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RESEARCH PAPER

Control of Spasticity in a Multiple Sclerosis Model is mediated by CB_1, not CB_2, Cannabinoid Receptors

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Background and Purpose: There is increasing evidence to suggest that cannabis can ameliorate muscle-spasticity in multiple sclerosis, as was objectively shown in experimental autoimmune encephalomyelitis models. The purpose of this study was to investigate further the involvement of CB_1 and CB_2 cannabinoid receptors in the control of experimental spasticity.
Experimental approach: Spasticity was induced in wildtype and CB_1-deficient mice following the development of relapsing, experimental autoimmune encephalomyelitis. Spastic-hindlimb stiffness was measured by the resistance to flexion against a strain gauge following the administration of CB_1 and CB_2 agonists.
Key Results: As previously suggested, some CB_2-selective agonists (RWJ400065) could inhibit spasticity. Importantly, however, the anti-spastic activity of RWJ400065 and the therapeutic effect of non-selective CB_1/CB_2 agonists (*R*(+)WIN55,212–2 and CP55, 940) was lost in spastic, CB_1-deficit mice.
Conclusions and Implications: The CB_1 receptor controls spasticity and cross-reactivity to this receptor appears to account for the therapeutic action of some CB_2 agonists. As cannabinoid-induced psychoactivity is also mediated by the CB_1 receptor, it will be difficult to truly dissociate the therapeutic effects from the well-known, adverse effects of cannabinoids when using cannabis as a medicine. The lack of knowledge on the true diversity of the cannabinoid system coupled with the lack of total specificity of current cannabinoid reagents makes interpretation of *in vivo* results difficult, if using a purely pharmacological approach. Gene knockout technology provides an important tool in target validation and indicates that the CB_1 receptor is the main cannabinoid target for an anti-spastic effect.
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Keywords: cannabinoids; CB_1 receptor; CB_2 receptor; experimental autoimmune encephalomyelitis; spasticity; multiple sclerosis

Abbreviations: CB_1, cannabinoid type 1 receptor; CB_2, cannabinoid type 2 receptor; EAE, experimental autoimmune encephalomyelitis; FAAH, fatty acid amide hydrolase; MS, multiple sclerosis; TRPV1, transient receptor potential vanilloid type 1

Introduction

There has been recent interest in the therapeutic potential of cannabis for control for a number of symptoms, notably spasticity that often develops as a consequence of multiple sclerosis (MS. Consroe *et al.*, 1997; Pertwee, 2002). Using cannabinoid agonists and antagonists, we were the first to provide objective, experimental evidence for the tonic control of spasticity by the cannabinoid system in the experimental autoimmune encephalomyelitis (EAE) model of MS (Baker *et al.*, 2000, 2001). This supported patient claims for the use of medicinal cannabis (Consroe *et al.*, 1997) and has been validated by the modest improvements of symptoms in more recent clinical trials of cannabinoids in MS (Zajicek *et al.*, 2003, 2005; Vaney *et al.*, 2004; Brady *et al.*, 2004; Wade *et al.*, 2004; Freeman *et al.*, 2006). Although the exact cause of spasticity is not definitively known, it is clear that this results from alterations in the balance, possibly secondary to selective neuronal loss, between excitatory and inhibitory neural circuits (Brown, 1994; Dutta *et al.*, 2006). This results in loss of control of neurotransmission between the muscles and the central nervous system resulting in uncontrolled spastic movements, which in some instances can be treated using GABA receptor agonists (Brown, 1994; Ivanhoe and Reistetter, 2004). After the initial observations in EAE (Baker *et al.*, 2000), the cannabinoid type 1 (CB_1) receptor and endocannabinoid system has been shown to regulate synaptic neurotransmission (Howlett *et al.*, 2002; Wilson and Nicoll, 2002) and this action would be consistent with the cannabinoid control of spasticity. In contrast to CB_1, there is limited evidence to indicate that normal nerve tissues express cannabinoid type 2 (CB_2) receptors (Van Sickle *et al.*, 2005) and they appear to be restricted to

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leucocytes (Munro *et al*., 1993; Galiegue *et al*., 1995; Howlett *et al*., 2002), although they are expressed by glial cells and may be upregulated in inflamed brain tissue (Maresz *et al*., 2005; Wotherspoon *et al*., 2005) and therefore may not be anticipated to control problems of neurotransmission. Surprisingly, however, a CB_2 agonist ameliorated and an antagonist transiently worsened spasticity in EAE (Baker *et al*., 2000), suggesting that CB_2 agonists could provide therapies that avoid the psychoactive effects associated with CB_1 agonism (Baker *et al*., 2000; Howlett *et al*., 2002; Varvel *et al*., 2005). In animals, cannabimimetic potential is determined by activity in 'tetrad' (hypomotility, hypothermia, ring catelepsy analgesia) tests, which show no response owing to CB_2 agonism (Howlett *et al*., 2002). However, currently there are no absolutely specific cannabinoid reagents (agonists or antagonists) available, which solely act on either of the CB_1 or CB_2 receptors, and although they may be selective to one or other of the cannabinoid receptors *in vitro*, at the doses used *in vivo*, there is the potential for cannabinoids to crossreact with the other CB receptor (Pertwee, 1999; Howlett *et al*., 2002). Furthermore, there is increasing evidence for additional receptors that mediate cannabimemetic effects (Hajos *et al*., 2001; Howlett *et al*., 2002; Begg *et al*., 2005; Baker *et al*., 2006), which further complicates the interpretation of pharmacological data. Therefore, receptor–deletion using transgenic technology (Zimmer *et al*., 1999, Brooks *et al*., 2002) provides a level of certainty of the role of the CB receptor subtype that is not provided by the CB receptor antagonism alone. This was used to re-evaluate the CB_2-mediated control of spasticity during EAE.

Methods

Animals

Biozzi ABH and ABH mice lacking the CB_1 receptor (*Cnr1)* gene were generated as described previously (Brooks *et al*., 2002, Pryce *et al*., 2003). The congenic ABH.*Cnr1*$^{-/-}$used for breeding were produced by intercrossing after seven generations of backcrossing. Genomic screening for the absence of wild-type *Cnr1*, using polymerase chain reaction (Brooks *et al*., 2002), was performed on parental animals, and the functional deletion of CB_1 protein was confirmed in animals used in these studies by the resistance to sedative doses ($20\,mg\,kg^{-1}$ intraperitoneal (i.p.)) of *R*(+)WIN-55,212–2. These were from in-house bred stock that was maintained in a 12 h-light/dark cycle with controlled humidity and temperature. Animals were fed RM-1E diet and water *ad libitum*. All animal studies conformed to the United Kingdom Animals (Scientific Procedures) Act 1986.

Chemicals

The full CB_1/CB_2 agonists *R*(+)WIN-55,212–2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(-4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthale-nylmethanone mesylate) (K_i $CB_1 = 9.9$ nM, K_i $CB_2 = 16.2$ nM (Rinaldi-Carmona *et al*., 1994)) and CP55,940 ((1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol) (K_i $CB_1 = 1.4$ nM, K_i $CB_2 = 1.4$ nM (Rinaldi-Carmona *et al*., 1994)) were purchased from RBI/Sigma (Poole, UK) and Tocris Ltd (Bristol, UK). The CB_2-selective agonist JWH056 (1-deoxy-δ-8-tetrahydrocannabinol; receptor affinity. K_i $CB_1 = 8770$ nM, K_i $CB_2 = 32$ nM) was provided by Dr J Huffman, Clemson University, South Carolina, USA (Huffman *et al*., 1996). The CB_2-selective agonist RWJ400065 (binding affinity. K_i $CB_1 = 600$ nM, K_i $CB_2 = 10$ nM), forskolin-stimulated cyclic adenosine monophosphate (cAMP) agonism half-maximal inhibitory concentration (IC_{50}) $CB_1 = 6600$ nM, IC_{50} $CB_2 = 6.6$ nM (Dr D Argentieri and Dr D Ritchie, unpublished observations) and non-selective CB_1/CB_2 agonist RWJ352303 (K_i $CB_1 = 0.6$ nM K_i $CB_2 = 0.3$ nM), forskolin-stimulated cAMP agonism in SKN cells IC_{50} $CB_1 = 0.64$ nM, IC_{50} $CB_2 = 0.14$ nm (Dr D Argentieri and Dr D Ritchie, unpublished observations) compounds were provided by RW Johnson] (Raritan, NJ, USA). These were suspended in intralipid 30% (Pharmacia, Milton Keynes, UK) before i.p. or intravenous (i.v.) injection in 0.1 ml. Unanaesthetized animals were placed in a $87 \times 112 \times 60$ mm box (Alpha laboratories, Eastleigh, UK) that had a slot cut to allow exit of the tail from the box. The tail was transiently immersed in warm water (about 45°C) to induce vasodilatation, before injection into the tail vein using 30 g needles. The RWJ compounds were initially injected i.v. to avoid effects of first-pass metabolism encountered with some compounds, such as tetrahydrocannabinol (Baker *et al*., 2000), using dose ranges based on pharmacokinetic data (Dr D Argentieri and Dr D Ritchie, unpublished observations). The i.p. route was selected for CP55,950 and *R*(+)WIN-55,212–2 as it was already known that these agents were rapidly active following i.p. delivery (Baker *et al*., 2000).

Induction of spasticity

Young adult mice were injected subcutaneously with 1 mg of freeze-dried mouse spinal cord homogenate in Freund's adjuvant on days 0 and 7 to induce EAE as described previously (Baker *et al*., 1990). Animals developed relapsing-remitting episodes of limb paralysis and spasticity typically developed after 2–3 relapses, about 80–100 days postinduction. This was assessed during remission from active paralytic episodes by the force required to bend the hind limb to full flexion against a strain gauge (Baker *et al*., 2000).

Assessment of body temperature

Temperature was monitored using a thermocouple placed under the hindlimb as previously described (Brooks *et al*., 2002).

Data analysis and statistical procedures

Each group contained a minimum of five different animals and the results represent the mean ± s.e. resistance to flexion force (N) or individual limbs, which were compared using repeated measures analysis of variance or paired *t* tests using Sigmastat software (Baker *et al*., 2001). Changes in tempera-

ture were compared using paired *t* tests using Sigmastat Software (Brooks *et al*., 2002).

Results

In an attempt to validate our previous studies showing an anti-spastic activity of CB_2 agonists (JWH133 receptor affinity. K_i $CB_1 = 680$ nM, $CB_2 = 3$ nM. Baker *et al*., 2000), additional compounds were investigated. Surprisingly, 10 mg kg^{-1} i.v. JWH056, which is less potent at CB_2, but with a lower affinity for CB_1 ($K_i > 8\ \mu$M) than JWH133, failed to inhibit spasticity at 10–60 min after injection i.v. (Figure 1a), whereas RWJ352303, a potent non-selective CB_1 agonist, inhibited spasticity (Figure 1a). However, a dose-dependent anti-spastic activity was detectable following injection i.v. of a potent CB_2 agonist RWJ400065 (Figure 1b). This compound has similar binding affinities to JWH133 and failed to induce observable sedation (unpublished observations) and hypothermia (Figure 2),

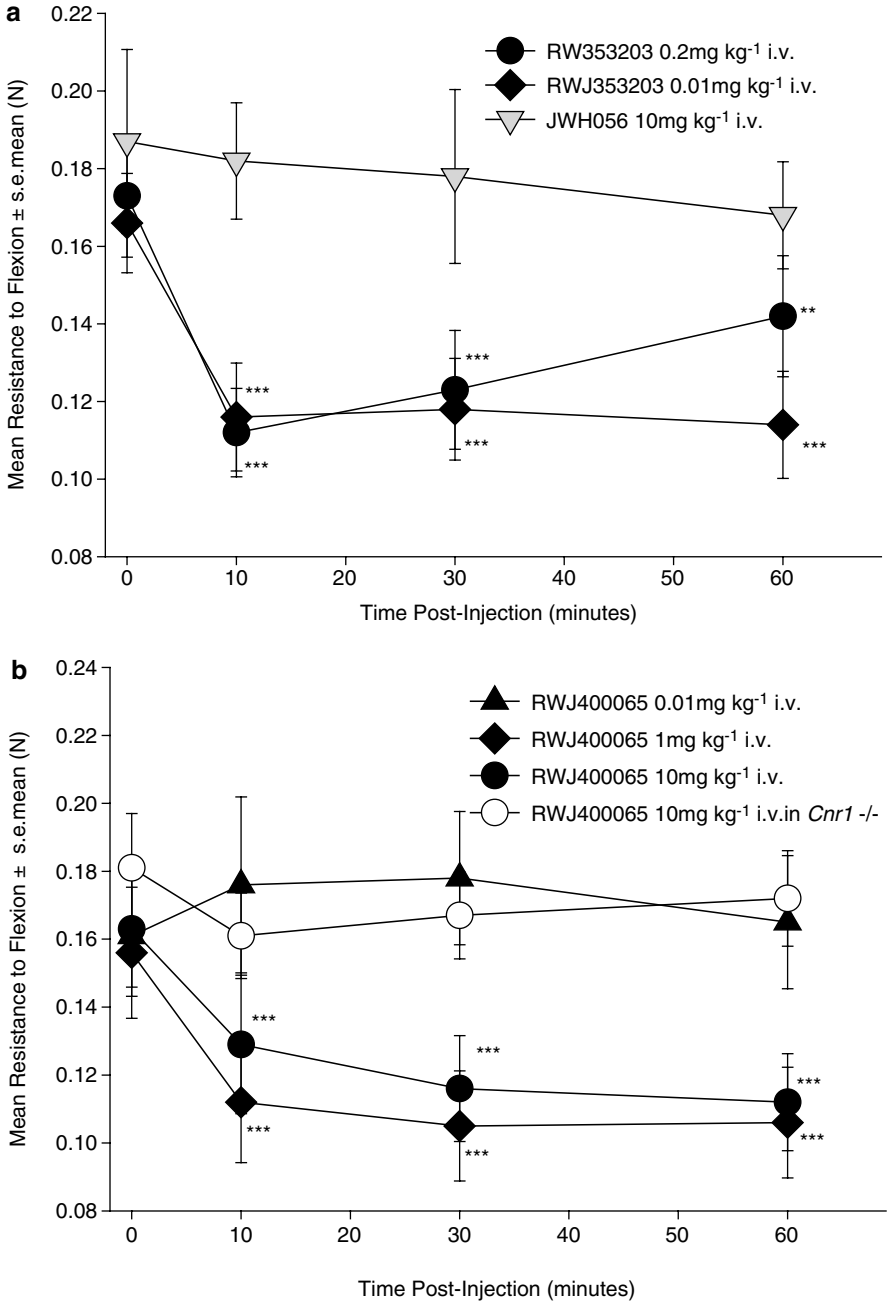

Figure 1 Inhibition of spasticity, with CB_2 agonists is CB_1-mediated. Following the development of spasticity ABH mice were injected i.v. with either: (**a**) the non-selective agonist RWJ35320 or the CB_2-selective agonist JWH056 or (**b**) the CB_2-selective agonist, RWJ400065. These received 0.2 mg kg^{-1} ($n = 17$ limbs), 0.01 mg kg^{-1} ($n = 13$ limbs) RWJ353203 or 10 mg kg^{-1} JWH056 ($n = 7$ limbs) or 0.01 mg kg^{-1} ($n = 12$ limbs), 1 mg kg^{-1} ($n = 16$ limbs) or 10 mg kg^{-1} ($n = 16$ limbs) RWJ400065 in wild-type or CB_1-deficient mice ($n = 12$ limbs) in intralipid. The resistance to flexion was measured against a strain gauge. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to baseline.

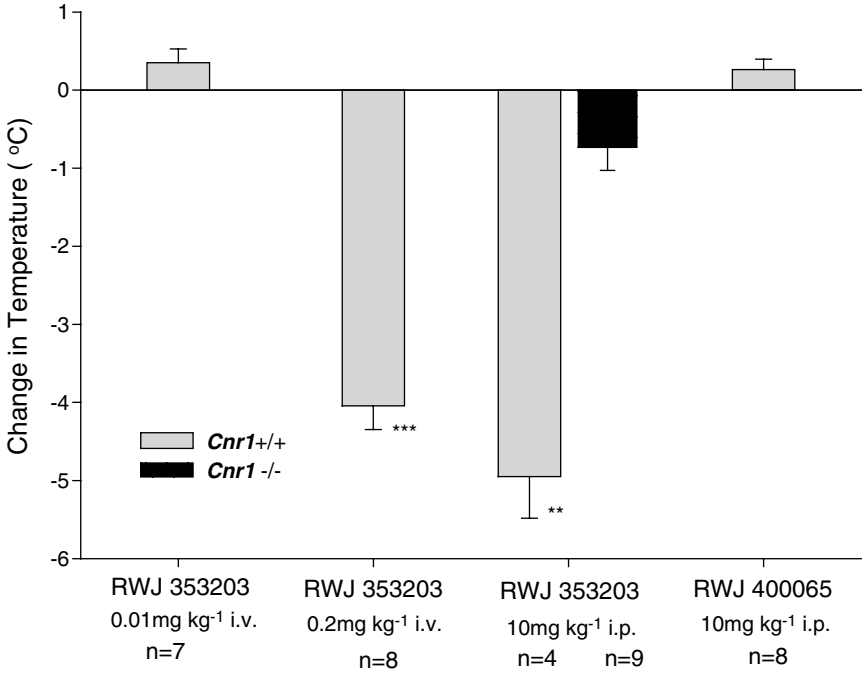

Figure 2 Hypothermia induced by cannabinoids. Wild-type or CB_1-deficient mice were injected either i.v. or i.p. with the non-selective agonist RWJ35320 or CB_2-selective agonist RWJ400065 in intralipid. The change in body temperature (mean ± s.e.mean (vertical lines)) 20 min following injection compared to baseline was assessed. $^{**}P<0.01$, $^{***}P<0.001$ compared to baseline by paired *t* tests.

indicative of CB_1 receptor-mediated effects. In contrast, RWJ352303 had the potential to induce 'tetrad-like' effects (Figure 2), but was still active as an anti-spastic agent (Figure 1a), at doses that did not induce 'tetrad-type' effects, shown here by hypothermic responses (Figure 2). However, when 10 mg kg^{-1} i.v. RWJ400065 was injected into *Cnr1*−/− mice, there was no apparent anti-spastic activity (Figure 1B). To clarify this further, commonly used high-affinity CB_1/CB_2 non-selective agonists were examined. However, there was no evidence of inhibition of spasticity in CB_1-deficient mice with either CP55,940 or *R*(+)WIN-55, 212–2 compared to significant ($P<0.001$) inhibitory activity in wild-type mice (Figure 3). This suggested that CB_1 and not the CB_2 receptors were actually mediating the inhibitory effects of some CB_2 agonists.

Discussion and conclusions

Although this study confirms our previous observation (Baker *et al.*, 2000) that 'tetrad inactive' apparent CB_2 agonists can show anti-spastic activity, this does not appear to be owing to the direct activity of CB_2 receptors. This most likely occurs because CB_2 agonists/antagonists (Baker *et al.*, 2000), or possibly their *in vivo* metabolites, have some affinity for CB_1 receptors that may actually mediate the inhibitory effects. The biology of cannabis and the cannabinoid system now indicates that both tetrahydrocannabinol and CB_1 receptors are the major mediators for both therapy in spasticity and also the adverse side effects (Howlett *et al.*, 2002; Wilkinson *et al.*, 2003; Varvel *et al.*, 2005). It will be virtually impossible to truly dissociate these two effects, using cannabis. Clinical studies indicate that there is a substantial variability of individuals to tolerate cannabis and tetrahydrocannabinol (Zajicek *et al.*, 2003; Brady *et al.*, 2004; Wade *et al.*, 2004). The apparent therapeutic window, before psychoactive effects, appears to be very small and is consistent with the modest effects in symptom control observed so far (Zajicek *et al.*, 2003, 2005; Brady *et al.*, 2004; Wade *et al.*, 2004; Freeman *et al.*, 2006), which nevertheless validate our original observations in animal models (Baker *et al.*, 2000, 2001; Wilkinson *et al.*, 2003). This variability of individuals to tolerate cannabinoids means that it will be difficult to dose–titrate adequately with potent CB_1 agonists and that weak CB_1 agonists, such as at the level found in some CB_2 agonists, may be preferable for clinical use.

Currently there are two recognized cannabinoid receptors, but there is pharmacological evidence (Breivogel *et al.*, 2001; Hajos *et al.*, 2001; Howlett *et al.*, 2002; Baker *et al.*, 2006; Oz, 2006), some of which is disputed (Kawamura *et al.*, 2006; Takahashi and Castillo 2006), for additional receptors or pathways that mediate cannabimimetic effects. Although the use of gene knockout technology is not without its own limitations, it provides an important tool in target validation. The loss of anti-spastic activity of *R*(+) WIN55,212–2 and CP55,940, both full CB_1/CB_2 agonists, in CB_1-deficient mice supports the indication that CB_1 and not CB_2 is mediating the therapeutic anti-spastic effect. Nevertheless, anti-spastic control is feasible in CB_1-deficient animals as shown previously with arvanil (Brooks *et al.*, 2002). Arvanil, a potent transient receptor potential vanilloid type 1 (TRPV1) receptor and weak CB_1 agonist, can also inhibit spasticity in the presence of CB_1/CB_2 antagonists and high doses of the TRPV1 antagonist capsazepine (Brooks *et al.*, 2002). It can also induce cannabimemetic 'tetrad-type' responses, such as hypothermia, and hypomotility, in wild-

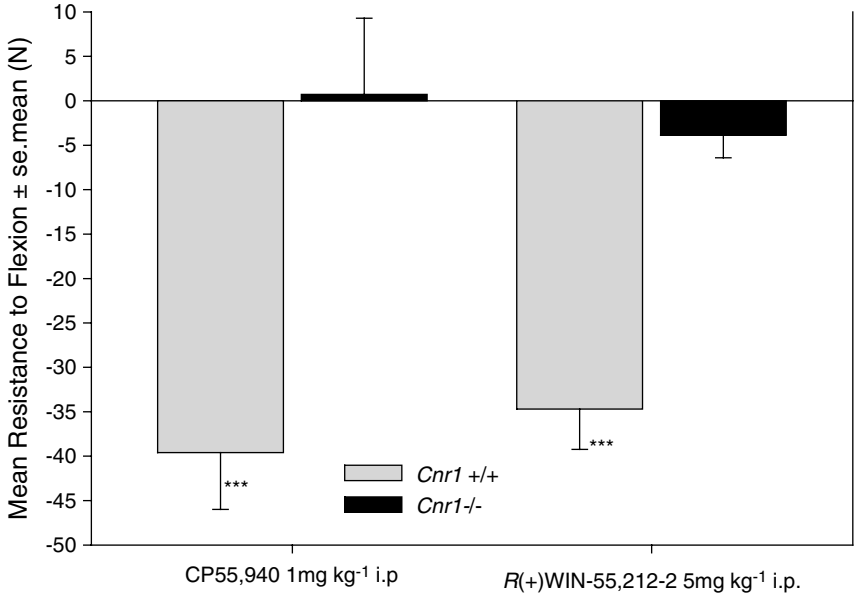

Figure 3 Spasticity is controlled by the CB_1 receptor. Wild-type or CB_1-deficient mice (*Cnr1*−/−) were injected intraperitoneally with the full CB_1/CB_2 agonists CP-55,940 ($n=8$/group) or *R*(+)WIN-55,212–2 ($n=14$/group). To facilitate visualization of differences between groups, results are expressed as the mean ± s.e.mean (vertical lines) percentage change in the resistance to hindlimb flexion compared to baseline, 10 min after the injection of compound. ***$P<0.001$ compared to baseline by paired *t* tests.

type and *Cnr1*−/− mice (Brooks *et al.*, 2002). However, capsazepine is a weak TRPV1 antagonist in mice (Correll *et al.*, 2004) and the hypothermia and marked hypomotility induced by 0.5 mg kg^{-1} i.v. arvanil is lost in *Trpv1*−/− mice (unpublished observations), further indicating the value of receptor knockout animals in target validation. However, cannabinoid receptors can exist as homodimers, and novel heterodimer formations between CB_1 receptors and other G-protein-coupled receptors are assumed or are generated (Kearn *et al.*, 2005; Wager-Miller *et al.*, 2002; Rios *et al.*, 2006). Therefore, CB_1/CB_2 receptor heterodimers or heterodimers between CB_1 and any other molecule to which the CB_2 agonists may bind would not exist in *Cnr1*−/− mice and this may have accounted for the loss of activity of RWJ400065 in CB_1-deficient mice. Therefore, similar studies in *Cnr2*−/− mice are required to exclude definitively a role for CB_2 in the control of spasticity.

However, the results from this study suggest that our previous data showing control of spasticity with endocannabinoid degradation inhibitors may need to be more cautiously interpreted (Baker *et al.*, 2001). Many of these inhibitors, often based on the structural modifications of anandamide, have low affinity for CB_1 receptors and are inactive in 'tetrad' tests, just as CB_2-selective agonists appear to be. Compounds believed to inhibit the anandamide transporter, including AM404, VDM11 (Baker *et al.*, 2001), OMDM-1, OMDM-2 (de Lago *et al.*, 2004), UCM707 (de Lago *et al.*, 2006) 0–2093 and 0–3246 (Ligresti *et al.*, 2006) all exhibit anti-spastic activity. However, many of these agents have activity on additional molecules such as TRPV1 vanilloid receptors and the cannabinoid degrading enzyme: fatty acid amide hydrolase (FAAH), which could account for their biological activity (Ralevic *et al.*, 2001; Fowler *et al.*, 2004). Although a site for membranous diffusion of endocannabinoids has been suggested (Moore *et al.*, 2005), the existence of a specific transporter for anandamide, independent of FAAH, has been questioned (Glaser *et al.*, 2003; Ortega-Gutierrez *et al.*, 2004; Kaczocha *et al.*, 2006). Therefore, until the putative endocannabinoid transporter(s) are identified and cloned, it must be considered possible that the therapeutic, anti-spastic effect of cannabinoid re-uptake inhibitors may be explained by alternative mechanisms. However, using FAAH gene knockout mice (ABH.*Faah*−/−), we have been able to verify the activity of some FAAH inhibitors (Boger *et al.*, 2000) as anti-spastic agents (unpublished observations). The lack of true understanding of diversity of the cannabinoid system and importantly the lack of absolute specificity of current cannabinoid agonists and antagonists (Pertwee, 1999) means that it may be difficult to correctly interpret results, particularly *in vivo,* if using a purely pharmacological approach.

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Conflict of interest

The authors state no conflict of interest.

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