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INVITED REVIEW

Neuroprotection in Experimental Autoimmune Encephalomyelitis and Progressive Multiple Sclerosis by Cannabis-Based Cannabinoids

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Abstract Multiple sclerosis (MS) is the major immune-mediated, demyelinating, neurodegenerative disease of the central nervous system. Compounds within cannabis, notably Δ9-tetrahydrocannabinol (Δ9-THC) can limit the inappropriate neurotransmissions that cause MS-related problems and medicinal cannabis is now licenced for the treatment of MS symptoms. However, the biology indicates that the endocannabinoid system may offer the potential to control other aspects of disease. Although there is limited evidence that the cannabinoids from cannabis are having significant immunosuppressive activities that will influence relapsing autoimmunity, we and others can experimentally demonstrate that they may limit neurodegeneration that drives progressive disability. Here we show that synthetic cannabidiol can slow down the accumulation of disability from the inflammatory penumbra during relapsing experimental autoimmune encephalomyelitis (EAE) in ABH mice, possibly via blockade of voltage-gated sodium channels. In addition, whilst non-sedating doses of Δ9-THC do not inhibit relapsing autoimmunity, they dose-dependently inhibit the accumulation of disability during EAE. They also appear to slow down clinical progression during MS in humans. Although a 3 year, phase III clinical trial did not detect a beneficial effect of oral Δ9-THC in progressive MS, a planned subgroup analysis of people with less disability who progressed more rapidly, demonstrated a significant slowing of progression by oral Δ9-THC compared to placebo. Whilst this may support the experimental and biological evidence for a neuroprotective effect by the endocannabinoid system in MS, it remains to be established whether this will be formally demonstrated in further trials of Δ9-THC/cannabis in progressive MS.

Keywords Cannabinoid · Cannabidiol · Experimental autoimmune encephalomyelitis · Multiple sclerosis · Neuroprotection · Δ9-tetrahydrocannabinol

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Introduction

Multiple sclerosis (MS) is a major immune-mediated, demyelinating and neurodegenerative disease of the central nervous system (CNS), which affects about 2-3 million people worldwide (Compston and Coles 2002, 2008). Disease is often associated with relapsing-remitting neurological attacks and the progressive, slow worsening of disability, typically over many years. Demyelination and axonal and neuronal loss leads to a variety of different cognitive, sensory and motor problems that accumulate as disease progresses due to lesions within different neural pathways of the CNS (Compston and Coles 2002). At present there is no cure, although there are some disease modifying therapies (DMT) that can slow down the development of CNS lesions and neurological relapses

caused by the entry of cells of the peripheral immune system into the CNS. These however, have relatively low efficacy, as occurs with the beta interferons, glatiramer acetate and teriflunomide, or higher efficacy which can be associated with significant, sometimes life-threatening side effects, which has been reported with fingolimod, natalizumab and alemtuzumab (Marta and Giovannoni 2012). These can limit the nerve loss that occurs as a consequence of these lesions (Gunnarsson et al. 2011), however, these treatments if not started sufficiently quickly following diagnosis, do not appear to control the nerve loss associated with progressive MS. This is driven by central inflammatory and other neurodegenerative effects that underlie irreversible disability (Compston and Coles 2002; Marta and Giovannoni 2012). Dysregulation of effective neurotransmission leads to a number of troublesome symptoms dependent on lesion location and include: incontinence; spasms; spasticity and pain (Compston and Coles 2002). These are controlled by a variety of different drugs, which are often associated with significant sedating side effects (Compston and Coles 2002). The failure to find adequate treatments, leads people with MS (PwMS) to often seek complementary or alternative medicines (CAM) to supplement their prescribed medicines (Yadav et al. 2014; Masullo et al. 2015). With the advent of the internet, use of CAM can be widely publicised and adopted even before scientific evidence can support or refute the claims of efficacy. Indeed PwMS perceived benefit from taking cannabis for the control of sleep disturbances, pain and spasticity (Consroe et al. 1997; Clark et al. 2004; Chong et al. 2006). This was subsequently supported by biology, experimental and clinical class I evidence in humans to support the role of cannabinoid control of spasticity and pain in PwMS (Baker et al. 2000, 2012; Novotna et al. 2011; Zajicek et al. 2012; Langford et al. 2013).

Symptom Control by Cannabinoids

The endocannabinoid systems regulates synaptic neurotransmission and it is therefore not surprising that compounds within cannabis can stimulate neuronal CB_1 cannabinoid receptors (CB_1R) to control the excessive or inappropriate neurotransmission that leads to symptoms of MS (Corey-Bloom et al. 2012; Zajicek et al. 2012). Some places are now supporting the use of medical marijuana, and cannabis extracts (Sativex/nabiximols) have become licensed medicines for the treatment of spasticity and pain in MS (Novotna et al. 2011; Langford et al. 2013). Early reports from Europe and the USA failed to distinguish any perceived therapeutic efficacy in symptom control of MS (Consroe et al. 1997). However, in experimental models of MS-related spasticity that occurs due to CNS autoimmunity, it could be shown that delta9 tetrahydrocannabinol (Δ9-THC) and the CB_1R controlled symptoms, with no apparent effect of cannabinol (CBD) on spasticity (Baker et al. 2000; Wilkinson et al. 2003; Pryce and Baker 2007; Pryce et al. 2014). This could suggest that Δ9-THC is the major therapeutic chemical within cannabis, based on the reports that cannabis in North America may have a low CBD content (ElSohly et al. 2000; Wilkinson et al. 2003; EMCDD 2008). However, pharmaceutical, medical cannabis extracts being developed (Sativex & Cannador) contain essentially equal proportions of Δ9-THC and CBD (Novotna et al. 2011; Zajicek et al. 2012; Langford et al. 2013). Although it has been reported that CBD may limit the side-effect potential of Δ9-THC within cannabis (Dalton et al. 1976; Russo and Guy 2006), little direct evidence has been provided for such a specific ratio and contrasts with the low CBD:Δ9-THC ratio (1:10–1:200) in many recreational cannabis extracts (Burgdorf et al. 2011). However, it appears that CBD is not inert and may have some medicinal value (Mechoulam et al. 2002; Russo and Guy 2006). Whilst medicinal cannabis has become a licensed treatment for symptom control, the question arises whether compounds within cannabis have additional properties that could be useful in the control of MS. We review the current literature and present data to suggest that cannabis may have utility in the control of nerve loss and disease progression due to neuroimmunological disease.

Lack of Marked Immunosuppressive Effects of Cannabinoids in EAE

Some studies have suggested that Δ9-THC and CBD may have an immunosuppressive activity that could provide some DMT function (Lyman et al. 1989; Maresz et al. 2007; Kozela et al. 2011). This is seen by a reduction in the incidence and severity of disease and/or a delay in the onset of disease in experimental autoimmune encephalomyelitis (EAE) models of MS (Baker et al. 2011). In contrast to some immunosuppressive action of 5 mg/kg CBD reported in myelin-peptide induced EAE in C57BL/6 mice (Kozela et al. 2011) and the observation that 5–10 mg/kg CBD, but not 2.5 or 20 mg/kg CBD, can inhibit the development of collagen-induced arthritis in DBA-1 mice (Malfait et al. 2000), we have consistently failed to detect any immunosuppressive effect in tissue homogenate induced EAE in ABH mice in multiple experiments across a range of doses from 0.5 to 25 mg/kg. The lack of immunosuppressive effects were found in an initial EAE attack (Maresz et al. 2007) or as found here (Fig. 1) in an induced-relapse; in the latter, essentially all the animals developed EAE of comparable severity and day of onset as found in vehicle treated animals. This suggests that CBD is unlikely to prevent relapsing neuroimmune-autoimmunity in MS.

Differences in the ease of immunosuppression in C57BL/6 (relatively EAE-resistant) and ABH (EAE susceptible) mice have been seen previously (Sisay et al. 2013). As such,

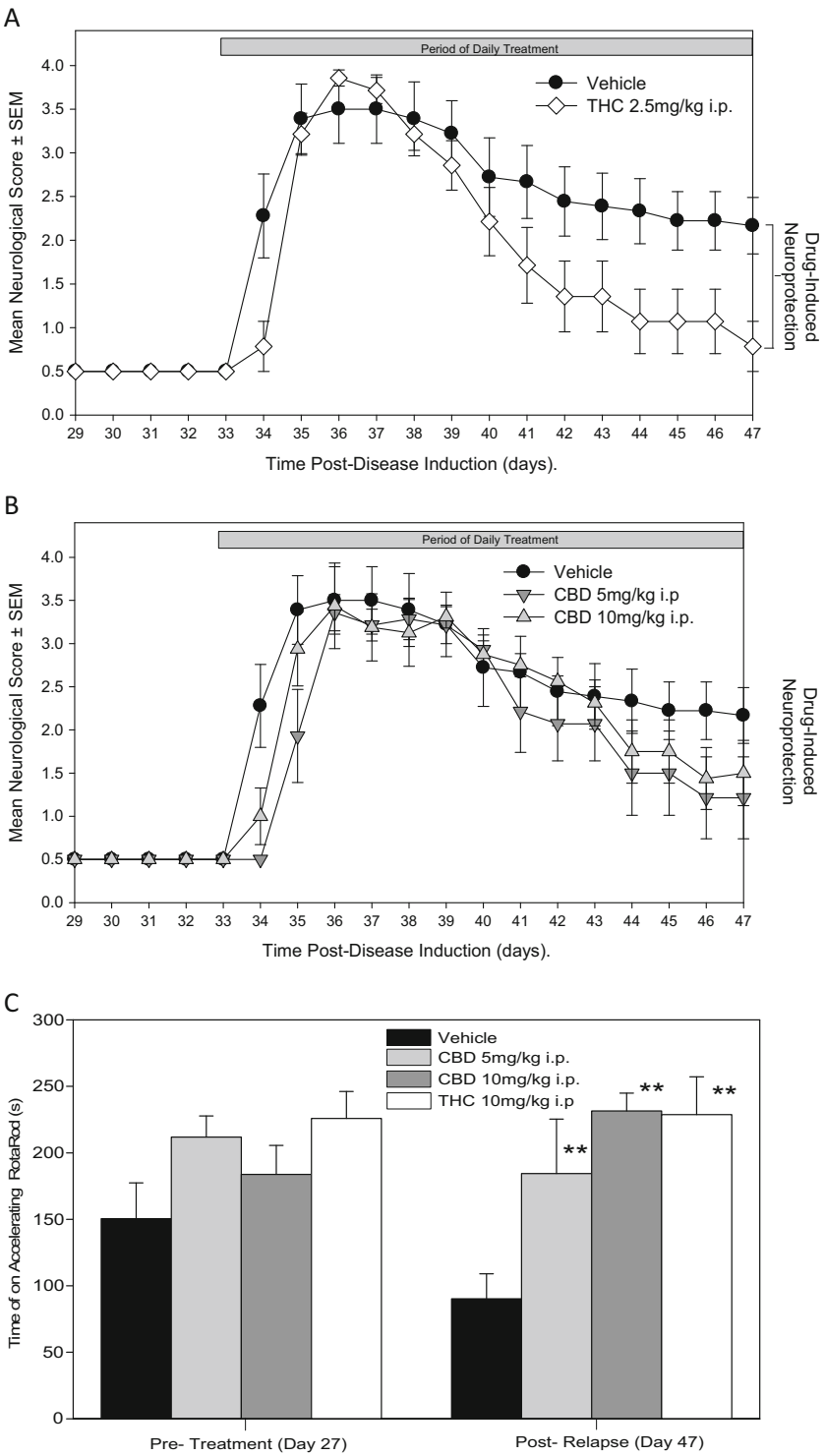

Fig. 1 Neuroprotective potential of cannabinoids during induce-relapsing autoimmune encephalomyelitis. EAE was induced in Biozzi ABH mice following immunization with spinal cord homogenate emulsified in Freunds complete adjuvant on day 0 and 7. (Al-Izki et al. 2012). Animals were allowed to undergo a paralytic inflammatory attack (all animals scored 3–4) and a relapse was induced by re-immunization on day 28 during the first remission (RM1). Animals (7–9/group) were injected i.p. with either: (A, C) 2.5 mg/kg Δ9-THC, (B, C) 5 mg/kg CBD, 10 mg/kg CBD or ethanol:cremophor:phosphate buffered saline from day 33 onwards. (**a**, **b**) The results represent the mean±SEM daily score based on a 0–5 scoring scale (Al-Izki et al. 2012). The differences between the minimal disease score at the termination of the experiment were analysed using Mann Whitney U statistics. (**c**) The mean±SEM activity on an accelerating rotorod (4–40 rpm) (Al-Izki et al. 2012) measured on day 27 and day 47 during the second remission (RM2). Differences between vehicle and treatment groups were analysed using Students *t* tests ** $P<0.01$ compared to vehicle treated animals

apparent immunosuppression observed in EAE induced in C57BL/6 mice, where disease induction can be inconsistent, is lost once tested in ABH mice where robust and consistent disease is induced (Sisay et al. 2013). Furthermore, in contrast to the relapsing-remitting nature of EAE in ABH mice (Al-Izki et al. 2012), myelin oligodendrocyte glycoprotein-induced EAE in C57BL/6 is typically monophasic with poor recovery (Sisay et al. 2013). This is related to inflammation induced neurodegeneration. Thus the benefit of CBD may relate to a neuroprotective effect rather than an immunosuppressive effect. Nevertheless, it is possible to induce immunosuppression of EAE with ≥5 mg/kg Δ9-THC in both SJL (Lyman et al. 1989) and ABH mice, via a neuronal CB_1R-dependent mechanism (Maresz et al. 2007; Croxford et al.

2008; de Lago et al. 2012). However, we do not believe that these effects are particularly relevant to the clinical use of cannabis (Baker et al. 2012). This is because the immunosuppression only occurs as doses of CB_1R agonists that cause marked sedative, cannabimimetic (hypomotility; hypothermia and sometimes seizures) effects (Croxford et al. 2008). These probably cause significant stress-responses that are known to be immunosuppressive in EAE (Bolton et al. 1997). Importantly, there is no solid data to suggest that doses of medical cannabis cause significant immunosuppressive effects in MS, following analysis of peripheral immune responses (Killestein et al. 2003; Katona et al. 2005; Sexton et al. 2014).

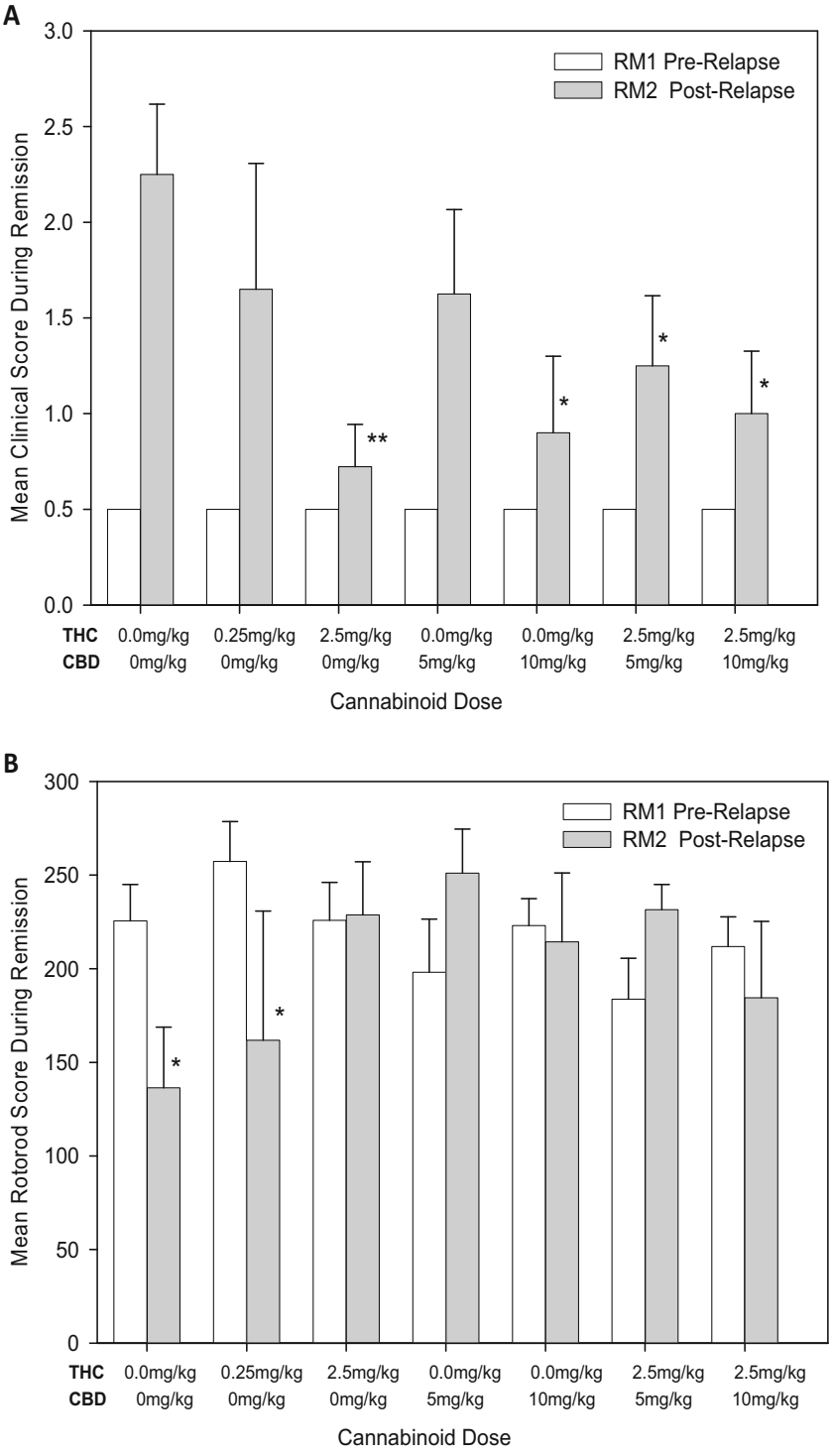

Fig. 2 Neuroprotective potential of cannabinoids during induce-relapsing autoimmune encephalomyelitis. EAE was induced in Biozzi ABH mice following immunization with spinal cord homogenate emulsified in Freunds complete adjuvant on day 0 and 7. (Al-Izki et al. 2012) Animals were allowed to undergo a paralytic inflammatory attack (all score 3–4) and a relapse was induced by re-immunization on day 28 during the first remission (RM1). Animals (5-9/group) were injected i.p. with either 0.25 mg/kg Δ9-THC, 2.5 mg/kg Δ9-THC, 5 mg/kg CBD, 10 mg/kg CBD a combination of Δ9-THC and CBD or ethanol:cremophor:phosphate buffered saline from day 33 onwards. (**a**) The results represent the mean minimum score during remission±SEM daily score based on a 0–5 scoring scale (Al-Izki et al. 2012). These were analysed using Mann Whitney U statistics. (**b**) The mean±SEM activity on an accelerating rotorod (Al-Izki et al. 2012) measured on day 27 and day 47 during the second remission (RM2). *$P<0.05$, ** $P<0.01$ compared to vehicle treated animals, using Students *t* test

Neuroprotective Effect of Cannabis-Plant Based Cannabinoids in EAE

In contrast to the limited immunosuppressive action of cannabis-based cannabinoids that inhibit the development of paralytic EAE (Maresz et al. 2007; Baker et al. 2011), we and others have shown that CB_1R agonists, including Δ9-THC and endocannabinoids, can induce a neuroprotective effect (Pryce et al.2003; Croxford et al. 2008; Webb et al. 2008; Hasseldam and Johansen 2010; Hernández-Torres et al. 2014; Bernal-Chico et al. 2015). In EAE, this is seen by a better functional recovery and a reduced accumulation of disability following paralytic attack (Croxford et al. 2008; Baker et al. 2011; Al-Izki et al. 2014). As such, Δ9-THC facilitated a dose-dependent enhanced recovery from the effects of the inflammatory penumbra (Al-Izki et al.2014) developing during paralytic, relapsing EAE (Figs. 1 and 2) at a stage that allows us to dissociate neuroprotective and immunosuppressive effects (Baker et al. 2011; Al-Izki et al. 2012). This was seen by significantly ($P<0.01$) less accumulation of neurological disability, as assessed using neurological score (Figs. 1a and 2a) and less loss of motor co-ordination as assessed using rotorod activity (Figs. 1c and 2b), which is consistent with neuroprotection observed in previous studies (Pryce et al. 2003). This is probably mediated by multiple mechanisms including; alterations in neural excitotoxicity, oxidative stress and changes in the glial neuroimmune responses (Pryce et al. 2003; Docagne et al. 2007; Rossi et al. 2011a; Ribeiro et al. 2013; Musella et al. 2014). Whilst we have not found any symptomatic benefit or immunosuppressive action of CBD in acute (Baker et al.2000; Maresz et al. 2007) and relapsing EAE (Fig. 1b), interestingly there was a significantly ($P<0.05$) better clinical recovery, indicative of a neuroprotective effect (Figs. 1b and 2a) following administration of 5 mg/kg and particularly 10 mg/kg CBD i.p. (Fig. 2a) when administered shortly before relapse. This clinical effect was also reflective of better rotorod activity (Figs. 1c and 2b). For technical reasons, it was not possible to measure spinal nerve content in these experiments to definitively demonstrate an effect on nerve survival. However, based on the clinical score and notably the rotorod score, which has a very strong positive correlation with spinal nerve content (Al-Izki et al. 2012, 2014), it appeared evident that both Δ9-THC and CBD alone had neuroprotective potential.

A neuroprotective effect of Δ9-THC was not surprising as CB_1R agonists, including Δ9-THC, have previously been shown to have neuroprotective effects as shown histologically, in other stages of EAE (Pryce et al.2003; Croxford et al. 2008). Likewise, this is consistent with the observation that CB_1R-deficient mice have been shown to accumulate more neurodegeneration and disability as a consequence of neuroimmune attack than wild type animals (Pryce et al. 2003; Rossi et al. 2011a). Here it may be associated with the inhibition of excessive glutamatergic signals that can lead to downstream, excitotoxic nerve damage, via neural CB_1R-dependent mechanisms (Pryce et al. 2003; Docagne et al. 2007; Musella et al. 2014). In addition, Δ9-THC also exhibits anti-oxidant properties (Hampson et al. 1998) and inhibits calcium and sodium ion channels (Howlett et al. 2002) that can also limit nerve cell death due to toxic ion concentrations within the disease CNS. In addition, there may be actions through the inhibition of microglial cell activity, which could be via CB_2R (Howlett et al. 2002; Docagne et al. 2007; Correa et al. 2009). Likewise, CBD has been found to offer neuroprotective potential in a variety of different experimental paradigms (Mecha et al. 2012, 2013). Although a number of studies indicate that CBD has neuroprotective anti-oxidant effects (El-Remessy et al. 2003; Hayakawa et al. 2007), we show here that CBD also appears to inhibit Na^+ ion channel activity (Fig. 3).

We used veratrine-evoked uptake of [^{14}C]-guanidine flux assay and inhibition of scorpion venom facilitated [^{12}H]-batrachotoxin-B (a sodium channel ligand; BTX-B) binding using rat cerebral cortex synaptosomes to measure sodium channel activity (Fig. 3.Garthwaite et al. 2002). In this assay, veratrine holds the Na^+ channels in an open state and the influx of [^{14}C] guanidine through the channels and into the synaptosomes is measured (Garthwaite et al. 2002). YC-1, a soluble guanylylcyclase activator and Na^+ channel inhibitor (Garthwaite et al. 2002) served as a positive control. This had an IC_{50} of 16.2 μM in veratrine-evoked uptake of [^{14}C]-guanidine flux assay (Fig. 3a). This is comparable to the response reported previously for YC-1 and showed a similar activity to Sipatrigine (BW619C89. IC_{50}=14.8 μM). Lamotrigine (IC_{50}=186.2 μM), which is a clinical sodium channel inhibitor used as an anti-convulsive agent to treat epilepsy, had a lower inhibitory capacity (Garthwaite et al. 2002). Cannabidiol, surprisingly, was over ten times more active than YC-1, in this veratrine-evoked uptake of [^{14}C]-guanidine flux assay with an IC_{50} of 0.9 μM (Fig. 3a). In contrast, nabilone, a potent CB_1R/CB_2R receptor agonist, used to treat emesis in humans was inactive (>100 μM) in the assay (Fig. 3a). Likewise nabilone was relatively inactive in blockage of titrated BTX-B binding, whereas cannabidiol potently (IC_{50} of 3–4 μM) inhibited binding of BTX-B to synaptosomes (Fig. 3b) and was again about ten times more potent than YC-1 (IC_{50} of 3–45.7 μM) and compared well with Sipatrigine (IC_{50} of 14.8 μM) and Lamotrigine (IC_{50} of 159.6 μM Garthwaite et al. 2002). Our studies on sodium channel blockage are supported and extended by other recent studies that have also shown activity of CBD on sodium channels including $Na_v1.1$, $Na_v1.2$ and others. (Hill et al. 2014). Sodium channel inhibitors can exhibit marked neuroprotective effects in induced-relapsing EAE through effects on nerves and microglia (Waxman 2002; Al-Izki et al. 2014; Morsali et al. 2013). Sodium channel blockers used to treat epilepsy may also have some neuroprotective potential in MS (Gnanapavan et al. 2013). This is perhaps consistent with the mechanism of action of CBD, although clinically CBD does not appear to have the

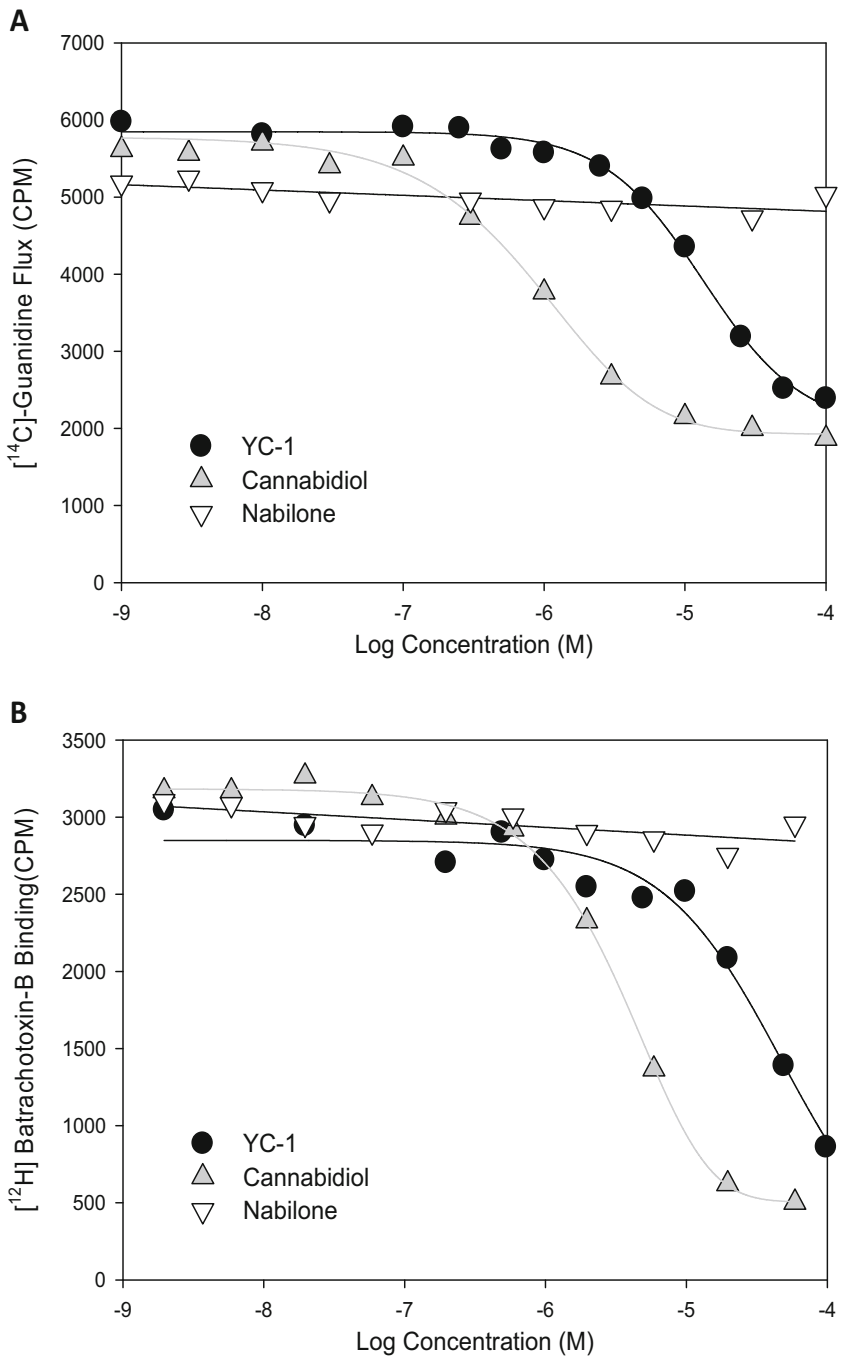

Fig. 3 Sodium ion channel Inhibitory activity of cannabidiol. Various concentrations of CBD, nabilone or YC-1 were incubated with rat cerebral cortex synaptosomes in the presence of (**a**) veratrine and [^{14}C]-guanidine or (**b**) Scorpion venon and [^{12}H]-Batrachotoxin-B (Garthwaite et al. 2002). Uptake of guanidine or inhibition of binding of batrachotoxin-B was assessed using liquid scintillation spectroscopy (Garthwaite et al. 2002). The results of CBD affinities were repeated with comparable results

side-effect potential of some clinical sodium channel blockers, which limited drug compliance and the perceived success of clinical trials of this compound class in MS (Kapoor et al. 2010; Gnanapavan et al. 2013). However, CBD is reported to have additional ionic effects within mitochondria, and inhibits pro-inflammatory activities of microglia and other modes of action (Ryan et al. 2009; Mecha et al. 2012; Espejo-Porras et al. 2013; Iannotti et al. 2014), which could also add to a neuroprotective potential and in addition CBD has been reported to limit oligodendrocyte damage (Mecha et al. 2012).

It was interesting however that there appeared to be no additive effect following co-administration of both CBD with Δ9-THC in 2:1–4:1 CBD:Δ9-THC ratios (Fig. 2). Furthermore, the neuroprotective effect of Δ9-THC appeared to be abrogated by the presence of CBD (Fig. 2). It has been reported that the presence of CBD limits the sedative and psychoactive effect of Δ9-THC (Dalton et al. 1976; Russo and Guy 2006). This may be compatible with the report that CBD has some CB_1R antagonist potential, which could alter some behavioural effects of Δ9-THC (Thomas et al. 2007; Vann et al. 2008). As

such this could counteract the CB_1R-mediated beneficial effects of Δ9-THC. Whether this impacts dosing of medical cannabis is difficult to properly address in the absence of human data. Although it been suggested that the influence of CBD on CB_1R-mediated effects of Δ9-THC are of marginal significance at the concentrations of CBD in typical US of smoked marijuana (Varvel et al. 2006), it may be relevant that long-term follow-up in symptom-control trials suggested that oral Δ9-THC but not oral 1:1 CBD:Δ9-THC cannabis extracts (Cannador) containing comparable levels of Δ9-THC, could limit the accumulation of disabilities in MS (Zajicek et al. 2005). This would be consistent with a neuroprotective effect of Δ9-THC (Carroll et al. 2012), which was formally investigated in a recent trial in MS (Zajicek et al. 2013).

Cannabinoids for the Control of Progression in Multiple Sclerosis

Genetic depletion of CB_1R in mice is associated with the development of neurodegeneration (Pryce et al. 2003; Rossi et al. 2011a). Furthermore, antagonism of CB_1R in humans

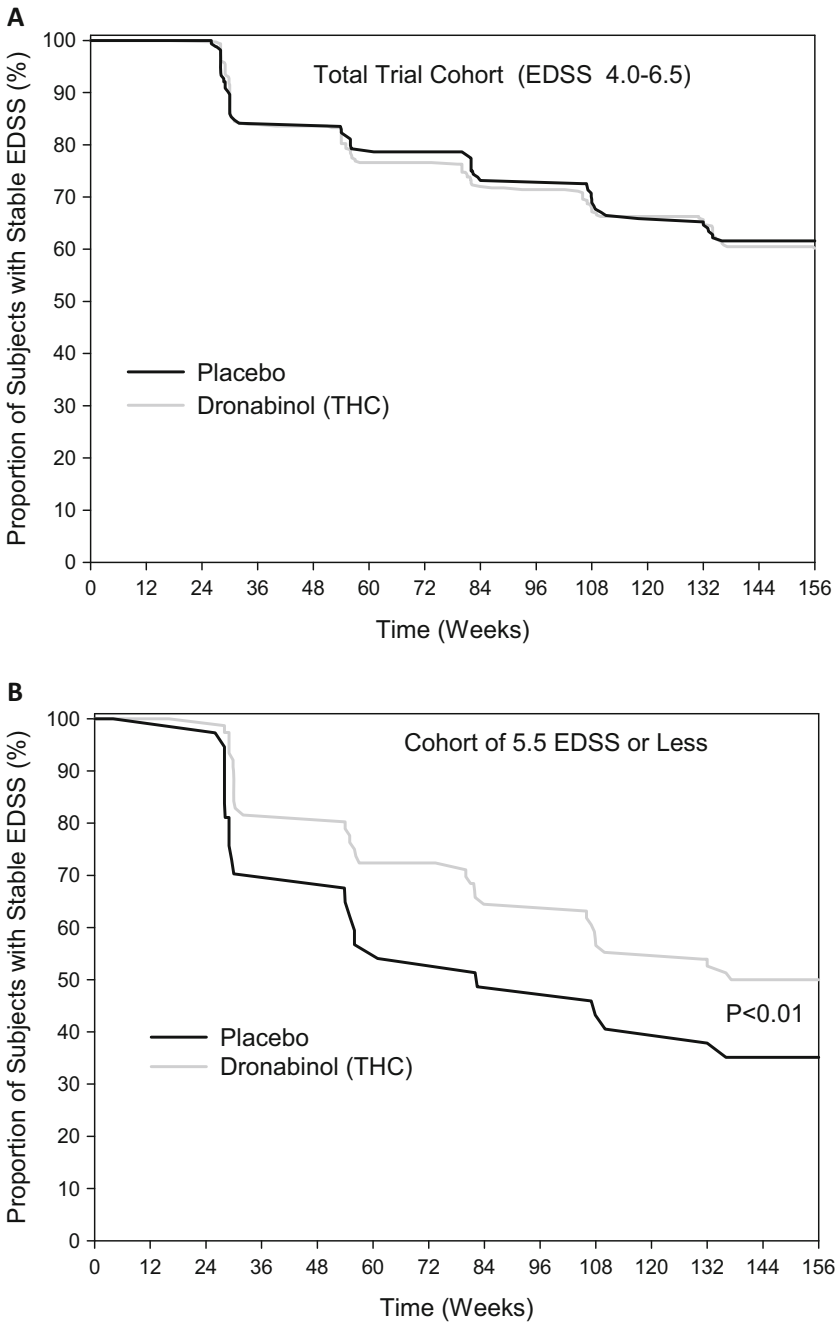

Fig. 4 Inhibition of progressive multiple sclerosis by oral tetrahydrocannabinol. People with progressive MS were enrolled into a placebo-controlled double blind clinical trial to assess efficacy of twice daily oral tetrahydrocannabinol (*Grey*) to a maximum of 28 mg/day verses vegetable oil placebo (*Black*) capsules (Zajicek et al. 2013). The probability of progression of EDSS was performed for the (**a**) total population (n=320 Δ9-THC and n=162 placebo) or (**b**) participants with a baseline EDSS score of 5·5 or lower (n=76 Δ9-THC and n=34 placebo). The results represent Kaplan-Meier estimates of the proportion of people with stable EDSS over time weekly. The plot shows timings of first events of progression. Figures have been presented and analysed in Zajicek J. et al. Lancet Neurol 2013:12:857–865+online supplement. Reproduced with permission from Elsevier

augments glutamatergic excitability (Oliviero et al. 2012), which is known in excess to cause excitotoxicity in nerves. It is of interest therefore that a non-coding; genetic variant of the CB_1R gene has been be associated with more rapid progression and neurodegeneration during inflammatory attack in MS (Rossi et al. 2011b, 2013). Endocannabinoid stimulation of cannabinoid receptors has also been reported to be neuroprotective (Eljaschewitsch et al. 2006). This suggests that agonism of the CB_1R by exogenous delivered cannabinoids should have neuroprotective potential. However, unfortunately, it was reported that daily treatment with oral Δ9-THC had no overall effect on the progression of MS in the progressive phase of MS (Zajicek et al. 2013. Fig. 4a). This may question previous suggestions of a neuroprotective effect in MS (Zajicek et al. 2005) and the accumulated experimental biology (Baker et al. 2012). However, similar to the failure of Δ9-THC in this progression trial, likewise, it was originally reported that oral Δ9-THC and cannabis extract (Cannador) had no effect on spasticity in a symptom-control trial (Zajicek et al. 2003). Likewise, other cannabis extracts (Sativex) failed to alter the Ashworth scale as a measure of spasticity in symptom control trials in MS (Wade et al. 2003). However, through adapting clinical trial design, duration and the outcome measures, it has been found that cannabis can indeed control symptoms of MS (Zajicek et al. 2005, 2012; Novotna et al. 2011; Corey-Bloom et al. 2012). This indicates that trial design is critical in the detection of therapeutic effects and the translation of animal studies into human benefit (Baker and Amor 2014). In the trial of Δ9-THC in progressive MS, lower than expected progression rates occurred and so may have affected the ability to detect clinical change, which would require longer trials (Zajicek et al. 2013). Clinical progression in MS is assessed using the Expanded Disability Status Score (EDSS). This is a motor score ranging from health (EDSS Score 0); walking without aid or rest for 500 m (EDSS Score 4), walking with an aid/cane (EDSS score 6); essentially restricted to a wheel chair (EDSS 7); to death (EDSS 10). However, this neurological rating system is not linear and PwMS can progress at variable rates (Leray et al. 2010). However, progression between from EDSS 3 to EDSS 6 is more consistent and potentially more rapid (Leray et al. 2010). Therefore, trials enriched for this subset of PwMS may have a greater chance of detecting differences. Whilst it was clear that daily Δ9-THC, at the doses tested, did not slow progression (Fig. 4a), it is of immense interest that analysis of a subset of people with MS with an EDSS≤5.5 demonstrated that Δ9-THC significantly ($P<0.01$) slowed disease progression (Fig. 4b; Zajicek et al. 2013). This would be consistent with the accumulating biological knowledge and supportive experimental evidence in animal models (Baker et al. 2000, 2012) and would strongly suggest that cannabinoids indeed have the potential to control neuroimmune processes that lead to neurodegeneration. Furthermore, the presence of Δ9-THC in the blood, presumably due to recreational cannabis use, was associated with a better prognosis following traumatic brain injury (Nguyen et al. 2014). This possibly suggests a neuroprotective effect from cannabinoid use in humans.

Conclusions

Despite the potential promise of cannabis to control progression in MS, it remains to be established whether similar trials of Δ9-THC in progressive MS, using a revised trial design, will be repeated to deliver licensed treatments. This may be difficult for investigator-led academic studies due to the perceived failure of the original trial (Zajicek et al. 2013), making it difficult to raise the significant funds required to undertake similar studies. This failure in humans probably also stifles support for further basic science research in this area. Following the initial failure of academic led-trials in spasticity (Zajicek et al. 2003), interest was maintained because of commercial development of alternative products. However, in contrast to the short symptom control trials of a few weeks duration (Novotna et al. 2011; Corey-Bloom et al. 2012), phase III trials in progressive MS will probably require a further 5–6 years to do a 3 year trial that recruits sufficiently large numbers of people for the trials (estimated to be $n=375$ with 90 % power to detect an 18 % treatment effect. Zajicek et al. 2013). Such a study would suffer from competitive recruitment to other pharmaceutical company and academic-investigator led trials in progressive MS, where a perceived failure has not yet occurred. Importantly, unless pharmaceutical companies are involved, it will be difficult to perform further studies, typically two phase III trials, to a level actually required for regulatory approval and licensing (Giovannoni et al. 2015). However, because of poor patent protection of oral Δ9-THC as a potential medicine for MS, coupled with MS drug-pricing structures, where symptom control drugs are significantly cheaper than current DMT, it means that there will probably be little major pharmaceutical interest in funding and undertaking these studies using a symptom control drug. The proliferation of outlets supplying relatively cheap, legalised, medical marijuana that is occurring, notably in the USA, which is the major commercial market in MS, undermines both the chance of commercial development in this area and the chances of recruiting to placebo-controlled trials, in regions where medical cannabis is readily available. Whether retrospective analysis of large numbers of long-term cannabis users and non-users for symptom control can detect effects on progression remains to be determined. However, development of patent-protected formulations of cannabinoids or non-cannabis pharmaceuticals such as endocannabinoid modulators may be one way to develop commercial interest that could exploit the cannabinoid biology to help deliver a treatment of progression in MS.

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Conflicts of Interest None.

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Materials and Methods

Induction of autoimmune experimental encephalomyelitis protocols consistent with the ARRIVE guidelines have been published previously (Al-Izki et al. 2012; Baker and Amor 2012). Animal studies were approved following local ethical and United Kingdom Government, Home Office review in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Full working protocols of the methods and doses and use of cannabinoids in animals have been reported previously (Pryce et al. 2003; Croxford et al. 2008; Al-Izki et al. 2012). Briefly, 6–8 week adult Biozzi ABH mice (Al-Izki et al. 2012) were injected with spinal cord homogenate in Freunds adjuvant on day 0 and 7 to induce experimental autoimmune encephalomyelitis with onset around day 15–19 post-inoculation (p.i.) and again during remission from paralytic attack on day 28 p.i. to induce a relapse 7–8 days later (Al-Izki et al. 2012). Animals were scored daily: 0=normal; 1=limp tail; 2=impaired righting reflex; 3=hindlimb paresis; 4=hindlimb paralysis; 5=moribund. Scores were assessed using Mann Whitney U statistics (Al-Izki et al. 2012). The motor co-ordination was assessed on an accelerating (0–40 rpm/5 min) rotorod and analysed using Students *t* test following normality and equal variance tests (Al-Izki et al.2012). Synthetic Δ9-THC and CBD were purchased from Δ9-THC Pharm GmbH. Frankfurt, Germany and were diluted in alcohol:cremophor:phosphate buffered saline (1:1:18). Various doses injected in 0.1 ml intraperitoneally (i.p). as described previously (Pryce et al. 2003; Croxford et al. 2008). These were administered shortly before anticipated relapse (Al-Izki et al. 2012, 2014).

Veratrine induced flux of [^{14}C]guanidine in synaptosomes has been reported previously (Pauwels et al. 1986; Garthwaite et al. 2002). Briefly veratrine (100 μg/ml final concentration), and rat cerebral cortex synaptosomes (4 mg/ml, wet weight) were incubated in the absence or presence of compound at 37 °C for 5 min in polypropylene test tubes. Uptake was initiated by the addition of pre-warmed [^{14}C]-guanidine (final concentration 1 μCi/ml) and stopped 2 min later by the addition of 10 ml of ice-cold wash medium as described previously (Pauwels et al. 1986). Incubates were immediately filtered under vacuum through GF/C filters by using a Brandel harvester. The incubation tubes were rinsed with 5 ml of ice-cold wash buffer, which was then used to wash the filter. Filters were transferred to minivials (Beckman Coulter, Fullerton, CA) with the use of a Brandel deposit/dispense system and subsequently counted by liquid scintillation spectroscopy with Picofluor40 liquid scintillator (Garthwaite et al. 2002). Cannabidiol; YC-1 (5-[1-phenylmethyl)-1H-indazol-3-yl]-2-furanmethanol (Cayman, Chem Ann Arbor, Michigan, USA); Nabilone (Cambridge Labs; Newcastle, UK); Lamotrigine (6-(2,3-Dichlorophenyl)-1,2,4-triazine-3,5-diamine. Tocris, Bristol, UK) and Sipatrigine (BW619C89. 2-(4-Methyl-1-piperazinyl)-5-(2,3,5-trichlorophenyl)-4-pyrimidinamine. Tocris Ltd) were diluted with medium from 10 mM stock solutions.

Batrachotoxin-B (BTX-B) Binding. This was performed as described previously (Garthwaite et al. 2002). Binding was initiated by the addition of synaptosomes (final concentration 10 mg/ml, wet weight) to a mixture of test compound and 10 nM [^{3}H]*Batrachotoxin-B i*n the absence or presence of scorpion venom (25 μg/ml final concentration). Samples

were mixed and incubated for 90 min at 25 °C. Ice-cold wash medium (5 ml) was added and then the samples subjected to vacuum filtration through GF/C filters by using a Brandel harvester. Incubation tubes were rinsed with 5 ml of ice-cold wash buffer, which was then used to wash the filter. Radioactivity in the filter was counted as described above.

Randomised, double-blind, placebo-controlled trial of Δ9-THC in people with progressive MS has been reported previously (Zajicek et al. 2013), with an International Standard Randomised Controlled Trial number 62942668. Human studies were approved by the South and West Devon Research Ethics Committee and done in accordance with Good Clinical Practice guidelines. Eligible patients provided written informed consent before participation as International Standard Randomised Controlled Trial (ISRCTN 62942668). Briefly, 18–65 year old humans with primary or secondary progressive MS (Expanded disability status scale (EDSS) Score4.0–6.5), not on current disease modifying therapy (DMT), were enrolled into the study. These were randomised to oral dronabinol (Δ9-THC) starting at 3.5 mg twice a day escalated to a maximum of 28 mg/day depending on tolerability (n=329) or vegetable oil placebo in gelatin capsules (n=164). These were supplied by Insys Therapeutics (Phoenix, AZ, USA). Analysis of the total population (n=493) or subgroup analysis on time to progression in those participants with a baseline EDSS score of 5·5 or lower (n=110) was performed using a log-rank test to compare probability of progression between treatment groups (Zajicek et al. 2013).