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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature. For additional sound examples, see http://www.nsi.edu/users/patel/tone_sequences.

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Cannabinoids control spasticity and tremor in a multiple sclerosis model

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Chronic relapsing experimental allergic encephalomyelitis (CREAE) is an autoimmune model of multiple sclerosis¹. Although both these diseases are typified by relapsing-remitting paralytic episodes, after CREAE induction by sensitization to myelin antigens¹ Biozzi ABH mice also develop spasticity and tremor. These symptoms also occur during multiple sclerosis and are difficult to control. This has prompted some patients to find

alternative medicines, and to perceive benefit from cannabis use². Although this benefit has been backed up by small clinical studies, mainly with non-quantifiable outcomes^{3–7}, the value of cannabis use in multiple sclerosis remains anecdotal. Here we show that cannabinoid (CB) receptor agonism using R(+)-WIN 55,212, Δ⁹-tetrahydrocannabinol, methanandamide and JWH-133 (ref. 8) quantitatively ameliorated both tremor and spasticity in diseased mice. The exacerbation of these signs after antagonism of the CB₁ and CB₂ receptors, notably the CB₁ receptor, using SR141716A and SR144528 (ref. 8) indicate that the endogenous cannabinoid system may be tonically active in the control of tremor and spasticity. This provides a rationale for patients' indications of the therapeutic potential of cannabis in the control of the symptoms of multiple sclerosis², and provides a means of evaluating more selective cannabinoids in the future.

High doses of Δ⁹-tetrahydrocannabinol THC; (the major psychoactive component of cannabis) can inhibit the development of CREAE in rodents^{9,10}, but this has been attributed to immunosuppression preventing the conditions that lead to the development of paralysis, rather than to a direct effect on the paralysis itself^{9,10}. However, the action of cannabinoids on experimental spasticity and tremor remains uncertain because there have so far been no behavioural data on the effects of cannabinoids in animal models relevant to these symptoms of multiple sclerosis.

It is well established that repeated neurological insults occur during CREAE; these are associated with increasing primary demyelination and axonal loss in the central nervous system (CNS)¹. However, it was also evident that CREAE animals can develop additional clinical signs, including unilateral or bilateral fore- and hindlimb tremor (Fig. 1) and hindlimb spasticity (Fig. 2). These accumulate with disease duration and activity. Tremor was associated with voluntary limb movements, but in more severe cases it was persistent at a frequency of ~40 Hz (Fig. 1e). Although considerably faster than encountered in humans (~6 Hz), this frequency is consistent with tremor electromyography in mutant spastic (*Glr^bSp^a*) mice¹¹. These animals develop episodes of rapid tremor and rigidity of the limb and trunk muscles¹². However, unlike the *Glr^bSp^a* mouse, spasticity in CREAE mice need not be triggered by sudden disturbance¹². The effects of cannabis are mediated through the CB₁, CB₂ and putative CB₂-like receptors^{13,14}. CB₁ is predominant in the CNS and is the main target for psychoactivity, but it is also expressed at lower levels in many

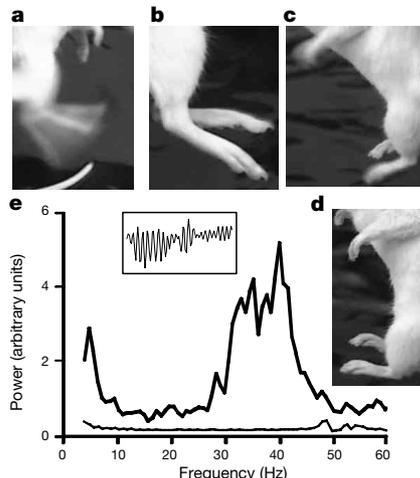


Figure 1 Cannabinoid receptor agonism inhibits tremor in autoimmune encephalomyelitis¹. Mice with hindlimb (a, b) or fore- and hindlimb (c, d) tremor both before (a, c) and after (b, d) treatment with 5 mg kg⁻¹ i.p. with R(+)-WIN 55,212. e, Power spectra of hindlimb tremors recorded with the foot suspended above a strain gauge before (thick line) and after (thin line) 5 mg kg⁻¹ i.p. R(+)-WIN 55,212 injection. Inset, snapshot of raw record over 0.5 s.

peripheral tissues. The CB₂ receptor is expressed at high levels on leucocytes, but there is also evidence for limited CB₂ receptor expression in mouse brain^{13,4}. The administration of a full CB₁ and CB₂ agonist, R(+)-WIN 55,212 (ref. 8), to post-relapse remission mice resulted in a rapid (within 1–10 min) amelioration of the frequency and amplitude of tremor in both the fore- and hindlimbs of CREAE mice (Fig. 1). This was visually evident at 5 mg kg⁻¹ (Fig. 1a–d; *n* = 10/10) and 1 mg kg⁻¹ intraperitoneal (i.p.) (*n* = 6/6). In addition, Δ⁹-THC (10 mg kg⁻¹ intravenous (i.v.)) also ameliorated this response (*n* = 5/5). Tremor returned within hours after treatment. As Δ⁹-THC was observed to be relatively ineffective when injected intraperitoneally (i.p.), as seen in other studies¹⁰, all subsequent compounds were injected intravenously. Furthermore, as Δ⁹-THC is a partial CB₁ agonist but provides more limited CB₂ agonist activity, these results suggest that the effect on tremor is mainly mediated by the brain CB₁ receptor⁸.

Pretreatment (10 min) of animals with 5 mg kg⁻¹ i.v. of both selective CB₁ (SR141716A) (ref. 15) and CB₂ (SR144528) (ref. 16) receptor antagonists eliminated the capacity of 5 mg kg⁻¹ i.p. R(+)-WIN 55,212 to inhibit tremor (*n* = 5/5). However animals with residual paresis and mild spasticity became significantly more spastic after such CB receptor antagonism (Fig. 3). This was associated with uncontrolled leg crossing (Fig. 3c and d) and severe tail spasms. These showed gross curling which is typical of post-remission animals, in which the tail generally hangs limply (Fig. 3e). Animals also show hindlimb extension (Fig. 3c), including a significant (*P* < 0.0001) increase in resistance to flexion (Fig. 3a, f). This was not observed in vehicle-treated controls (Fig. 3a). These signs were also not evident in similarly injected normal mice (*n* = 0/5) or normal-appearing pre-acute EAE animals (hindlimb resistance to flexion 0.159 ± 0.013N compared with 0.206 ± 0.022N in treated mice (*n* = 12 limbs, *P* > 0.05) and in animals with paresis/paralysis without evidence of spasticity (*n* = 0/5 treated with SR141716A and SR144528, *n* = 0/4 treated with SR141716A or SR144528 alone). When mildly spastic animals without tremor were injected with 5 mg kg⁻¹ i.v. CB₁ antagonist, not only did significant hindlimb (*P* < 0.001; Fig. 3a) and tail spasticity (*n* = 18/18, *P* < 0.001) develop compared with vehicle treated

controls (*n* = 0/6), but forelimb tremor also became evident in 3 out of 10 mice. This suggests a role for CB₁ in the control of tremor. After injection of 5 mg kg⁻¹ i.v. CB₂ antagonist, some animals (*n* = 10/14) seemed to show a mild increase in tail spasticity (*P* < 0.02) and showed a small but significant (*P* < 0.05) increase in resistance to hindlimb flexion (Fig. 3a). However, when the CB₂ antagonist was injected into animals previously made more spastic (*P* < 0.01) by CB₁ antagonism, spasticity increased significantly (*P* < 0.001) compared with animals treated with SR141716A alone, whereas this was resolved in animals treated with vehicle. This suggests that both CB receptors may control spasticity (Fig. 3f). However, it is possible that the effects of SR144528 could be mediated by CB₂-like (rather than CB₂) receptors as previously proposed¹⁷, or that at the dose used, SR144528 may have produced additional CB₁ antagonism because it has some limited capacity to bind to CB₁ (ref. 8). These observations may indicate the continual release of endogenous cannabinoid receptor agonists such as anandamide and 2-arachidonylglycerol which are present within the brain and exhibit neurotransmitter function¹⁸. Alternatively, or in addition, they may reflect the presence of precoupled, constitutively active cannabinoid receptors, as there is evidence that SR141716A and SR144528 are both inverse agonists that are capable of producing inverse cannabinimetic effects by reducing the proportion of cannabinoid receptors that exist in a precoupled state^{8,15,16}. In comparison to some studies in which the antagonists affected the exogenous agonists¹⁷, the actions of the antagonists seen here were relatively short-lived (Fig. 3f). This may reflect the fact that the animals were attempting to compensate for the antagonist effect, and would be consistent with tonic control of the endogenous cannabinoid system. These data provide compelling evidence that CB receptors are involved in the control of spasticity in an environment of existing neurological damage, and that exogenous agonism may be beneficial.

Indeed, in mice with significant spasticity, 5 mg kg⁻¹ i.p. R(+)-WIN 55,212 reduced severity both visually (*n* = 7/7; Fig. 3g, h and i) and after assessment of resistance to hindlimb flexion (*P* < 0.001) (Fig. 3a and i). This was also evident with 2.5 mg kg⁻¹ i.p. R(+)-WIN 55,212 (Resistance of flexion of both limbs being reduced (*P* < 0.05) from 0.384 ± 0.096N to 0.276 ± 0.063N, *n* = 7, *P* < 0.05). Similar treatment with 5 mg kg⁻¹ i.p. of the inactive enantiomer S(-)-WIN 55,212 failed to significantly affect the spastic response (Fig. 3a). In contrast, 10 mg kg⁻¹ i.v. Δ⁹-THC and 5 mg kg⁻¹ i.v. methanandamide (CB₁-selective; *K*_i for CB₁ ≈ 20 nM and *K*_i for CB₂ ≈ 815 nM)⁸ induced a significant (*P* < 0.001) amelioration in spasticity (Fig. 3g). Coupled with the observations using SR141716A, this may suggest further that CB₁ is a main target for control of spasticity. Currently there are no compounds which are totally CB₁ or CB₂ receptor specific, but the lack of effect after 10 mg kg⁻¹ i.v. cannabidiol (main non-psychoactive component of cannabis. *K*_i for CB₁ = 4350 nM)⁸ suggested a subthreshold dose for CB₁ stimulation for treatment of spasticity. Using the CB₂-selective agonist JWH-133 (1.5 mg kg⁻¹ i.v. *K*_i for CB₁ ≈ 680 nM and *K*_i for CB₂ ≈ 3 nM)^{8,19} spasticity was reduced both 10 min (*P* < 0.05) and 30 min (*P* < 0.001) after injection at a time when 0.05 mg kg⁻¹ i.v. (dose selected to exhibit similar CB₁ activity to JWH-133) methanandamide was not active (Fig. 4). It is possible that sedative effects may have contributed (though CB₁ receptors) to cannabinoid-mediated effects in these assays, but there was no hypothermia, indicative of 'sedation' after JWH-133 administration (37.1 ± 0. °C (baseline), 37.2 ± 0.4 °C (10 min) 37.1 ± 0.2 °C (30 min)). That non-CB₁ receptors may also control spasticity is further indicated by the transient inhibition of spasticity with the endocannabinoid palmitoylethanolamide (Fig. 4). This compound has no significant affinity for CB₁ but may have activity for CB₂-like receptors⁸. The involvement of non-CB₁ receptors may be definitively resolved through the use of CB receptor subtype-specific compounds or CB-receptor-deficient mice.

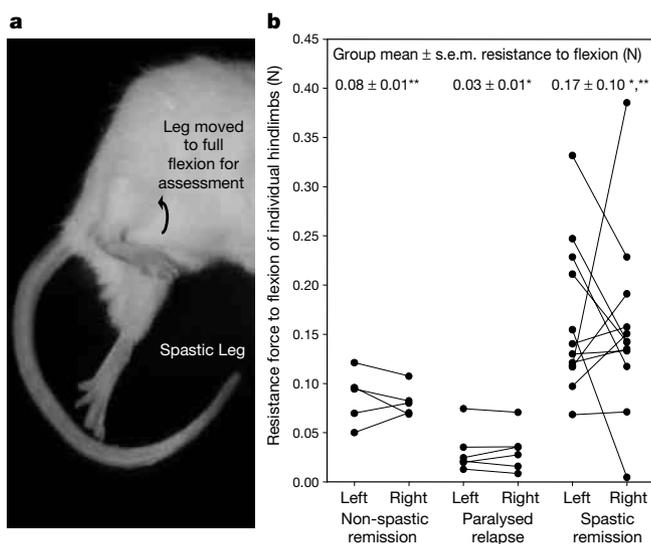


Figure 2 Spasticity develops in autoimmune encephalomyelitis. **a**, Spastic hindlimb showing full extension, including the digits. These were pressed against a strain gauge to measure the force required to bend the leg to full flexion. **b**, Increased resistance to flexion in post-relapse remission animals with spasticity (*n* = 12 mice) compared with age-matched mice without evidence of spasticity (*n* = 5 mice; asterisk, = *P* < 0.001), or during active paralytic relapse episodes (*n* = 6; two asterisks, = *P* < 0.001).

Spasticity in patients with multiple sclerosis can be very difficult to control despite the use of oral baclofen, dantrolene, diazepam and tizanidine, continuous intrathecal baclofen infusion, and selective injection of botulinum toxin²⁰. There is a need for more effective oral or systemic antispasticity agents. The hydrophobic nature of cannabinoids allows their rapid access to the CNS. Although the effects of chronic administration and dose dependency of CB receptor agonists on experimental spasticity remain to be

investigated further, the data presented here provide evidence for the rational assessment of cannabinoid derivatives in the control of spasticity and tremor in multiple sclerosis, in placebo-controlled trials. The observation that CB₁ appears to be the main therapeutic target suggests that it may be difficult to dissociate the full benefit from undesirable psychoactive elements using Δ⁹-THC or cannabis. It is also consistent with the unpleasant side effects experienced by some patients at the doses required for potential therapy by existing

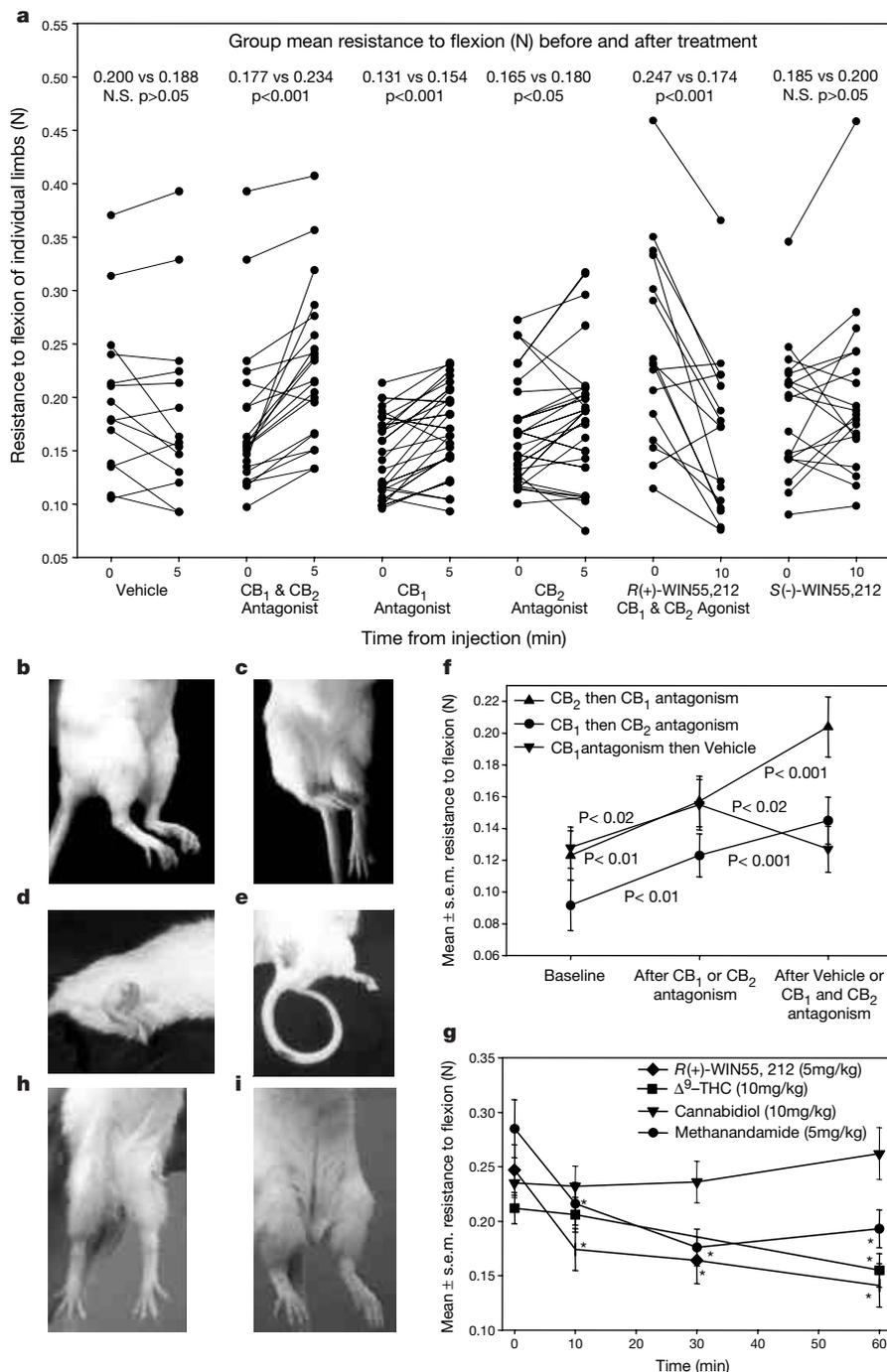


Figure 3 Control of spasticity by the cannabinoid system. **a**, Forces required to flex individual spastic hindlimbs against a strain gauge before and after injection with vehicle (0.1 ml i.v., $n = 14$), SR141716A (5 mg kg⁻¹ i.v., $n = 32$), SR144528 (5 mg kg⁻¹ i.v., $n = 32$), SR141716A and SR144528 ($n = 21$ limbs), R(+)-WIN 55,212 (5 mg kg⁻¹ i.p., $n = 16$) or S(-)-WIN 55,212 (5 mg kg⁻¹ i.p., $n = 19$). **b–e**, Cannabinoid receptor antagonism increased spasticity. Before (**b**) and after (**c, e**) SR141716A and SR144528 or after SR141716A (**d**) administration. **c, d**, Extension and crossing of limbs; **e**, spastic tail.

f, Resistance to flexion forces 5 min after SR141716A or SR144528 administration. 10 min later, mice were re-injected (5 mg kg⁻¹ i.v.) with either SR144528 ($n = 10$), vehicle ($n = 15$) or SR141716A ($n = 18$ limbs) and the resistance to flexion assessed after 5 min. **g**, Cannabinoid receptor agonism in spastic mice after either R(+)-WIN 55,212 ($n = 16$ limbs), Δ⁹-THC ($n = 18$), methanandamide ($n = 23$) or cannabidiol ($n = 22$). Asterisk, $P < 0.001$ compared with baseline. **h**, Spasticity was ameliorated (**i**) by treatment with R(+)-WIN 55,212.

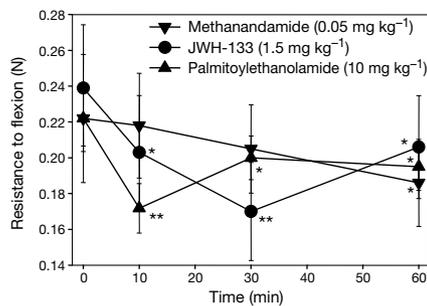


Figure 4 Treatment of spasticity in autoimmune encephalomyelitis¹ with non-CB₁ receptor agonists. Forces (mean ± s.e.m.) required to flex individual spastic hindlimbs against a strain gauge after i.v. injection with either low-dose methanandamide (*n* = 9 limbs), JWH-133 (*n* = 9) or palmitoylethanolamide (*n* = 14). Asterisk, *P* < 0.05; two asterisks, *P* < 0.001 compared with baseline.

cannabinoids³. The use of selective CB₂ agonists may provide some symptomatic benefit without significant psychoactive effects. Furthermore, it may be possible to upregulate endogenous produced cannabinoids¹⁸ to mediate therapeutic benefit. This CREAE model provides a means of evaluating and controlling the pathophysiology of spasticity in a chronic inflammatory environment relevant to the control of multiple sclerosis. □

Methods

Induction of CREAE

Biozzi ABH mice, bred at the Institute of Ophthalmology, were injected with 1 mg of mouse spinal cord homogenate emulsified in Freund's complete adjuvant on days 0 and 7 (ref. 1). Animals injected for CREAE, before the onset of acute phase CREAE¹ (usually occurring 15–20 days post inoculation (p.i.)) were used as normal CREAE controls. Paralysed CREAE animals were selected during the acute phase or first relapse (typically occurring 34–45 days p.i.), and remission animals used for the assessment of tremor and spasticity were used after the second or third relapse 40–80 days p.i.).

Chemicals

R(+)-WIN 55,212, S(-)-WIN 55,212, Δ⁹-THC, methanandamide and cannabidiol were purchased from RBI/Sigma (Poole, UK). Palmitoylethanolamide was purchased from Tocris Cookson Ltd (Bristol, UK). SR141716A (ref. 15) and SR144528 (ref. 16) were supplied by M. Mossé and F. Barth (Sanofi Research, Montpellier, France). JWH-133 (3-(1'-1'-dimethylbutyl)-1-deoxy-Δ⁸-THC) was synthesised as described¹⁹. All compounds were dissolved at 0.5 mg ml⁻¹ in ethanol containing 1 mg ml⁻¹ Tween 80 (Sigma). The ethanol was removed by vacuum drying, and samples were reconstituted with phosphate buffered saline to a concentration of 2 mg ml⁻¹. Similar preparations without active drugs were used as vehicle controls. Suspensions (0.1 ml) were injected either i.v. or i.p. after CREAE induction.

Assessment of Clinical Signs

Spasticity and tremor were initially assessed by blinded analysis of video recordings. Digital images were sampled from video at 0.04 s. Signs of tail spasticity (flicking and curling) were assessed visually as being either present or absent. Spasticity was confirmed by assessing limb spasticity against a small purpose-built strain gauge. Limbs of animals without clinical evidence of spasticity (propensity to full extend the limb after tension on the leg) or the propensity to cross were not examined in drug studies. The analogue signal was amplified and digitally converted using an Amplicon card (Brighton, UK). This was captured using dacquire V10 software (D. Buckwell, MRC HMBU, Institute of Neurology) and analysed using Spike 2 software (Cambridge Electronic Design, UK). The hindlimbs were fully extended twice then moved to full flexion against the strain gauge. Each hindlimb was individually assessed by a blinded operator. The mean of 4–8 individual readings per limb was taken. Tremor frequency and severity were also recorded by holding the limb ~5 mm above the strain gauge. Tremor lead to the foot knocking the strain gauge. The strain gauge output was notch filtered at 50 Hz. The device had a resonance frequency of 95 Hz. The frequency of limb tremor was also confirmed using a lightweight unidirectional accelerometer (EGA XT-50, Entrain, UK) mounted over the foot.

Statistical Analysis

Results are expressed as means of individual feet or animals ± s.e.m. per group. The data were assessed using either a *t*-test, paired *t*-test for flexion data or nonparametric Mann-Whitney *U*-test using SigmaStat 2.0 software (Jandel Corp, San Rafael, California, USA).

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Light acts directly on organs and cells in culture to set the vertebrate circadian clock

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The expression of clock genes in vertebrates is widespread and not restricted to classical clock structures^{1,2}. The expression of the Clock gene in zebrafish shows a strong circadian oscillation in many tissues in vivo and in culture, showing that endogenous oscillators exist in peripheral organs³. A defining feature of circadian clocks is that they can be set or entrained to local time, usually by the environmental light–dark cycle^{4,5}. An important question is whether peripheral oscillators are entrained to local time by signals from central pacemakers such as the eyes or are themselves directly light-responsive. Here we show that the