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RESEARCH PAPER

The phytocannabinoid Δ^9-tetrahydrocannabivarin modulates inhibitory neurotransmission in the cerebellum

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Background and purpose: The phytocannabinoid Δ^9-tetrahydrocannabivarin (Δ^9-THCV) has been reported to exhibit a diverse pharmacology; here, we investigate functional effects of Δ^9-THCV, extracted from *Cannabis sativa*, using electrophysiological techniques to define its mechanism of action in the CNS.
Experimental approach: Effects of Δ^9-THCV and synthetic cannabinoid agents on inhibitory neurotransmission at interneurone-Purkinje cell (IN-PC) synapses were correlated with effects on spontaneous PC output using single-cell and multi-electrode array (MEA) electrophysiological recordings respectively, in mouse cerebellar brain slices *in vitro*.
Key results: The cannabinoid receptor agonist WIN 55,212-2 (WIN55) decreased miniature inhibitory postsynaptic current (mIPSC) frequency at IN-PC synapses. WIN55-induced inhibition was reversed by Δ^9-THCV, and also by the CB_1 receptor antagonist AM251; Δ^9-THCV or AM251 acted to increase mIPSC frequency beyond basal values. When applied alone, Δ^9-THCV, AM251 or rimonabant increased mIPSC frequency. Pre-incubation with Δ^9-THCV blocked WIN55-induced inhibition. In MEA recordings, WIN55 increased PC spike firing rate; Δ^9-THCV and AM251 acted in the opposite direction to decrease spike firing. The effects of Δ^9-THCV and WIN55 were attenuated by the $GABA_A$ receptor antagonist bicuculline methiodide.
Conclusions and implications: We show for the first time that Δ^9-THCV acts as a functional CB_1 receptor antagonist in the CNS to modulate inhibitory neurotransmission at IN-PC synapses and spontaneous PC output. Δ^9-THCV- and AM251-induced increases in mIPSC frequency beyond basal levels were consistent with basal CB_1 receptor activity. WIN55-induced increases in PC spike firing rate were consistent with synaptic disinhibition; whilst Δ^9-THCV- and AM251-induced decreases in spike firing suggest a mechanism of PC inhibition.
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Keywords: cannabinoids; CB_1 receptor; $GABA_A$ receptor; patch-clamp; multi-electrode array

Abbreviations: aCSF, artificial cerebrospinal fluid; AM251, *N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-multipyrazole-3-carboxamide; BMI, bicuculline methiodide; CB, cannabinoid; DCN, deep cerebellar nuclei; IEI, inter-event intervals; IN-PC, interneurone-Purkinje cell; MEA, multi-electrode array; mIPSC, miniature inhibitory postsynaptic current; PC, Purkinje cell; Δ^9-THCV, Δ^9-tetrahydrocannabivarin; TTX, tetrodotoxin; WIN 55, 212-2,(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

Introduction

Cannabinoids represent a diverse group of compounds, including plant-derived phytocannabinoids, aminoalkylindoles (such as the prototypic WIN55) and endocannabinoids, (Di Marzo *et al.*, 1999; Howlett *et al.*, 2002, 2004). Cannabinoids act on two major subtypes of G protein-coupled receptors, CB_1 and CB_2 (Alexander *et al*, 2007), both of which predominantly couple to inhibitory $G_{i/o}$ subunits. CB_1 is strongly expressed in the CNS, particularly in the cerebellum (Tsou *et al.*, 1998; Egertova and Elphick, 2000; Diana *et al.*, 2002). In contrast, CB_2 receptors are principally localized to peripheral, non-neuronal tissue (Howlett *et al.*, 2002; see also Van Sickle *et al.*, 2005; Ashton *et al.*, 2006). In addition to

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CB_1/CB_2-mediated effects, cannabinoids may act at further putative CB receptor subtypes, other non-CB receptors and may have other non-receptor-mediated actions (Howlett *et al.*, 2002; Pertwee, 2007). Plant-derived phytocannabinoids have recently attracted considerable interest as novel potential therapeutic agents (Mechoulam, 2005, Pertwee, 2005a); however, mechanisms of action remain poorly defined. In particular, Δ^9-tetrahydrocannabivarin (Δ^9-THCV), the propyl homologue of Δ^9-tetrahydrocannabinol, has recently been reported to have a diverse pharmacology and to display tissue-dependent effects (Pertwee, 2008). Δ^9-THCV was originally shown to share Δ^9-tetrahydrocannabinol-like properties, producing catalepsy in a mouse ring test, although at a lower potency (Gill *et al.*, 1970). More recently, [^{35}S]GTPγS-binding assays have shown that Δ^9-THCV exhibits an antagonist action at CB_1 and CB_2 receptors in whole mouse brain membranes and recombinant cells, respectively (Thomas *et al.*, 2005). Our preliminary data also show that Δ^9-THCV has regional effects in [^{35}S]GTPγS assays, acting as a CB_1 receptor antagonist in cerebellar and piriform cortical membranes (Dennis *et al*, 2007). Plant-derived Δ^9-THCV also acted as a functional antagonist *in vitro* in the isolated mouse vas deferens (Thomas *et al.*, 2005). Synthetic Δ^9-THCV shared these properties and also acted as an antagonist in anti-nociceptive and hypothermia tests *in vivo* (Pertwee *et al.*, 2007). In contrast, higher concentrations of Δ^9-THCV were shown to inhibit electrically evoked responses in vas deferens reportedly by a non-CB_1 receptor-mediated mechanism (Thomas *et al.*, 2005) and to be anti-nociceptive via an agonist effect at CB_1 receptors (Pertwee *et al.*, 2007). Despite this recent interest, the functional effects of Δ^9-THCV on neuronal excitability remain unknown.

In the cerebellum, activation of presynaptic CB_1 receptors at interneurone-Purkinje cell (IN-PC) synapses causes inhibition of GABA release (Takahashi and Linden, 2000; Diana *et al.*, 2002; Kreitzer *et al.*, 2002; Szabo *et al.*, 2004; Yamasaki *et al.*, 2006), whereas CB_1 receptor activation at excitatory synapses inhibits glutamate release (Takahashi and Linden, 2000; Kreitzer and Regehr, 2001; Yamasaki *et al.*, 2006). Importantly, PCs represent the sole output of the cerebellar cortex. The major inhibitory drive onto PCs is provided by basket cell interneurones, which form numerous synaptic contacts in the 'pinceau' region surrounding the PC axon initial segment (Palay and Chan-Palay, 1974). The prominent expression of CB_1 receptors within the pinceau (Tsou *et al.*, 1998; Egertova and Elphick, 2000) provides a unique opportunity for exogenously applied cannabinoids, as well as endogenously released endocannabinoids, to modulate the overall output of the cerebellum. Activation of CB_1 receptors has been shown to play a role in cerebellar dysfunction, causing severe motor incoordination, including forms of ataxia (DeSanty and Dar, 2001; Patel and Hillard, 2001), therefore novel modulators of CB_1 receptors have clear therapeutic potential.

Here, we perform single-cell and network-level recordings to show that Δ^9-THCV, extracted from *Cannnabis sativa*, acts in a similar manner to standard CB_1 receptor antagonists to increase inhibitory neurotransmission at IN-PC synapses and that these actions correlate with decreases in spontaneous PC output in the cerebellum.

Methods

Tissue preparation and solutions

All animal procedures complied with UK Home Office regulations (Animals (Scientific Procedures) Act 1986). Cerebellar slices were prepared according to methods described in detail previously (Harvey and Stephens, 2004). Three- to five-week-old male TO mice (Harlan, UK) were humanely killed by cervical dislocation and decapitated. The brain was removed and transferred to chilled, carboxygenated sucrose-based artificial cerebrospinal fluid (aCSF). The cerebellum was dissected out and parasagittal cerebellar slices (300 μm thick) were cut using a Vibratome (R. & L. Slaughter, Upminster, UK). Slices were transferred into standard aCSF solution at 37 °C for 1 h and then maintained at room temperature (20–24 °C). The standard aCSF contained NaCl 124 mM, KCl 3 mM, $NaHCO_3$ 26 mM, NaH_2PO_4 2.5 mM, $MgSO_4$ 2 mM, $CaCl_2$ 2 mM, D-glucose 10 mM, maintained at pH 7.3 by bubbling with 95% O_2/5% CO_2. The sucrose-based solution used for dissection and slicing was identical to standard aCSF, with the exception that NaCl was replaced by isosmotic sucrose.

Single-cell electrophysiological recording

Slices were placed in a recording chamber at room temperature and perfused at 2–4 ml min^{-1} with standard carboxygenated aCSF. Individual neurones were visualized via a ×60 water immersion lens with infrared differential interference contrast optics using an upright Olympus BX50WI microscope (Olympus, Tokyo, Japan). Whole-cell recordings were performed using an EPC-9 patch-clamp amplifier (HEKA Electronik, Lambrecht, Germany), controlled by Pulse software (HEKA) with a Macintosh G4 computer. Patch electrodes were made from borosilicate glass (GC150-F10, Harvard Apparatus, Kent, UK) and, when filled with an intracellular solution containing CsCl 140 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, EGTA 10 mM, MgATP 4 mM, NaGTP 0.4 mM, HEPES 10 mM, pH 7.3, had resistances between 3 and 7 MΩ. Series resistance was typically 5–10 MΩ and was monitored and compensated by 70–90% throughout. Data were sampled at 7 kHz and filtered at one-third of the sampling frequency. Spontaneous miniature inhibitory postsynaptic currents (mIPSCs), recorded in the presence of 1 μM tetrodotoxin (TTX), were identified as rapidly activating, inward currents at a holding potential of −70 mV. Agents were applied in the presence of the non-NMDA glutamate receptor antagonist 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2–3-dione (NBQX) and the $GABA_B$ receptor antagonist CGP 55845.

Data were analysed using Pulsefit (HEKA), Axograph (Molecular Devices, Sunnydale, CA, USA), Igor (Wavemetrics, Lake Oswego, OR, USA), Origin (Microcal, Northampton, MA, USA) and Excel (Microsoft, Redmond, WA, USA) software. Cumulative frequency plots were constructed for mIPSC inter-event intervals (IEIs) using 5 ms bins. Data are presented as mean value ± s.e.mean, where n = number of cells. Statistical significance was determined using a Student's paired *t*-test or a one-way ANOVA followed by Tukey's HSD test, as appropriate. Cumulative frequency plots were analysed by Kolmogorov–Smirnov tests.

MEA electrophysiological recording

Spontaneous spike activity was recorded from cerebellar slices (produced as described above) using substrate-integrated MEAs (Multi Channel Systems, Reutlingen, Germany) (Egert *et al.*, 2002a; Stett *et al.*, 2003). MEAs comprised of 60 electrodes of 30 μm diameter, arranged in an 8 × 8 array with 200 μm spacing between electrodes (Figure 5a(i)). MEAs were cleaned before each recording using series treatments of Terg-A-Zyme (Cole-Palmer, UK), methanol and, finally, distilled water before air drying and coating. Slices were adhered to the MEA surface using an applied (~4 μl) and evaporated cellulose nitrate solution in methanol (0.24% w/v; Fisher Scientific, Loughborough, UK) ensuring maximum contact between the tissue and recording electrodes. Slice position upon the MEA was determined by observation at magnification × 4 with a Nikon TS-51 (Nikon, Tokyo, Japan) inverted microscope and imaged via a Bresser Mikro-Okular camera (Meade, Rhede, Germany). Once placed, slices were maintained at room temperature, continuously superfused (2–4 ml min^{-1}) with carboxygenated aCSF and allowed to stabilize for at least 10 min before recordings. Only data acquired from electrodes visually confirmed as proximal to the PC layer were used for analysis. Signals were amplified (1200 × gain), band-pass-filtered (10–3200 Hz) by a 60-channel amplifier (MEA60 System, Multi Channel Systems) and simultaneously sampled at 50 kHz per channel on all 60 channels. Data acquisition was to a PC using MC_Rack software (Multi Channel Systems) to monitor and record data for offline analysis.

Data analysis was performed using Neuroexplorer3 (Nex Technologies, Littleton, MA, USA) and MATLAB (Mathworks Inc., Natick, MA, USA) plus MEA Tools (Egert *et al.*, 2002b). Negative spike events were identified by threshold detection (minimum amplitude, −15 μV; typical noise level, ⩽ ± 5 μV; threshold determined as ⩾3σ, where σ represents the s.d. of the mean of data sets without spike activity (Egert *et al.*, 2002a)) in MC_Rack from continuously recorded data sets of not less than 300 s duration. Spike data were exported to Neuroexplorer3 and spike rate histograms constructed using 100 ms bins. Mean spike firing rates were calculated and are presented as normalized mean ± s.e.mean. Inevitable dead cell debris on the slice surface and PC depth from recording electrodes produced some variability in the apparent spontaneous firing frequency in the PC layer (typical range, 20–80 Hz; see also Egert *et al.*, 2002a). Consequently, drug-induced changes are presented as percentage changes in firing frequency vs control firing frequency per experiment to provide normalized measures for pooled data. *n* numbers for MEA recordings are shown as *n* = total number of electrodes (total number of slices). Statistical significance was determined by a non-parametric Mann–Whitney *U*-test. In all cases, $P<0.05$ was considered significant.

Materials

The following agents were used: *N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-multipyrazole-3-carboxamide (AM251), CGP 55845, NBQX, 212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (Tocris Cookson, Bristol, UK) and bicuculline methiodide (BMI; Sigma, UK). TTX was from Alomone (Jerusalem, Israel). Rimonabant was kindly provided by NIDA/NIH, Bethesda, USA. Δ^9-THCV was a kind gift of GW Pharmaceuticals (Salisbury, Wilts, UK). All drugs, except Δ^9-THCV, which was supplied as a 58 mM stock solution in ethanol and stored at 4 °C, were made up as stock solutions in distilled water or dimethyl sulphoxide (AM251, CGP 55845, WIN55) and stored at −20 °C. Solvent was present at a maximum final concentration of 0.1%; solvent, applied alone at equivalent experimental concentrations, had no effect on synaptic responses in this preparation (see Bardo *et al.*, 2002 and the Results section). Aliquots were thawed and dissolved in carboxygenated aCSF immediately before use. In all experiments, drugs were bath-applied for a minimum of 20 min to achieve steady-state effects.

Results

Effect of cannabinoids on inhibitory synaptic transmission at IN-PC synapses

Whole-cell recordings were made from PCs in acute cerebellar brain slices in the presence of 1 μM TTX (plus 5 μM NBQX and 5 μM CGP 55845) to isolate action potential-independent GABAergic mIPSCs. Drugs were bath-applied for a minimum of 20 min to achieve steady-state effects. The CB_1/CB_2 agonist WIN55 (1 and 5 μM) caused a clear decrease in mean mIPSC frequency (Figures 1a, b, d, e). At 5 μM, WIN55 caused a steady-state reduction from 5.2 ± 0.8 to 3.3 ± 0.5 Hz, equivalent to 63.2 ± 3.1% of control values ($n=8$; $P<0.001$; paired *t*-test); this was reflected by an increase in the mIPSC IEI by WIN55 (Figure 1d; $n=8$; each replicant $P<0.05$; Kolmogorov–Smirnov). WIN55 had no effect on mean mIPSC amplitude (74.5 ± 10.7–71.4 ± 10.7 pA; $n=8$; $P=0.34$; paired *t*-test), as reflected in normalized data (Figure 1c). The selective CB_1 antagonist AM251 (2 μM) reversed the WIN55-induced decrease in mean mIPSC frequency (Figures 1a, b, e); this was to 7.1 ± 1.1 Hz, equivalent to 137.7 ± 9.6% of control values ($n=8$; $P<0.01$; ANOVA) and reflected by a decrease in the mIPSC IEI by AM251 that exceeded control levels (Figure 1d; $n=8$; each replicant $P<0.05$; Kolmogorov–Smirnov). AM251 had no effect on mean mIPSC amplitude (84.2 ± 14.8 pA; $n=8$; $P=0.19$; ANOVA), as reflected in normalized data (Figure 1c). In all subsequent experiments, no clear changes in mean mIPSC amplitude were seen. Reductions in mPSC frequency in the absence of effects on event amplitude are consistent with a reduction in quantal transmitter release from presynaptic sites. Although CB_2 receptor immunohistochemical labelling has recently been described in the rat cerebellum (Ashton *et al.*, 2006), there is, as yet, little evidence for functional CB_2 receptor activity in this region. Accordingly, the selective CB_2 agonist JWH-133 (10 μM) showed no effects on mean mIPSC frequency or amplitude ($n=3$; data not shown); these data were consistent with a lack of functional CB_2 receptors at IN-PC synapses. Overall, these data confirm previous studies showing that IN-PC synapses in mouse cerebellum possess functional presynaptic CB_1 receptors; moreover, the above results describing observed AM251 effects suggest that CB_1 antagonists act to increase inhibitory neurotransmission at these synapses.

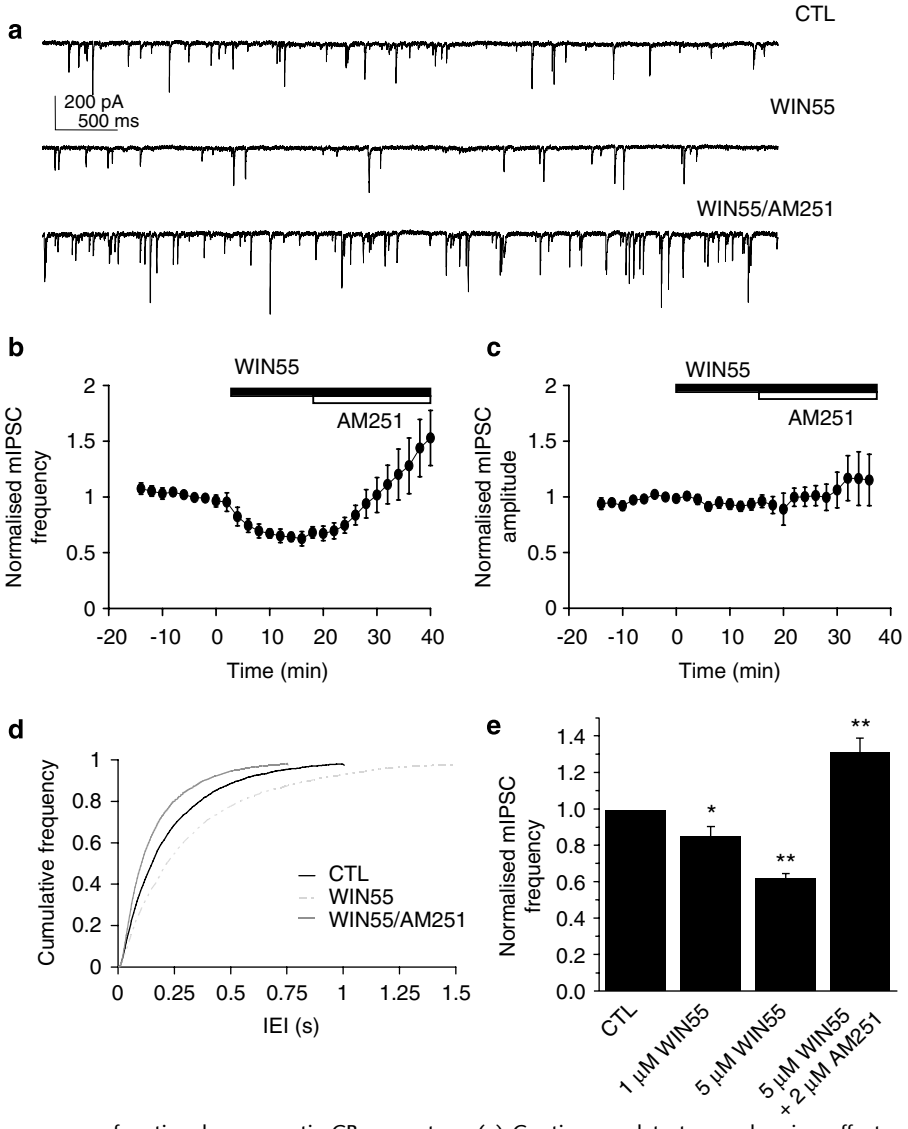

Figure 1 IN-PC synapses possess functional presynaptic CB_1 receptors. (**a**) Continuous data traces showing effects of WIN55 (5 μM) and subsequent application of AM251 (2 μM) in the continued presence of WIN55 (5 μM) on control (CTL) mIPSCs (holding potential (V_H) = −70 mV). Note that WIN55 decreased mIPSC frequency, whereas AM251 caused increases beyond basal levels. Time course for effect of WIN55 (5 μM) and AM251 (2 μM) on normalized mIPSC frequency (**b**) and normalized mIPSC amplitude (**c**) (both $n = 8 \pm$ s.e.mean). (**d**) Cumulative frequency plots for effect of WIN55 (5 μM) and subsequent application of AM251 (2 μM) on control (CTL) mIPSC IEI (bin widths 5 ms) (all $n = 8 \pm$ s.e.mean). (**e**) Bar graph showing summarized effects of WIN55 (1 μM; $n = 6 \pm$ s.e.mean and 5 μM; $n = 8 \pm$ s.e.mean) and of subsequent application of AM251 (2 μM, $n = 8 \pm$ s.e.mean) in the continued presence of WIN55 (5 μM) on control (CTL, $n = 8 \pm$ s.e.mean) normalized mIPSC frequency; *$P < 0.05$, **$P < 0.01$ (paired *t*-test or ANOVA).

Phytocannabinoids have recently attracted interest as potential therapeutic agents. Here, we examined functional effects of Δ^9-THCV on neuronal excitability for the first time. Δ^9-THCV reversed the WIN55-induced decrease in mean mIPSC frequency, causing increases in mean mIPSC frequency, which were beyond basal levels (Figure 2a). The actions of Δ^9-THCV (5–58 μM), each concentration for a minimum of 20 min to achieve steady-state effects, were concentration related (Figures 2a–c). Δ^9-THCV had no effect on mean mIPSC amplitude distribution at any concentration tested. When applied alone, AM251 (2 μM: 143 ± 8.7% of control values; $n = 6$; $P < 0.001$; Figure 3a), another synthetic CB_1 antagonist rimonabant (3 μM: 162 ± 22%; $n = 6$; $P < 0.05$; Figure 3b) or Δ^9-THCV (10 μM: 154 ± 21%; $n = 6$; $P < 0.01$; Figure 3c) all caused significant steady-state increases in mean mIPSC frequency vs control levels over the time periods shown (paired *t*-test used for each). In all cases, no changes in mean mIPSC amplitude distribution were seen. These data indicate that Δ^9-THCV acts to increase quantal GABA release at IN-PC synapses in a similar manner to selective CB_1 antagonists and, moreover, that such effects were independent of previous experimental activation of presynaptic CB_1 receptors. We next determined the effects of applying a single, maximal concentration of Δ^9-THCV (Ma *et al.*, 2006) before WIN55 application. Δ^9-THCV (58 μM) caused the expected increase in mean mIPSC frequency (Figures 4a and b); this increase was from 2.5 ± 0.6 to 4.2 ± 0.8 Hz (= 176 ± 21% of control values; $n = 5$; $P < 0.01$

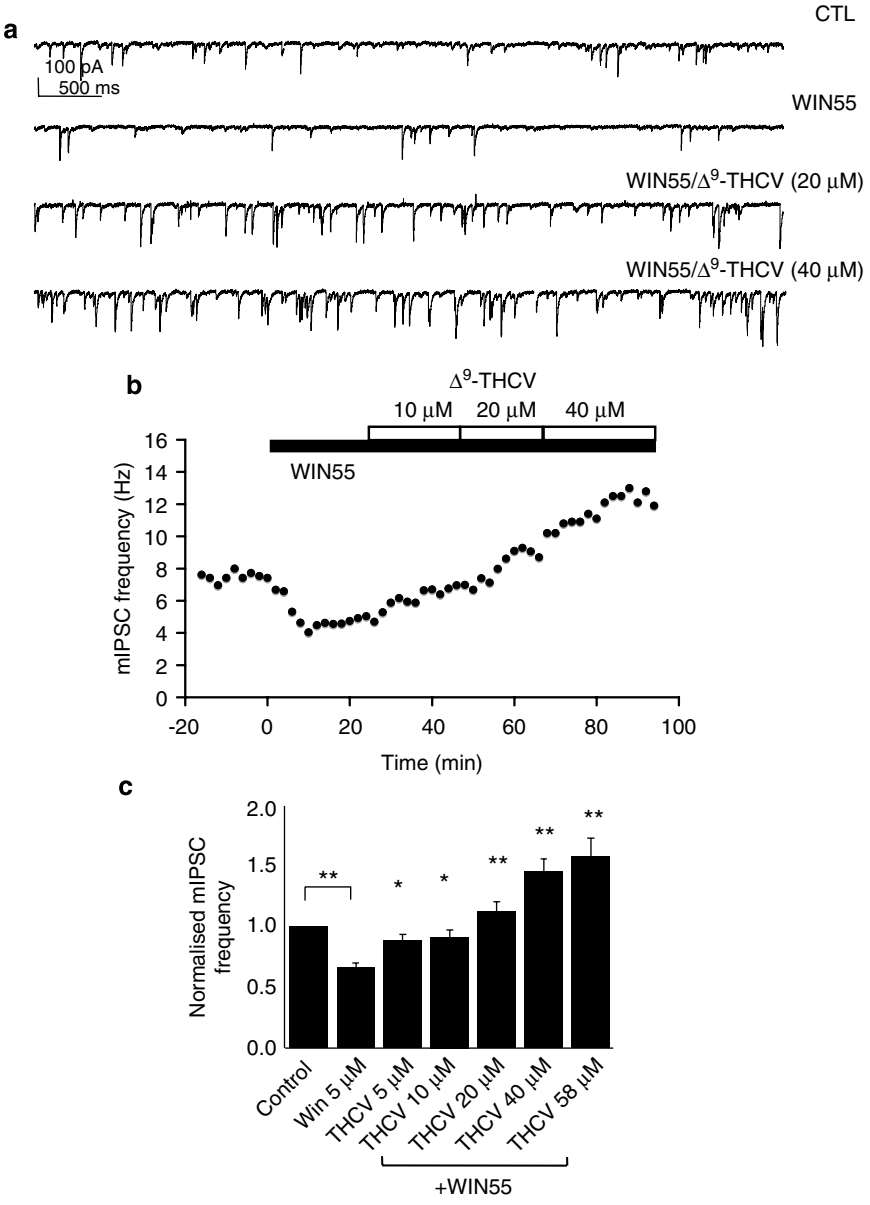

Figure 2 WIN55-induced inhibition at IN-PC synapses is reversed by Δ^9-THCV. (**a**) Continuous data traces showing effects of WIN55 (5 μM) and subsequent application of Δ^9-THCV (20 and 40 μM) in the continued presence of WIN55 (5 μM) on control (CTL) mIPSCs; $V_H = -70$ mV. Δ^9-THCV reversed WIN55-induced inhibition and increased mIPSC frequency beyond basal levels. (**b**) Example of time course for effects of WIN55 (5 μM) and subsequent application of Δ^9-THCV (10–40 μM) on mIPSC frequency. (**c**) Bar graph showing summarized effects of WIN55 (5 μM, $n = 8 \pm$ s.e.mean) and subsequent application of Δ^9-THCV (5–58 μM; $n = 3$–$8 \pm$ s.e.mean) in the continued presence of WIN55 (5 μM) on control (CTL, $n = 8 \pm$ s.e.mean) normalized mIPSC frequency; *$P < 0.05$, **$P < 0.01$ vs WIN55, or vs CTL as shown (paired *t*-test or ANOVA).

(paired *t*-test)). In the continued presence of Δ^9-THCV, the WIN55-induced inhibition was now blocked (Figures 4a and b; 4.1 ± 0.9 Hz ($= 99 \pm 10\%$ of Δ^9-THCV values) $n = 5$; $P = 0.82$; ANOVA). No changes in mean mIPSC amplitude distribution were seen. These data indicate that pre-incubation with Δ^9-THCV blocks WIN55 action at IN-PC synapses.

Effect of cannabinoids on PC output in cerebellar brain slices

Having demonstrated that Δ^9-THCV and synthetic CB_1 antagonists act to increase inhibitory neurotransmission at IN-PC synapses, we next used MEA recordings from acute cerebellar brain slices to determine whether Δ^9-THCV could also affect spontaneous PC output under conditions of basal synaptic transmission. Brain slices were cut in a sagittal plane, which maintains PC soma and dendritic tree structure and allows recording from intact PCs close to the slice surface (Egert *et al.*, 2002a, b). Recordings were made only from electrodes proximal to the PC layer (Figure 5a(i)) to correlate with intracellular recordings from single PCs (Figures 1–4). Here, spikes of negative polarity, representative of single-unit or multiple single-unit excitatory events (Egert *et al.*, 2002a), were identified by threshold detection. Under these conditions, we recorded robust, high-frequency

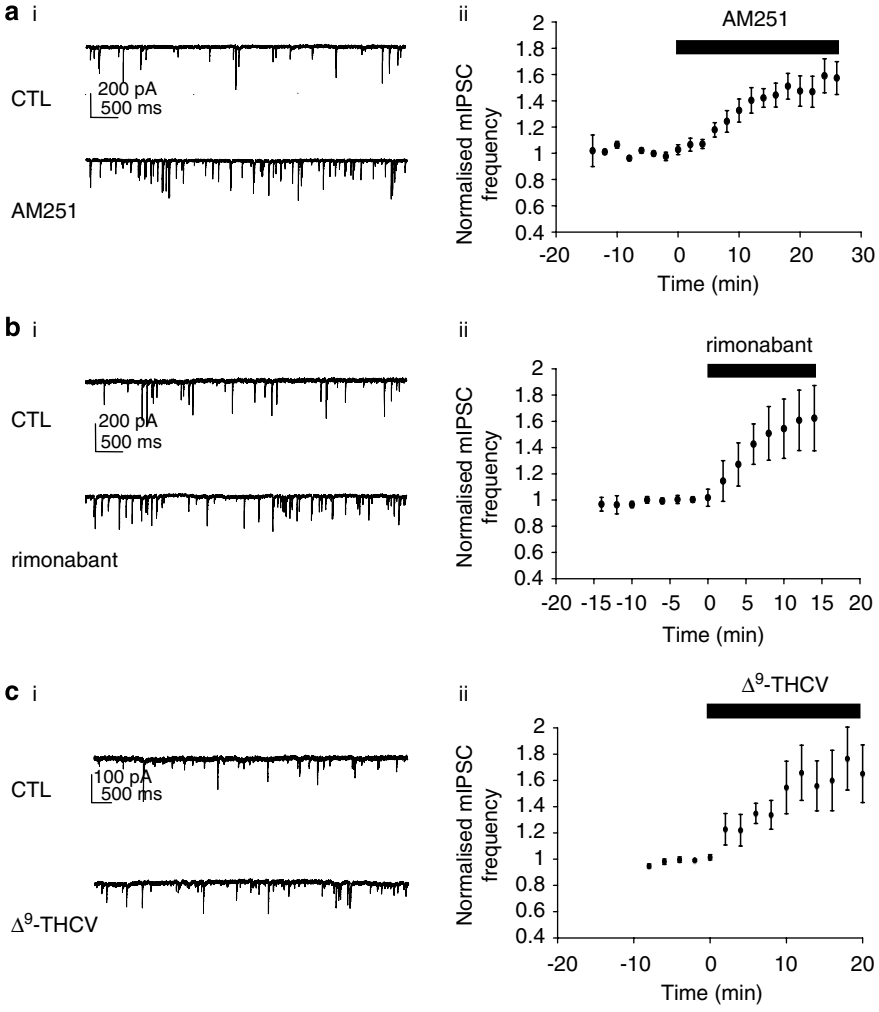

Figure 3 CB_1-selective antagonists and Δ^9-THCV applied alone increase inhibitory neurotransmission at IN-PC synapses. Continuous data traces showing effects of (**a(i)**) AM251 (2 μM), (**b(i)**) rimonabant (3 μM) and (**c(i)**) Δ^9- THCV (10 μM) on control (CTL) mIPSCs; in all cases, $V_H = -70$ mV. Note that each cannabinoid acted to increase mIPSC frequency. Corresponding time courses for effects of (**a(ii)**) AM251 (2 μM; $n = 6 \pm$ s.e.mean), (**b(ii)**) rimonabant (3 μM; $n = 5 \pm$ s.e.mean) and (**c(ii)**) Δ^9-THCV (10 μM; $n = 6 \pm$ s.e.mean).

spontaneous electrical activity from PCs (typical range, 20–80 Hz; $n = 34$ (from six separate slices); Figure 5a(ii)). All spontaneous activity recorded by the MEA was abolished in the presence of 1 μM TTX ($n = 3$; data not shown), and application of the vehicle (dimethyl sulphoxide) at concentrations used for drug dilution (~0.1% v/v) revealed no observable effects upon spike firing ($n = 3$; data not shown). Bath application of WIN55 (5 μM) caused a significant increase in normalized spontaneous mean spike firing rate (216 ± 21% of control values; $n = 28$ (6); $P < 0.05$; Mann–Whitney *U*-test; Figures 5a(ii), b(i), (ii), c, Table 1). Subsequent application of Δ^9-THCV (5–40 μM), each concentration for a minimum of 20 min to achieve steady-state effects, caused clear, concentration-dependent decreases in PC spontaneous spike firing (for example, 58 μM: 49 ± 17% of control values; $n = 28$ (6); $P < 0.05$; Mann–Whitney; Figures 5a(ii), b(iii), c, Table 1). These data demonstrate that WIN55-induced increases in PC spontaneous spike firing were opposed by Δ^9-THCV. We next examined the effects of cannabinoids without pre-treatment with the CB receptor agonist WIN55. AM251 (2 μM) alone caused a significant decrease in PC spontaneous spike firing rate (68 ± 4% of control values; $n = 24$ (6); $P < 0.05$; Mann–Whitney; Figures 6a–c). Similarly, Δ^9-THCV (5 μM) alone also decreased PC output (56 ± 4% of control values; $n = 30$ (6); $P < 0.05$; Mann–Whitney; Figure 6c).

MEA recordings demonstrate that WIN55-induced CB_1 receptor activation caused an increase in PC spontaneous excitatory discharge, whereas Δ^9-THCV and a CB_1 receptor antagonist acted to limit excitation. A clear possibility is that these data reflect modulation of inhibitory neurotransmission at IN-PC synapses (Figures 1–4). To test this hypothesis, we examined the effects of cannabinoids in the presence of the $GABA_A$ receptor antagonist BMI. Bath application of BMI itself caused expected large increases in spontaneous spike firing frequency, at 10 μM ($P < 0.05$; Mann–Whitney; Figure 7a(i), Table 1) and at 30 μM ($P < 0.05$; Mann–Whitney; Figure 7b(i), Table 1). These data demonstrate that functional inhibitory input modulates spontaneous activity of PCs and hence cerebellar output. We next examined the effects of $GABA_A$ receptor blockade on cannabinoid-induced changes in PC output. In the presence of 10 μM BMI, WIN55 (5 μM)

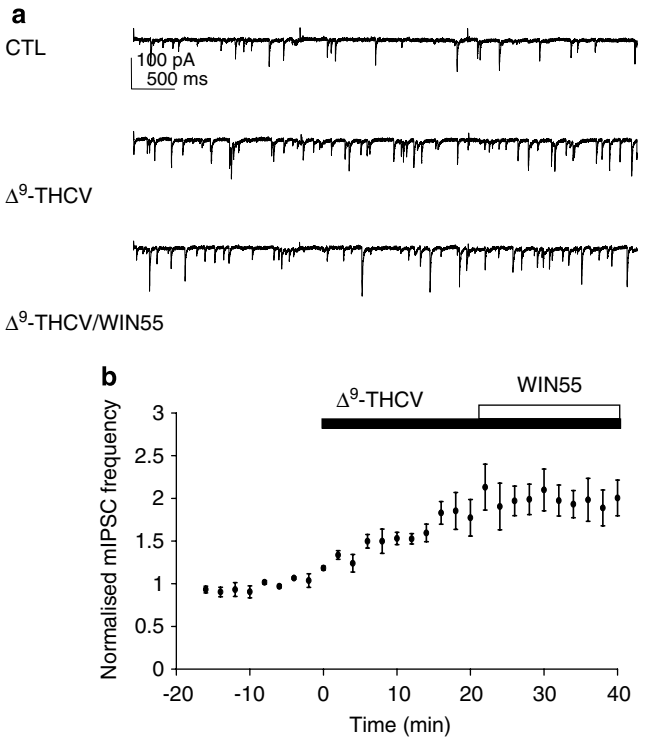

Figure 4 Δ^9-THCV blocks WIN55-induced inhibition at IN-PC synapses. (**a**) Continuous data traces showing effects of Δ^9-THCV (58 μM) and of subsequent application of WIN55 (5 μM) in the continued presence of Δ^9-THCV on control (CTL) mIPSCs; $V_H = -70$ mV. Δ^9-THCV increased mIPSC frequency and blocked WIN55-induced inhibition. (**b**) Time course for effects of Δ^9-THCV (58 μM; $n = 5 \pm$ s.e.mean) and of subsequent application of WIN55 (5 μM; $n = 5 \pm$ s.e.mean) in the continued presence of Δ^9-THCV on normalized mIPSC frequency.

still caused an increase in PC spontaneous spike firing (Mann–Whitney; Figure 7a(i), (ii), Table 1); however, WIN55 effects were now significantly reduced in comparison with previous control levels (216 ± 21% vs pre-BMI control compared with 155 ± 24% vs 10 μM BMI control; $P<0.05$; Mann–Whitney). In the presence of 10 μM BMI, Δ^9-THCV still caused an overall decrease in PC firing rate, which was significant for Δ^9-THCV concentrations $\geqslant 10$ μM (all $P<0.05$ vs BMI control; Mann–Whitney; Figure 7a(ii); Table 1). However, consistent with WIN55 data, Δ^9-THCV-induced inhibition of PC firing rate in the presence of 10 μM BMI was also significantly attenuated (for example, 20 μM Δ^9-THCV: 15 ± 8% vs pre-BMI control compared with 47 ± 3% vs 10 μM BMI control; $P<0.05$; Mann–Whitney). In the presence of 30 μM BMI, cannabinoid effects were now abolished: no further changes in PC spontaneous firing rates were seen upon subsequent addition of WIN55 (5 μM = 104 ± 3% vs 30 μM BMI control; $P=0.67$; Mann–Whitney; Figures 7b(i), (ii), Table 1) or Δ^9-THCV (5–40 μM) (for example, 20 μM = 110 ± 4% vs 30 μM BMI control; $P=0.73$; Mann–Whitney; Figures 7b(i), (ii), Table 1).

The sensitivity of Δ^9-THCV and WIN55 effects on spontaneous PC output to $GABA_A$ receptor blockade indicate that, under the experimental conditions used, cannabinoid effects are mediated by their action on inhibitory neurotransmission. More specifically, we show that Δ^9-THCV-induced increases in GABA release can affect PC output and hence that this phytocannabinoid has the potential to modulate the output of the cerebellar cortex.

Discussion and conclusions

We have correlated patch-clamp and MEA recordings to demonstrate that Δ^9-THCV acts in a manner similar to standard, selective CB_1 receptor antagonists to modulate inhibitory neurotransmission at a cellular (IN-PC synapses) and network (spontaneous PC output) level in the mouse cerebellum *in vitro*. We provide evidence that a CB receptor agonist reduces GABA release onto PCs leading to synaptic disinhibition, whereas Δ^9-THCV acts to produce opposing effects, promoting an inhibition of spontaneous PC output.

Δ^9-THCV modulates inhibitory neurotransmission at IN-PC synapses

We have recorded from IN-PC synapses in mouse cerebellar cortex as a suitable system to study modulation of inhibitory neurotransmission by cannabinoids. The CB receptor agonist WIN55 inhibited quantal GABA release; actions were consistent with a presynaptic effect downstream of Ca^{2+} entry, possibly via a direct action on the exocytotic release machinery, as proposed for other presynaptic GPCR agonists at IN-PC synapses (Harvey and Stephens, 2004). Standard CB_1 receptor antagonists reversed WIN55-induced inhibition and, when applied alone, they caused an increase in GABA release over basal levels. Similar effects at IN-PC synapses have been reported in some studies (Diana *et al*. 2002; Kreitzer *et al*., 2002; Galante and Diana, 2004; Yamasaki *et al*., 2006), but not others (Szabo *et al*., 2004). CB_1 antagonists also increase synaptic transmission at other synapses (Melis *et al*., 2004; Hentges *et al*., 2005; Zhu and Lovinger, 2005), suggesting that mechanisms of presynaptic regulation may be conserved between certain central synapses.

This study provides the first evidence that Δ^9-THCV modulates neuronal excitability in the CNS. Δ^9-THCV shared properties with synthetic CB_1 antagonists, reversing WIN55-induced inhibition of GABA release and increasing release over basal levels when applied alone; in addition, pre-incubation with Δ^9-THCV blocked subsequent WIN55-induced inhibition. These data were consistent with Δ^9-THCV acting as a functional CB_1 receptor antagonist in the CNS. CB_1 antagonist effects most likely involve removal of endocannabinergic tone, as proposed for studies at IN-PC and other central, synapses (Kreitzer *et al*., 2002; Galante and Diana, 2004; Melis *et al*., 2004; Hentges *et al*., 2005; Zhu and Lovinger, 2005; Neu *et al*., 2007). In particular, Kreitzer *et al*. (2002) reported that AM251 caused an increase in interneurone firing in cerebellar brain slices, suggesting basal inhibition due to CB_1 receptor activity. Thus, although differences in cannabinoid action are reported between synapses, our data confirm that IN-PC synapses are likely to be under strong endocannabinergic inhibition. In this regard, it has recently been shown that the major endocannabinoid 2-arachidonoyl glycerol is released from PCs to

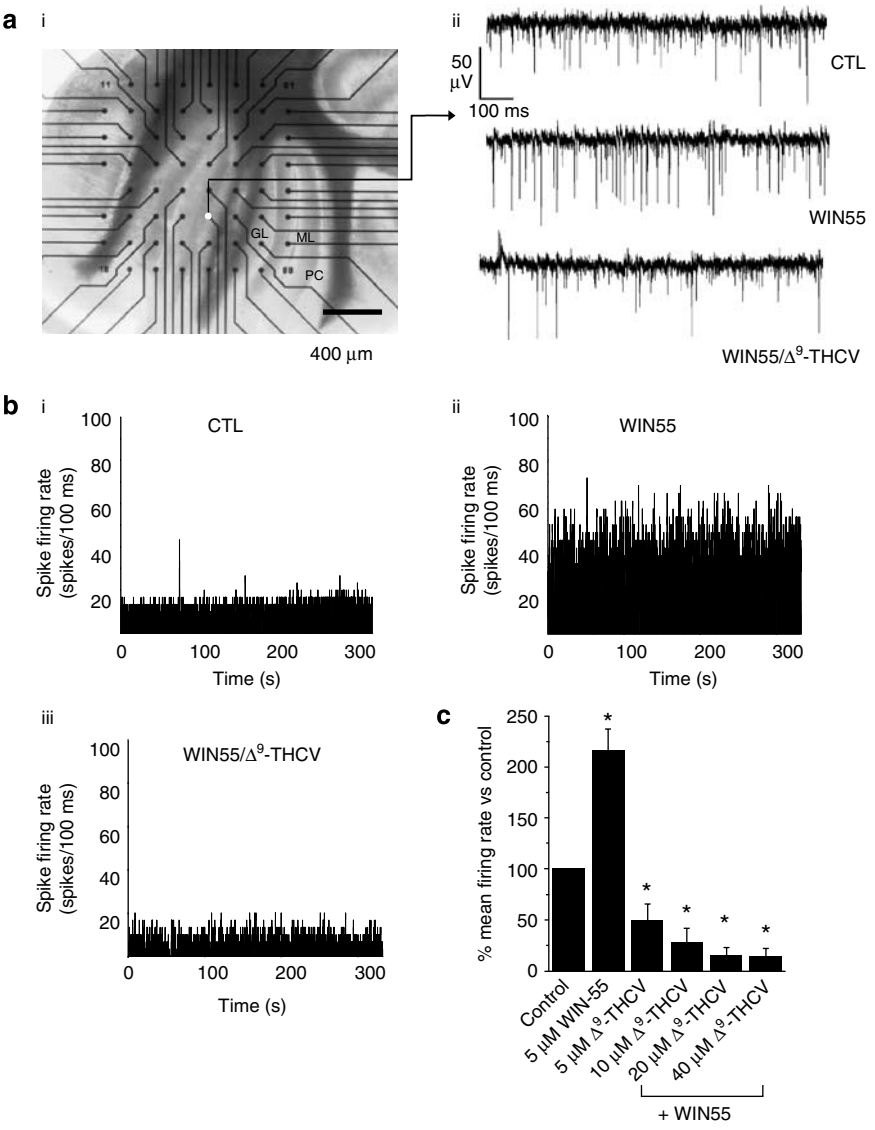

Figure 5 Cannabinoids modulate PC spontaneous excitatory spike firing frequency in cerebellar brain slices. (**a(i)**) Micrograph showing a cerebellar slice adhering to an MEA. Specific cerebellar layers: GL = granular layer, ML = molecular layer and PC = Purkinje cell layer. The filled white circle shows a recording electrode position in the PC layer. (**a(ii)**) Examples of continuous MEA recordings from a single electrode showing effects of WIN55 (5 μM) and subsequent application of Δ^9-THCV (5 μM) in the continued presence of WIN55 (5 μM) on PC control (CTL) spontaneous excitatory spike firing frequency; steady-state effects, drugs applied for a minimum of 20 min. Note the increase in PC firing rate upon application of WIN55 and consequential decrease below control levels upon subsequent application of Δ^9-THCV. (**b**) PC spike firing rate histograms (minimum 5 min continuous recording, 100 ms bin size) for a representative electrode for (**i**) control, (**ii**) WIN55 (5 μM) and (**iii**) subsequent application of Δ^9-THCV (5 μM). (**c**) Bar graph showing summarized WIN55 (5 μM) and Δ^9-THCV (5–40 μM; all $n = 28$ (6) ± s.e.mean) in the continued presence of WIN55 (5 μM) effects on PC control ($n = 36$ (6) ± s.e.mean) spontaneous excitatory spike firing rates; *$P < 0.05$ vs control; Mann–Whitney *U*-test.

Table 1 Effects of cannabinoids on spontaneous PC excitatory spike properties

PC firing rate (%)	*CTL*	*BMI*	*+5 μM WIN55*	*+5 μM Δ9-THCV*	*+10 μM Δ9-THCV*	*+20 μM Δ9-THCV*	*+40 μM Δ9-THCV*
CTL ($n = 28$ (6))	100	—	216 ± 21*	49 ± 17*	27 ± 15*	15 ± 8*	14 ± 8*
10 μM BMI ($n = 38$ (6))	100	346 ± 46*	536 ± 83*	224 ± 86	125 ± 7†	162 ± 10†	135 ± 10†
30 μM BMI ($n = 35$ (6))	100	564 ± 62*	587 ± 17[a]	598 ± 18[a]	581 ± 17[a]	620 ± 45[a]	626 ± 23[a]

Abbreviations: BMI = bicuculline methiodide; CTL = control; PC = Purkinje cell; Δ^9-THCV = Δ^9-tetrahydrocannabivarin; WIN55 = 212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate.
PC firing rate (expressed as a percentage of control firing rate) calculated using Neuroexplorer3 (Nex Technologies), MATLAB (Mathworks Inc.) plus MEA Tools (Egert *et al*., 2002b) as described in Methods. *Note*: Δ^9-THCV results were obtained in the continued presence of 5 μM WIN55.
[a]NS = no significant difference vs BMI control, Mann–Whitney *U*-test.[3] **P* < 0.05 *vs* control, †*P* < 0.05 *vs* BMI control.

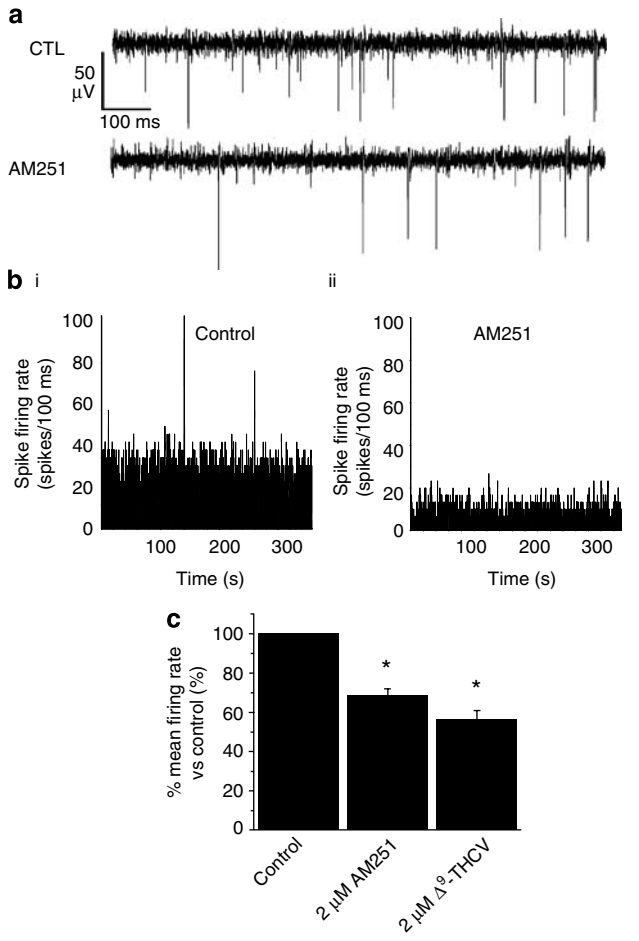

Figure 6 AM251 reduces PC spontaneous excitatory spike firing frequency in cerebellar brain slices. (**a**) Examples of continuous MEA recordings from a single electrode showing effects of AM251 (2 μM) on control (CTL) responses; steady-state effects, drugs applied for a minimum of 20 min. Note the decrease in PC spike firing rate upon application of AM251. (**b**) PC spike firing rate histograms (minimum 5 min continuous recording; 100 ms bin size) for a representative electrode in control (**i**) and (**ii**) AM251 (2 μM). (**c**) Bar graph showing summarized effects of AM251 (2 μM; $n=30$ (6)) on PC control ($n=36$ (6)) spontaneous excitatory spike firing rates. Also shown are effects of Δ^9-THCV alone (5 μM; $n=24$ (6)); $^*P<0.05$ vs control; Mann–Whitney *U*-test.

act retrogradely at presynaptic CB_1 receptors (Szabo *et al.*, 2006). Other putative mechanisms associated with receptor antagonism are less likely. The use of TTX in this study argues against mechanisms such as decreases in the rate of failures of action potential-elicited transmitter release (Neu *et al.*, 2007).

Δ^9-THCV and the synthetic analogue O-4394 have been reported variously to antagonize WIN55 action in isolated mouse vas deferens and Δ^9-tetrahydrocannabinol-induced anti-nociception and hypothermia *in vivo* (Thomas *et al.*, 2005; Pertwee *et al.*, 2007; Pertwee, 2008). [^{35}S]GTPγS-binding studies in mouse whole brain membranes have supported an antagonist action, with an apparent K_B <100 nM reported for Δ^9-THCV acting at CB_1 receptors (Thomas *et al.*, 2005); we report broadly similar K_B values and a rightward shift in WIN55 concentration–response curves induced by Δ^9-THCV in isolated cerebellar (and piriform cortical) membranes (Dennis *et al.*, 2007). We have also seen that higher concentrations of Δ^9-THCV (and AM251) can reduce [^{35}S]GTPγS binding to cerebellar membranes (Dennis *et al.*, 2007), which may suggest inverse agonism (MacLennan *et al.*, 1998). Accordingly, there is evidence that CB_1-selective compounds AM251 and rimonabant can both act as inverse agonists and hence that CB_1 receptors may possess constitutive activity (Bouaboula *et al.*, 1997; Pan *et al.*, 1998; Pertwee, 2005b). However, in our hands, decreases in [^{35}S]GTPγS binding occurred independently of CB_1 receptor expression. Although we cannot fully rule out that Δ^9-THCV actions here are mediated, at least in part, by a CB_1 receptor-independent mechanism, this pathway would also have to be affected by both WIN55 and AM251. In this regard, it is also important to note that drug concentrations necessary to elicit measurable responses in brain slice preparations are difficult to relate to equilibrium-binding constants. For example, despite nanomolar affinities for CB_1 receptors, clear WIN55 effects required millimolar concentrations here and in other studies (Brown *et al.*, 2004). Concentration-dependence issues are exacerbated for highly lipophilic cannabinoids, which are likely to partition into lipid membranes in brain slice preparations (Howlett *et al.*, 1989; Bloom *et al.*, 1997). Moreover, the end point of our assay (inhibition of vesicular GABA release) is several steps far from receptor binding/GTPγS turnover. Interestingly, higher Δ^9-THCV doses have also been reported to have agonist actions *in vivo*, indicating a complex pharmacology (Pertwee, 2008). In this study, Δ^9-THCV had no agonist effects at IN-PC synapses; in contrast, Δ^9-THCV acted in a manner opposite to the CB receptor agonist WIN55, but similar to standard CB_1 antagonists, to increase inhibitory neurotransmission.

Cannabinoids modulate spontaneous PC output in the cerebellum

The cerebellar cortex has a distinct, well-defined architecture and micro-circuitry (Ramon y Cajal, 1911; Eccles *et al.*, 1967), affording an ideal opportunity to study spatio-temporal network activity. We have used MEA recordings to correlate Δ^9-THCV effects at IN-PC synapses with those on spontaneous PC output. Firstly, our data confirm that mouse PCs exhibit robust spontaneous activity under conditions that maintain synaptic connectivity (Egert *et al.*, 2002a; Mapelli and D'Angelo, 2007). This is substantiated by PC firing rates measured here being in good agreement with those recorded *in vivo* (Hausser and Clark, 1997; Raman and Bean, 1999), suggesting that MEA recording in cerebellar brain slices represents an appropriate model to investigate PC output. This activity is likely to reflect spontaneous PC discharge, modulated by integrated input from spontaneously discharging inhibitory INs and recurrent PC collaterals (Palay and Chan-Palay, 1974; Hausser and Clark, 1997; Mann-Metzer and Yarom, 1999). We extend previous studies to provide the first examination of drug effects on spontaneous PC activity using MEA methods. WIN55 caused clear increases in excitatory spike firing rate consistent with a decrease in GABA release at IN-PC synapses leading to synaptic disinhibition. Conversely, Δ^9-THCV reversed WIN55 effects and when applied alone it decreased

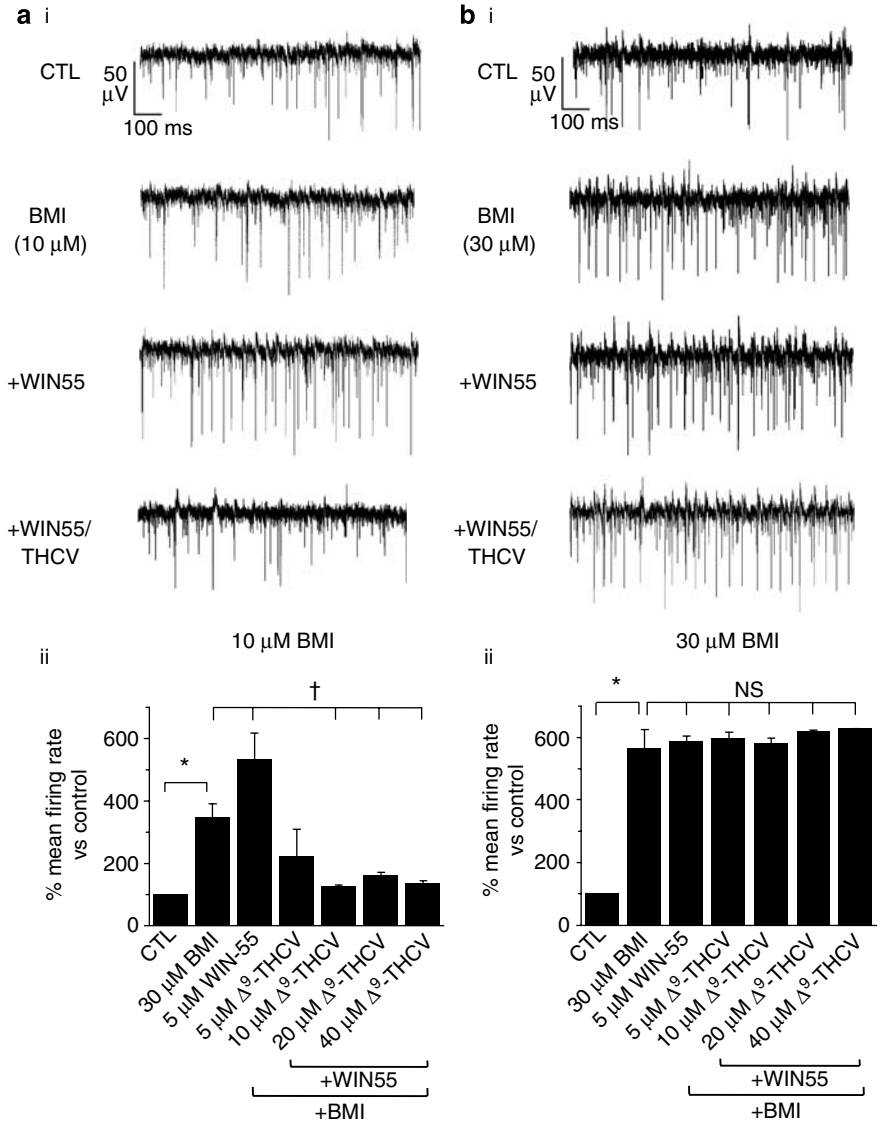

Figure 7 The $GABA_A$ antagonist BMI attenuates cannabinoid effects on PC spontaneous excitatory spike firing frequency. Examples of continuous MEA recordings from a single electrode showing effects of BMI at (**a**) 10 μM and (**b**) 30 μM on control (CTL) responses and on WIN55 (5 μM)- and Δ^9-THCV(5 μM)-induced changes in PC spontaneous excitatory spike firing frequency; steady-state responses, drugs applied for a minimum of 20 min. (**a(i)**) Control firing rate was increased by 10 μM BMI and was further increased by WIN55 in the continued presence of BMI; subsequent application of Δ^9-THCV decreased firing rate to below control levels. (**a(ii)**) Bar graph showing summarized effects of WIN55 (5 μM) and of Δ^9-THCV (5–40 μM) on PC spontaneous excitatory spike firing rates in the continued presence of 10 μM BMI (all $n = 38$ (6); * = $P < 0.05$ vs control, † = $P < 0.05$ vs 10 μM BMI as normalized control; Mann–Whitney *U*-test. (**b(i)**) BMI (30 μM) caused an increase in control spike firing rate, subsequent application of WIN55 (5 μM) and then Δ^9-THCV (5 μM) in the continued presence of BMI caused no further changes. (**b(ii)**) Bar graph showing summarized effects of WIN55 (5 μM) and of Δ^9-THCV (5–40 μM) on PC spontaneous excitatory spike firing rates in the continued presence of 30 μM BMI; (all $n = 35$ (6)). *$P < 0.05$ vs control, NS = no significant difference vs 30 μM BMI as normalized control; Mann–Whitney *U*-test.

excitatory spike firing; these data were consistent with Δ^9-THCV causing an increased GABAergic inhibition, manifest as an overall decrease in spontaneous PC activity. Again, Δ^9-THCV effects on PC output were similar to those seen for the CB_1 antagonist AM251. Direct CB_1 receptor-mediated postsynaptic effects (such as apparent reductions in firing rate due to spike amplitude falling below the detection threshold) are unlikely as PCs do not express significant CB_1 receptor numbers (Matsuda *et al.*, 1993; Egertova and Elphick, 2000; Freund *et al.*, 2003). Activation of presynaptic CB_1 receptors leads to reduction in transmitter release; the overall increase in excitability at a network level is consistent with cannabinoid-mediated modulation of inhibitory neurotransmission at IN-PC synapses being reflected in spontaneous PC output. This hypothesis is supported by the concentration-dependent abolition of WIN55 and Δ^9-THCV effects by the $GABA_A$ receptor antagonist BMI, which blocks phasic and tonic GABA currents at mouse IN-PC synapses (Harvey *et al.*, 2006). Interestingly, although cannabinoid actions were reduced by 10 μM BMI, it was necessary to use 30 μM BMI to ensure complete block; however, this concentration is well within ranges commonly

used in brain slice experiments. Overall, our data support a mechanism whereby Δ^9-THCV increases GABA release at inhibitory terminals (including IN-PC synapses) to cause a decrease in spontaneous PC output; thus, we demonstrate that Δ^9-THCV has the potential to modulate network activity within the cerebellar cortex.

Functional relevance

We show that Δ^9-THCV (and the CB_1 receptor antagonist AM251) acts to increase inhibitory neurotransmission to cause any overall decrease in PC output under the conditions used. In contrast, the CB agonist WIN55 decreased GABA release and caused synaptic disinhibition of PCs. PCs project inhibitory innervation to deep cerebellar nuclei (DCN) to control their intrinsic firing rate (Gauck and Jaeger, 2000); in turn, DCN supply areas such as the vestibular nucleus and motor cortex to coordinate movement, balance and posture. Abnormally high spiking activity can damage neurones and lead to degenerative disease. Moreover, excessive PC activation has been proposed to increase inhibition of DCN and lead to cerebellar dysfunction (Patel and Hillard, 2001). Importantly, CB_1 receptor agonists have been shown to promote cerebellar dysfunction in behavioural tests, in particular causing severe motor incoordination (DeSanty and Dar, 2001; Patel and Hillard, 2001). Therefore, decreases in GABA release at IN-PC synapses due to excessive endocannabinoid release would be expected to result in PC disinhibition and a corresponding increased inhibition of DCN, which may then precipitate cerebellar dysfunction. Agents that increase inhibitory neurotransmission, such as Δ^9-THCV, will cause opposing effects, reducing PC output and ultimately facilitating the control of posture and movement by DCN. These studies suggest that Δ^9-THCV, alongside standard CB_1 receptor antagonists, has therapeutic potential to combat diseases involving cerebellar dysfunction and hyperexcitability. For example, our preliminary studies suggest that Δ^9-THCV may be anti-convulsant in a developmental model of epilepsy (Weston *et al.*, 2006; see Pertwee, 2008). Our data support recent proposals that phytocannabinoids may represent important, but neglected, therapeutic agents (Mechoulam, 2005). It will be of interest in future studies to investigate how different phytocannabinoids may similarly modulate disease states in the CNS.

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Conflict of interest

The authors state no conflict of interest.

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