ion channels on demyelinated nerves (Foster *et al.*, 1980; Black *et al.*, 2000), loss of trophic support and the formation of compensatory neural pathways, which may contribute to excitotoxic stress and induce further degeneration. This could amplify as the disease progresses, possibly largely independent of inflammation (Compston and Coles, 2002), and may have similarities to nerve destruction in other neurodegenerative conditions, such as Huntington's chorea, Alzheimer's disease and amyotrophic lateral sclerosis, where nerve loss accumulates slowly. The cannabinoid system acts as a regulator of many different neurotransmitters and ion (K⁺ and particularly Ca²⁺) channels (Henry and Chavkin, 1995; Twitchell *et al.*, 1997; Howlett *et al.*, 2002) and appears to be particularly important when CNS homeostasis is in imbalance, as occurs in disease (Baker *et al.*, 2000). Therefore, CB₁ can act at many levels within the death cascade, which will ultimately lead to toxic ion influxes, cell metabolic failure and activation of death effector molecules, such as caspase-3 (Ahmed *et al.*, 2002). This would be consistent with the rapid neurodegeneration that accumulates in CB₁-deficient mice. This also implicates a role for endocannabinoids in neuroprotection. The nature of

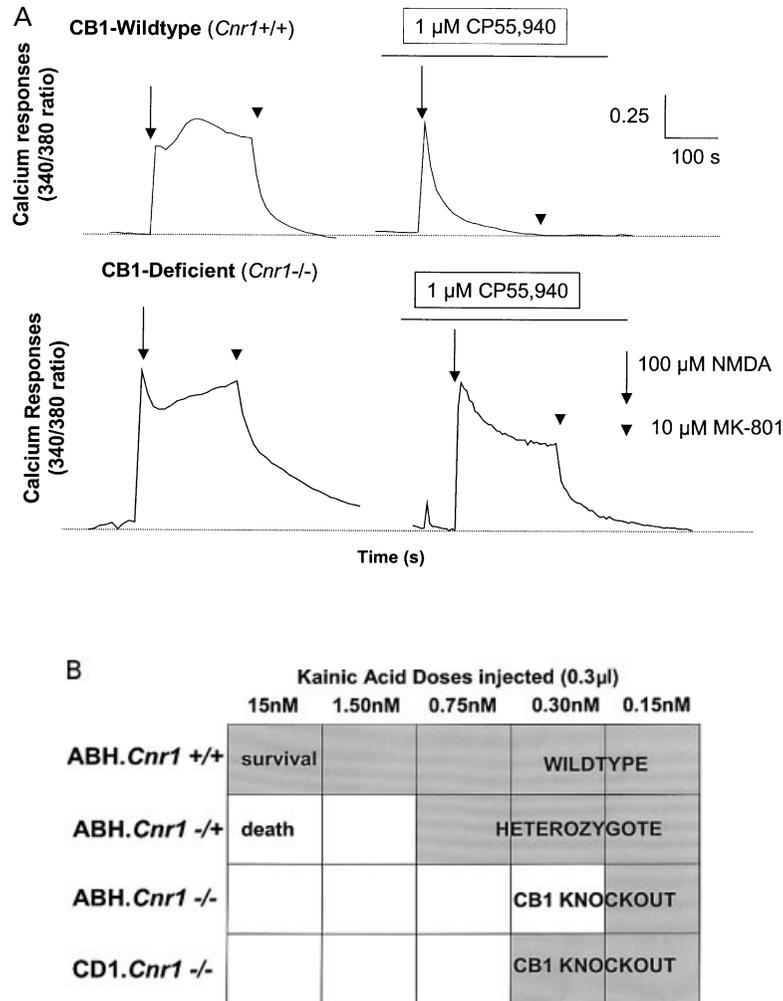


Fig. 5 Cannabinoids inhibit glutamate-induced excitotoxicity. **(A)** Lack of CB₁ receptors leads to loss of regulation of NMDA-induced calcium influx. Cerebellar neurons from wild-type and CB₁ knockout mice were loaded with the fluorescence Ca²⁺ indicator dye fura-2 prior to ionotropic glutamate receptor stimulation. Each trace is the mean somatic response of 40 individual neurons measured by single-cell fluorescence imaging. The NMDA receptor agonist, NMDA (100 μ M), was added at the arrow and the NMDA receptor antagonist, MK-801, was added at the arrowhead. Where indicated, the CB₁ receptor agonist CP55,940 (1 μ M) was added 5 min before imaging commenced and was present throughout the experiment. **(B)** Kainate-induced excitotoxicity *in vivo*. Halothane anaesthetized animals ($n = 5$ per group) were intracerebrally injected with 15–0.15 nmol of kainic acid. Rapid (1–10 min) mortality (open blocks) developed in ABH.*Cnr1*^{-/-} mice compared with the survival (0 out of 5 mortality; shaded blocks) that occurred in wild type (ABH.*Cnr1*^{+/+}). Seizures consistent with glutamate excitotoxicity were evident. CB₁ knockout animals died within 1–3 min of injection, whereas heterozygotes typically showed adverse effects 5–10 min after injection. Doses lower than the maximal survival dose for each strain were not tested.

the endogenous neuroprotective cannabinoid has yet to be definitively resolved and may involve more than one CB₁-mediated pathway, possibly dependent on the neural circuit involved. Whilst in head trauma it has been suggested that 2-AG may mediate neuroprotection (Panikashvili *et al.*, 2001), in a similar study anandamide, not 2-AG, was shown to be active (Hansen *et al.*, 2001). However, as both anandamide and 2-AG are elevated in chronic EAE lesions (Baker *et al.*, 2001) both may participate in endogenous neuroprotective

mechanisms. This will be elucidated once suitable agents to dissect these pathways become available.

Previous studies in non-demyelinating EAE models have demonstrated that high-dose Δ^9 -THC, often administered during the induction process, has clinical disease ameliorating effects, due to prevention of infiltrate reaching the CNS (Lyman *et al.*, 1989; Wirguin *et al.*, 1994). Furthermore, Δ^9 -THC had no effect on the clinical course, which in Lewis rats is usually naturally self-limiting, when treatment was initi-

ated at disease onset (Lyman *et al.*, 1989). Acute phase paralysis in most rodent EAE models is rapidly reversed and can occur largely independent of any demyelination and axonal loss, and more probably reflects conduction block (Wujek *et al.*, 2002). Therefore, inhibition of acute phase paralysis (Lyman *et al.*, 1989) may reflect inhibition of the immune process that leads to damage in addition to neural effects. Indeed, CB agonists have recently been reported to have immunomodulatory effects in a viral model of multiple sclerosis (Arevalo-Martin *et al.*, 2003; Croxford and Miller, 2003). Without the use of tissue-specific CB₁ conditional knockouts (Marsicano *et al.*, 2002), it is probably not possible to completely exclude some influence of cannabinoid inhibition of the immune response in the neurodegenerative process. However, in the context of multiple sclerosis, both would be of benefit for inhibition of disease.

The results of this study are important because they suggest that in addition to symptom management, cannabinoids offer the potential to slow the progression of a disease that as yet has no satisfactory treatment. Therefore, if trials are extended to monitor the long-term effects of cannabis use on symptom management, they should be designed to monitor neuronal loss and progression. If CB₁ agonism can be applied whilst limiting the unwanted psychoactive potential, such as through enhancement of endocannabinoid levels (Baker *et al.*, 2001), this may provide a new therapeutic route in multiple sclerosis and could be combined with therapies that target the immunological elements of disease. In neurodegenerative diseases including multiple sclerosis, signs appear once significant damage has already accumulated, slowing the degenerative process early following diagnosis may help improve quality of life for many more years.

Acknowledgements

The Multiple Sclerosis Society of Great Britain and Northern Ireland, the Brain Research Trust, Aims2Cure and the National Institute for Drug Abuse (NIDA) chemical supply program supported this work. Support from the Wellcome Trust and the Alzheimers Trust is also gratefully acknowledged. The authors have declared that they have no conflicting financial interests.

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Received February 19, 2003. Revised April 23, 2003

Accepted April 28, 2003