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Functional CB2 type cannabinoid receptors at CNS synapses

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ABSTRACT

To date, it has been thought that cannabinoid receptors in CNS are primarily of the CB1R subtype, with CB2R expressed only in glia and peripheral tissues. However, evidence for the expression of CB2 type cannabinoid receptors at neuronal sites in the CNS is building through anatomical localization of receptors and mRNA in neurons and behavioural studies of central effects of CB2R agonists. In the medial entorhinal area of the rat, we found that blockade of CB1R did not occlude suppression of GABAergic inhibition by the non-specific endogenous cannabinoid 2-AG, suggesting that CB1R could not account fully for the effects of 2-AG. Suppression could be mimicked using the CB2R agonist JWH-133 and reversed by the CB2R inverse agonist AM-630, indicating the presence of functional CB2R. When we reversed the order of drug application AM-630 blocked the effects of the CB2R agonist JWH-133, but not the CB1R inverse agonist LY320135. JTE-907, a CB2R inverse agonist structurally unrelated to AM-630 elicited increased GABAergic neurotransmission at picomolar concentrations. Analysis of mIPSCs revealed that CB2R effects were restricted to action potential dependent, but not action potential independent GABA release. These data provide pharmacological evidence for functional CB2R at CNS synapses.

1. Introduction

Cannabinoid receptors are a group of G-protein coupled receptors that mediate retrograde synaptic signalling via endogenous lipid molecules which together form the endocannabinoids. Since the original description of cannabinoid action at cholinergic terminals in guinea pig isolated ileum (Rosell and Agurell, 1975), a cannabinoid receptor, the CB1 receptor, was identified and cloned from rat brain (Devane et al., 1988; Matsuda et al., 1990). The existence of a second receptor subtype in rat spleen was reported by Munro et al. (1993), and these developments were complemented by identification of endogenous cannabinoid receptor ligands such as 2-arachidonyl glycerol (2-AG, Mechoulam et al., 1995) and anandamide (Devane et al., 1992).

In CNS, cannabinoids have been shown to exert powerful control over GABA release (Chan et al., 1998), reducing the amplitude of both inhibitory postsynaptic potentials (IPSPs; Szabo et al., 1998) and inhibitory postsynaptic currents (IPSCs; Hájos et al., 2000). Cannabinoids are believed to mediate the phenomenon of depolarisation-induced suppression of inhibition (DSI; Llano et al., 1991; Pitler and Alger, 1992; Alger et al., 1996), indicating a role in retrograde regulation of synaptic function.

While the abundance of CB1 receptors in CNS is undisputed, there has been a relative paucity of immunohistochemical evidence for CB2R in neuronal tissue (e.g. Schatz et al., 1996; Griffin et al., 1999), and this has led to the perception that expression of CB2 receptors is restricted to non-neuronal peripheral systems e.g. lymphocytes (Lynn and Herkenham, 1994). However, recent investigations have shown CB2R expression in neuronal populations. For example, CB2R have been reported in cultured sensory neurons (Ross et al., 2001), human sensory nerve fibres (Ständer et al., 2005) and rodent brain stem (Van Sickle et al., 2005). Gong et al. (2006) have demonstrated CB2R protein in rat brain using immunocytochemistry and shown that CB2R mRNA is also expressed in cerebellar neuronal elements. Similarly, Beltramo et al. (2006) have shown CB2R mRNA in spinal cord neurons. In both of the latter two studies, knockout technology has allowed the exclusion of CB1R mediated effects and confounding influences.

Recent developments in cannabinoid receptor pharmacology have delivered agonists, antagonists and inverse agonists at CB1R and CB2R that have nanomolar potency and a high degree of selectivity. Hence, the aim of the present study was to explore the possibility that functional CB2R exist in medial entorhinal cortex (mEC) using the newly developed, highly specific pharmacological agents. These studies were performed in the mEC, a part of the temporal lobe which shows a high degree of expression of cannabinoid receptors (Tsou et al., 1998; Moldrich and Wenger, 2000; Liu et al., 2003), and is well-placed to mediate cannabinoid effects on

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memory (see Pattij et al., 2008 for review) and emotion (for review see Viveros et al., 2007).

We have previously reported that CB1R modulate both spontaneous GABA release and neuronal network oscillations in mEC (Morgan et al., 2008). Here, we report that suppression of GABAergic spontaneous inhibitory postsynaptic current (sIPSC) amplitude in mEC shows a pharmacological profile indicating expression of functional CB2R.

2. Materials and methods

Combined EC-hippocampal slices were prepared from young male Wistar rats (50–110 g) as previously described (Woodhall et al., 2005). All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act 1986 and European Communities Council Directive 1986 (86/609/EEC). Rats were anaesthetized with isoflurane and N_2/O_2, until cardio-respiratory arrest, and decapitated. The brain was rapidly removed and immersed in oxygenated artificial cerebrospinal fluid (ACSF) chilled to 4 °C. Slices (450 μm) were cut using a vibrating microtome (MicroM, Germany), and stored in ACSF continuously bubbled with 95% O_2/5% CO_2, at room temperature. Following a recovery period of at least 1 h, individual slices were transferred to a recording chamber mounted on the stage of an Olympus (BX50WI) upright microscope. The chamber was continuously perfused with oxygenated ACSF at 30–32 °C, at a flow rate of approximately 2 ml/min. The ACSF contained the following (in mM): NaCl (126), KCl (3.25), NaH_2PO_4 (1.25), $NaHCO_3$ (24), $MgSO_4$ (2), $CaCl_2$ (2.5), and D-glucose (10). The solution was continuously bubbled with 95% O_2/5% CO_2 to maintain a pH of 7.4. Neurons were visualized using differential interference contrast optics and an infrared video camera (Hitachi).

Patch-clamp electrodes were pulled from borosilicate glass (1.2 mm OD, 0.69 ID; Harvard Apparatus), had open tip resistances of 2–4 MΩ, and were filled with a solution containing the following (in mM): CsCl (90), HEPES (33), QX-314 (5), EGTA (0.6), $MgCl_2$ (5.0), TEA-Cl (10), phosphocreatine (7) ATP (4), GTP (0.4). The solution was adjusted to 290 mOsmol with sucrose and to pH 7.4 with CsOH. IEM 1460 (1–3 mM) was added to the internal solution to block AMPA and NMDA receptors from inside the neuron. This method removed the need to use bath application of CNQX and 2-AP5, thus enhancing action potential dependent IPSCs, which show the greatest sensitivity to the actions of presynaptic cannabinoid receptors. Whole-cell voltage clamp recordings were made from neurons in layers II and V of the medial division of the EC, using an Axopatch 700 series amplifier (Molecular Devices, USA). The holding potential in all cases was −80 mV. Under these experimental conditions, layer II/V neurons exhibited sIPSCs, mediated by GABA acting at $GABA_A$ receptors.

Data were recorded directly to computer hard disk using AxoScope software (Molecular Devices, USA). MiniAnalysis (Synaptosoft, USA) was used for analysis of sIPSCs off-line. sIPSCs were detected automatically using a threshold-crossing algorithm, and their frequency and amplitude analysed. sIPSCs were sampled during a continuous recording period for each neuron under each condition. Differences between drug and control situations in studies of sIPSCs were assessed by means of a one-way ANOVA. All error values stated in the text refer to the S.E.M. Inhibitory charge transfer (ICT, measured in pA ms) is a measure of how much charge has crossed the membrane and this is representative of the amount of neurotransmitter that has been released. It can be calculated by measuring the area under the curve of an event (Hollrigel and Soltesz,, 1997) and is directly proportional to the amplitude multiplied by the decay time of an event. In our analysis we used normalised ICT (NICT) facilitating determination of changes in GABA release over time upon application of CB ligands.

All salts used in preparation of ACSF were Analar grade and purchased from Merck/BDH (UK). LY320135, AM-630, JWH-133 and JTE-907 were obtained from Tocris Cookson (UK).

3. Results

3.1. PSCs recorded using intracellular IEM 1640 are primarily GABAergic

Numerous previous studies have shown that CBR act primarily through voltage-gated calcium channels (VGCC) to inhibit the release of GABA (e.g. Wilson et al., 2001). In order to maximise the action potential (AP) dependent release of GABA, involvement of VGCC and therefore detect cannabinoid effects at presynaptic terminals reliably, we inhibited postsynaptic NMDA and AMPA receptors using the adamantane derivative IEM 1460 (1–3 mM), which was added to the internal recording solution just prior to whole-cell recording. Since the use of intracellular blockade of both AMPAR and NMDAR is a novel technique, we sought to underpin its utility and validity in isolating purely GABAergic postsynaptic currents. Fig. 1A and B shows the effects of the $GABA_A$ receptor antagonist picrotoxin (50 μM) on PSCs recorded in layer II in the presence of intracellular IEM 1460. As can be seen, $GABA_A$ receptor blockade reduces sIPSC frequency to near-zero. Further evidence that PSCs recorded in layer II using IEM 1460 consist mostly of $GABA_A$ receptor mediated inhibitory sIPSCs is the observation that 10–90% rise times and decay time constants are similar to values reported previously (Woodhall et al., 2005), when glutamate receptors were blocked. Fig. 1C shows scaled and superimposed PSCs (average of 10 in each case) recorded using either IEM 1460 or a combination of CNQX and 2-AP5. There is little difference in the kinetics of the events and we conclude that both are GABAergic IPSCs. Table 1 shows kinetic values for layer II sIPSCs measured in the presence of either IEM 1460 or CNQX/2-AP5. As can be seen, sIPSCs recorded in IEM 1460 have comparatively large amplitude and lower IEI with respect to those recorded in CNQX/2-AP5, consistent with their greater AP-dependent origin, but rise times, decay time and area values are consistent. By contrast, excitatory events in layer II have much more rapid decay (3–5 ms, Berretta and Jones, 1996; Woodhall et al., 2001) and IEI on the order of 1000 ms. Hence, while we cannot claim to have reduced the presence of EPSCs to zero in all recordings (since we did not use PTX in every case), it is reasonable to infer that the great majority of PSCs are IPSCs mediated by $GABA_A$ receptors.

3.2. An antagonist at CB1R does not fully occlude the actions of 2-AG

During recordings of sIPSCs in pyramidal neurons located in layer V of the medial EC, we applied the CB1R selective inverse agonist LY320135, at 500 nM. This concentration is approximately 3 times its reported K_i value of 141 nM at CB1R, but well below that for CB2R (K_i > 14,900 nM; and below the reported threshold (2.1 μM)) for muscarinic receptor action (Felder et al., 1998), hence, CB1R would be subject to near-maximal antagonism/inverse agonism within the constraints imposed by selectivity. As the raw traces in Fig. 2A show, at 500 nM, LY320135 caused an increase in the amplitude of sIPSCs in layer V. When we calculated normalised inhibitory charge transfer (NICT, Fig. 2C) this increased by 133.9 ± 67.6% ($P < 0.01$, ANOVA, $n = 6$), an effect mediated primarily by increased sIPSC amplitude from mean 42.1 ± 3.1 to 79.5 ± 7.3 pA (Fig. 2B; $P \leq 0.003$, ANOVA, $n = 6$), which was not related to alterations in sIPSC frequency since mean inter-event interval (IEI) was not significantly altered (448.7 ± 75.7 ms in control to 325.9 ± 67.1 in LY320135; $P = 0.9$, ANOVA, $n = 6$).

In the continued presence of LY320135 we then co-applied the non-selective endocannabinoid, 2-AG, at a concentration (500 nM) close to its reported K_i value of 472 nM for CB1R (Mechoulam et al., 1995). Under these conditions, we observed a decrease in sIPSC amplitude (Fig. 2A) in all recordings, and NICT decreased −130 ± 26.1% compared to the LY320135 condition, with mean peak sIPSC amplitude also decreased from 79.5 ± 7.3 pA to 45.2 ± 3.2 pA ($P \leq 0.05$ ANOVA, $n = 6$). Hence, blockade of CB1R using LY320135 did not occlude the actions of 2-AG on sIPSCs recorded in the deep mEC *in vitro*. We subsequently added a CB2R-selective antagonist, AM-630, at 50 nM, to the bath already containing LY320135 and 2-AG. As the raw traces in Fig. 2A show, after 20 min application of AM-630, there was a significant increase in sIPSC amplitude from 45.2 ± 3.2 pA to 82.3 ± 4.0 pA ($P \leq 0.01$ ANOVA, $n = 6$), whilst NICT increased by 80.5 ± 52.3% of control ($P \leq 0.02$ ANOVA, $n = 6$). These data indicated that whilst LY320135 did not block the effects of 2-AG, the CB2R antagonist AM-630, at low concentration, was able to reverse the residual effects of 2-AG in the presence of LY320135.

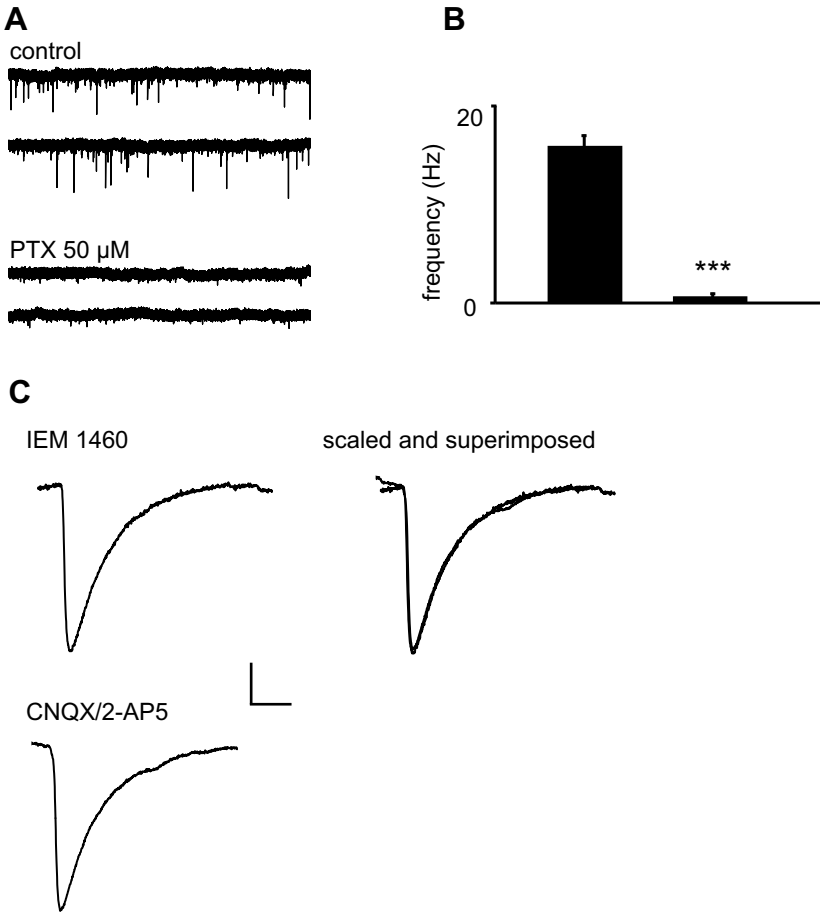

Fig. 1. PSCs recorded using intracellular IEM 1460 are GABAergic. A, Raw traces showing that PSCs are abolished by addition of picrotoxin (50 μM). B, Pooled data from 8 recordings indicating that PSC frequency is reduced to near-zero. C, Averaged PSCs from 10 events randomly selected from recordings using either IEM 1460 or CNQX+2-AP5 (scale bar 200 pA × 10 ms upper trace, 300 pA × 10 ms lower trace). Scaled and overlaid traces show very similar rise and decay kinetics.

We attempted a similar series of experiments in the superficial layers of the mEC (layer II), which has a fundamentally different profile of GABAergic inhibition in comparison to layer V, being much less dependent on action potential driven release (Woodhall et al., 2005). Here, application of LY320135 at 500 nM (Fig. 3A–C) induced a relatively small increase in mean sIPSC amplitude from 59.3 ± 3.1 pA to 67.4 ± 4.87 pA, and this did not reach statistical significance (NS, ANOVA, $n = 6$). However, whilst amplitude effects alone were subtle, when we analysed inhibitory charge transfer, a robust increase by 76.5 ± 21% of control value was revealed ($P \leq 0.005$ ANOVA, $n = 6$). When we repeated the pattern of experiments performed previously, we found that once again, 2-AG was able to depress mean sIPSC amplitude from 67.4 ± 4.8 pA to 47.1 ± 1.3 pA ($P \leq 0.03$ ANOVA, $n = 6$), and NICT decreased by −20.1 ± 16.4% compared to control (net change −96.6% compared to LY320135 alone). When we compared NICT between LY320135 and 2-AG periods, this decrease was highly significant ($P \leq 0.005$ ANOVA, $n = 6$). As was the case in layer V, application of AM-630 at 50 nM reversed the effects of 2-AG (Fig. 3A–C), such that mean sIPSC amplitude increased from 47.1 ± 1.3 pA to 67.4 ± 4.3 pA ($P \leq 0.005$ ANOVA, $n = 6$), whilst NICT increased by 89.5 ± 27.8% of control value, and net change of +109% compared to the previous drug condition ($P \leq 0.007$ ANOVA, $n = 6$). Again, these data indicated that the depression of GABAergic neurotransmission by 2-AG which was not blocked by LY320135 could be reversed by a CB2R antagonist. It was notable that when CB1 and CB2 inverse agonists were co-applied, 'bursts' of sIPSCs were often seen (e.g. Figs. 3 and 6). These bursts were not evident when a single CBR ligand was applied.

Table 1
Kinetics of PSCs recorded under different conditions.

	Amplitude (pA)	Rise time (ms)	Tau-decay (ms)	Area (pA ms)	IEI (ms)
+IEM 1640	184.8 ± 7.5	2.9 ± 0.04	15.5 ± 0.46	371 ± 10	63.2 ± 1.7
CNQX+2AP-5	60.8 ± 1.7	3.75 ± 0.08	13.3 ± 0.33	354 ± 15	90.2 ± 2.2

3.3. An agonist at CB2R mimics the actions of 2-AG

We repeated the previous experimental paradigm, substituting the CB2R-specific agonist JWH-133 in place of 2-AG. JWH-133, which has a K_i for CB2R and CB1R of 3.4 and 677 nM respectively (Huffman et al., 1999; for review see Marriott and Huffman, 2008), and is reported to be much more potent at inhibiting cAMP accumulation at CB2R compared to CB1R (Pertwee, 2000). We recorded sIPSCs in layer V of the mEC and applied LY320135 at 500 nM, again observing (Fig. 4A–C) a doubling of sIPSC amplitude (control mean amplitude in control 47.0 ± 3.1 pA increasing to 87.9 ± 7.4 pA in LY320135; $P \leq 0.0001$ ANOVA, $n = 6$) and a profound increase in inhibitory charge transfer (407 ± 170%; $P \leq 0.03$ ANOVA, $n = 6$). When we added JHW-133 (50 nM), we noted a similar response to that seen with 2-AG, such that mean sIPSC amplitude decreased from 87.9 ± 7.4 pA in LY320135 to

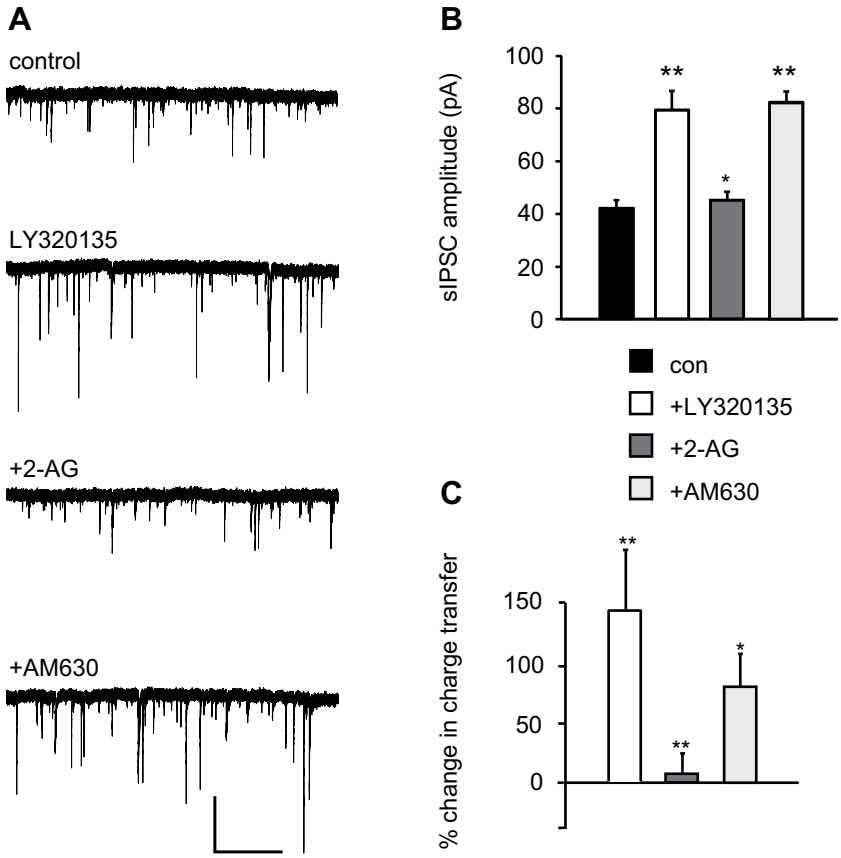

Fig. 2. Blockade of CB1R does not occlude the actions of the endogenous cannabinoid 2-AG in layer V of the mEC. A, Representative data showing the effect of cannabinoid ligands on sIPSCs recorded in layer V of the mEC. sIPSC amplitude was increased by initial application of the CB1R inverse agonist LY320135 (500 μM), but this did not prevent reduction in sIPSC amplitude by 2-AG (500 μM). Subsequent application of the CB2R-selective inverse agonist AM-630 (50 nM) reversed the effects of 2-AG on sIPSC amplitude. Scale bar 80 pA × 2 s. B, Summary bar graph showing sIPSC amplitude during each period of drug application. C, Summary bar graph showing normalised inhibitory charge transfer during each period of drug application.

38.1 ± 2.4 pA in LY320135 + JWH-133 ($P \leq 0.0001$ ANOVA, $n = 7$) and NICT decreased by −19.7 ± 31.5% of the control value ($P \leq 0.03$ ANOVA, $n = 7$), a net decrease of 425% compared to LY320135. Once again, we applied the CB2R-selective antagonist, AM-630 (50 nM) and sIPSC amplitude was increased from 38.1 ± 2.4 pA to 80.0 ± 7.2 pA ($P \leq 0.0001$ ANOVA, $n = 7$), with NICT increasing by 172 ± 83.1% of control ($P \leq 0.04$ ANOVA, $n = 7$).

When we repeated these experiments in layer II of the mEC, we observed a similar pattern of drug effects (Fig. 5A–C), such that LY320135 increased mean sIPSC amplitude from control (51.7 ± 3.6 pA to 69.0 ± 5.9 pA in LY320135; $P \leq 0.01$ ANOVA, $n = 6$) and NICT increased by 62.5 ± 27.1% of control ($P \leq 0.05$ ANOVA, $n = 6$). Again, JWH-133 depressed mean sIPSC amplitude from 69.0 ± 5.9 pA in LY320135 to 44.9 ± 1.4 pA in LY320135 + JWH-133, and NICT was reduced by −27.6 ± 9.5% compared to control ($P \leq 0.025$ ANOVA, $n = 6$; net decrease 89% compared to LY320135). Once again, AM-630 reversed the effects of JWH-133, increasing amplitude from 44.9 ± 1.4 pA in LY320135 + JWH-133 to 72.5 ± 4.7 pA in LY320135 + JWH-133 + AM-630 ($P \leq 0.0001$ ANOVA, $n = 6$), with NICT increasing by 108.6 ± 50.1% with respect to control (net increase 136% compared to previous condition; $P \leq 0.025$ ANOVA, $n = 6$).

These experiments suggested that a non-CB1 type cannabinoid receptor could be activated by application of an endogenous agonist, or by a synthetic agonist showing a high degree of selectivity for CB2R. In both cases, in the presence of the CB1R antagonist, LY320135, application of the agonist depressed GABAergic inhibition, and this was reversible on application of the selective CB2R inverse agonist, AM-630. To control for possible non-specific effects related to the order in which we applied cannabinoid receptor ligands, we next attempted to block the effects of the CB2R-specific agonist JWH-133, by prior application of AM-630, reversing the order of drugs applied in previous experiments.

3.4. An antagonist at CB2R occludes the actions of JWH-133, but not LY320135

We recorded from a further two groups of neurons in layers V and II of the mEC, as previously described, but applying CBR ligands in reverse order to the experiments described so far. We applied AM-630 at 100 nM, prior to addition of JWH-133 (50 nM) to the recording chamber (in the continued presence of AM-630). Under these conditions, as Fig. 6A–C shows, in layer V of the mEC we noted that the initial application of AM-630 alone caused an increase in sIPSC amplitude from a baseline of mean 47.3 ± 2.7 pA to 64.1 ± 4.5 pA ($P \leq 0.04$, ANOVA, $n = 9$). When we subsequently applied JWH-133 (50 nM), amplitude was not significantly altered (59.9 ± 11.7 pA; $P = 0.98$ compared to AM-630 alone, ANOVA, $n = 9$). Further addition of LY320135 increased mean sIPSC amplitude from 59.9 ± 11.7 pA to 73.1 ± 4.2 pA, and in this case, the increase was significant compared to the previous condition ($P \leq 0.006$, ANOVA, $n = 9$). We obtained similar results when we analysed NICT values, which indicated that charge transfer was not suppressed by JWH-133 in the presence of AM-630. Here, AM-630

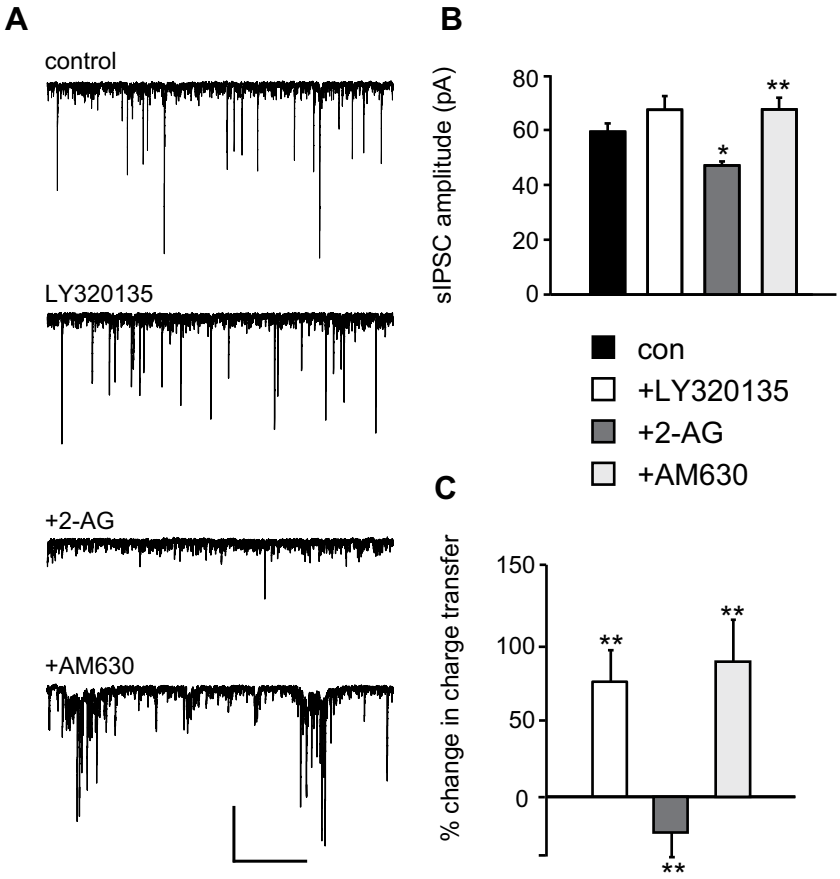

Fig. 3. Blockade of CB1R does not occlude the actions of the endogenous cannabinoid 2-AG in layer II of the mEC. A, Representative data showing the effect of cannabinoid ligands on sIPSCs recorded in layer II of the mEC. sIPSC amplitude was increased by initial application of the CB1R inverse agonist LY320135 (500 μM), but this did not prevent reduction in sIPSC amplitude by 2-AG (500 μM). Subsequent application of the CB2R-selective inverse agonist AM-630 (50 nM) reversed the effects of 2-AG on sIPSC amplitude. Scale bar 400 pA × 2 s. B, Summary bar graph showing sIPSC amplitude during each period of drug application. C, Summary bar graph showing normalised inhibitory charge transfer during each period of drug application.

alone increased charge transfer by 58.9 ± 12.44% ($P \leq 0.003$, ANOVA, $n = 9$), and this value continued to increase in the presence of JWH-133 (by 118.4 ± 30.4% of control) and LY320135 (227.4 ± 124.8% of control). Changes in NICT were not significant between JHW-133 and LY320135 conditions, although they were between AM-630 and JWH-133 ($P \leq 0.01$, ANOVA, $n = 9$). These data indicated that JWH-133 did not reduce GABAergic neurotransmission in the presence of AM-630, and that LY320135 was still able to increase GABAergic sIPSC amplitude in the presence of AM-630. When we repeated these experiments in layer II of the mEC, we observed a similar pattern of effects. Hence, AM-630 alone increased (Fig. 7) the amplitude of sIPSCs from mean 43.8 ± 1.7 pA to 57.4 ± 3.0 pA ($P \leq 0.003$, ANOVA, $n = 5$), but JWH-133 failed to significantly depress sIPSC amplitude (mean 58.9 ± 4.5 pA in JWH-133; NS, ANOVA, $n = 5$). When LY320135 was subsequently applied, a significant increase in amplitude (to mean 78.4 ± 3.0 pA) was again observed ($P \leq 0.003$, ANOVA, $n = 5$). In both layer II and layer V, the degree of occlusion of the effects of JWH-133 by AM-630 was dose-dependent, being fully apparent at 100 nM AM-630, and partial at 50 nM (data not shown). When we analysed NICT values, we found that AM-630 alone increased charge transfer by 34.2 ± 16.1% of control, and this was just significant ($P \leq 0.043$, ANOVA $n = 4$). Subsequent addition of JWH-133 had no significant effect (NICT 57.26 ± 17.5%, NS $n = 4$), however, addition of LY320135 did significantly increase NICT (to 105.8 ± 25.8% of control, $P \leq 0.029$, $n = 4$).

The effects of AM-630 described above indicate a dose-dependent blockade of the effects of a specific CB2R agonist on the amplitude of sIPSCs recorded in both deep and superficial layers of the mEC. Any response obtained by use of CB2R ligands in our experiments could come from non-specific effects of such ligands at non-cannabinoid receptors. We sought to minimise these possibilities through careful choice of drug concentrations such that interactions would be limited, and through use of different exogenous and endogenous ligands. We also altered the order of drug application. To further challenge our evidence for CB2R-mediated responses in the mEC, we applied a structurally novel ligand, JTE-907, which bears little similarity to AM-630, and hence might be less likely to have a similar profile of effects at non-cannabinoid receptors. Given the potency of this particular ligand, we bath applied JTE-907 at concentrations ranging from 500 pM to 1 nM, again minimising the potential for cross-reactions with non-cannabinoid receptors.

3.5. JTE-907 a CB2R antagonist structurally dissimilar to AM-630, enhances GABA transmission at picomolar concentrations

The quinolinecarboxamide compound, JTE-907, is reported to have high selectivity for CB2R ($K_i = 0.38$ nM at rat CB2R; Iwamura et al., 2001). As shown in Fig. 8A–C, when we applied JTE-907 at 500 pM in layer V, a robust increase in the amplitude and apparent frequency of sIPSC was observed. In fact, in layer V mean control sIPSC

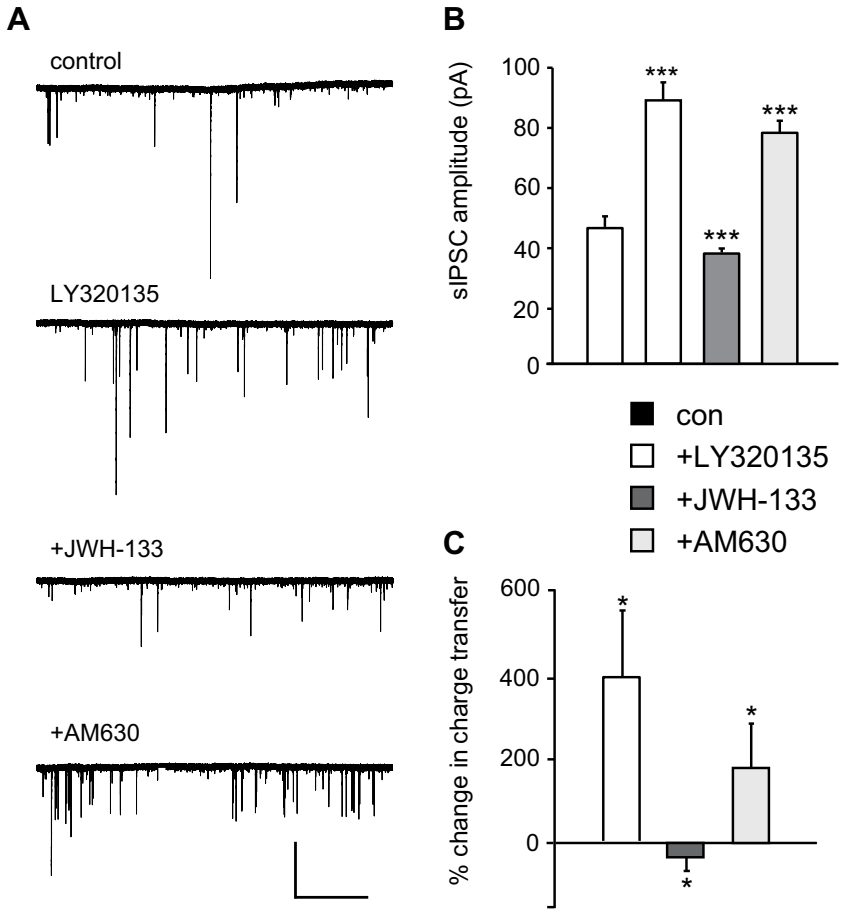

Fig. 4. A CB2R-selective agonist, JWH-133, mimics the effects of 2-AG in layer V of the mEC. A, Representative data showing the effect of cannabinoid ligands on sIPSCs recorded in layer V of the mEC. sIPSC amplitude was increased by initial application of the CB1R inverse agonist LY320135 (500 μM), but this did not prevent reduction in sIPSC amplitude by JWH-133 (50 nM). Subsequent application of the CB2R-selective inverse agonist AM-630 (50 nM) reversed the effects of JWH-133 on sIPSC amplitude. Scale bar 300 pA × 2 s. B, Summary bar graph showing sIPSC amplitude during each period of drug application. C, Summary bar graph showing normalised inhibitory charge transfer during each period of drug application.

amplitude essentially doubled from 86.1 ± 13.6 pA to 156.1 ± 20.7 pA ($P \leq 0.0001$, ANOVA, $n = 6$), and charge transfer increase by 83.4 ± 21.7% of control ($P \leq 0.003$, ANOVA, $n = 6$). Similar experiments in layer II provided significant changes (control sIPSC amplitude 61.8 ± 4.0 pA, JTE-907 86.7 ± 6.7 pA ($P \leq 0.01$, *t*-Test, $n = 6$)). Similarly, charge transfer was increased in JTE-907 (by 119.8 ± 70.2%) although this was of similar magnitude to the change seen in layer V, it was not significant, presumably due the high variance in NICT values in this layer. The similarity of these data to those observed with AM-630 indicates strongly that a non-CB1R response could be elicited at synapses in both deep and superficial mEC.

3.6. Effects of CB ligands on kinetics of sIPSCs

The location of putative CB2R effects at either pre- or postsynaptic elements is a matter of importance in consideration of any functional role for CB2R. As changes in sIPSC frequency were inconsistent across recordings, we analysed the decay time constants of sIPSCs in the presence of CB1R and CB2R ligands in the experiments reported above since a postsynaptic action might be revealed in altered decay kinetics. Data from a total of >15,000 IPSCs revealed no significant change in decay time constant (tau) when LY320135 was applied following control or a previous drug (or drugs). For example, in layer II, a drug-naïve control tau value of 10.9 ± 0.4 ms was seen, and this remained unchanged at 10.1 ± 0.3 ms in LY320135 (92.7 ± 2.7% of control, $P > 0.05$, *t*-Test, $n = 6$ recordings). However, AM-630 showed a small, but consistent, increase in tau values (by 14.6 ± 2.5% compared to control), and this was statistically significant ($P < 0.05$, *t*-Test). Similar effects were seen in layer V, but LY320135 had no such effect (4.3 ± 6.7% in LY320135, NS, $n = 6$ recordings). In support of a unique role for putative CB2R in affecting decay times, JTE-907 increased tau values in both layer II (by 16.7 ± 4.3%, $P < 0.05$, *t*-Test, $n = 6$) and layer V (by 20.2 ± 5.0% $P < 0.05$, *t*-Test, $n = 6$).

If changes in sIPSC decay times reflected a postsynaptic locus for the effects of CB2R, this should be visible in recordings of miniature IPSCs (mIPSCs) made in the presence of the voltage-gated sodium channel inhibitor, tetrodotoxin (TTX). In layer II recordings, we added TTX (1 μM) to the bath 15–30 min prior to addition of a high concentration of AM-630 (100 nM). In pooled data from 3200 mIPSCs from 8 recordings (Fig. 9A and B), we found no effect of AM-630 on mIPSC frequency (control mean IEI 107.1 ± 3.5 ms versus 102.8 ± 3.2 ms) or charge transfer (102.3 ± 3.3% of control in AM-630). Rise and decay times were not significantly different (99.6 ± 1.2% and 98.1 ± 1.8% of control in AM-630 respectively), and although amplitude was slightly reduced after bath application of AM-630 (control mean amplitude 41.7 ± 0.7 pA versus 39.3 ± 0.5 pA in AM-630) this proved to be insignificant using a *t*-Test ($P = 0.06$). Similarly, the non-parametric Kolmogorov–Smirnov test revealed no significant difference in the distribution of mIPSC amplitudes between the two conditions ($P = 0.2$, $Z = 1.06$, $n = 3200$). We also applied the CB2R agonist JWH-133 at 100 nM

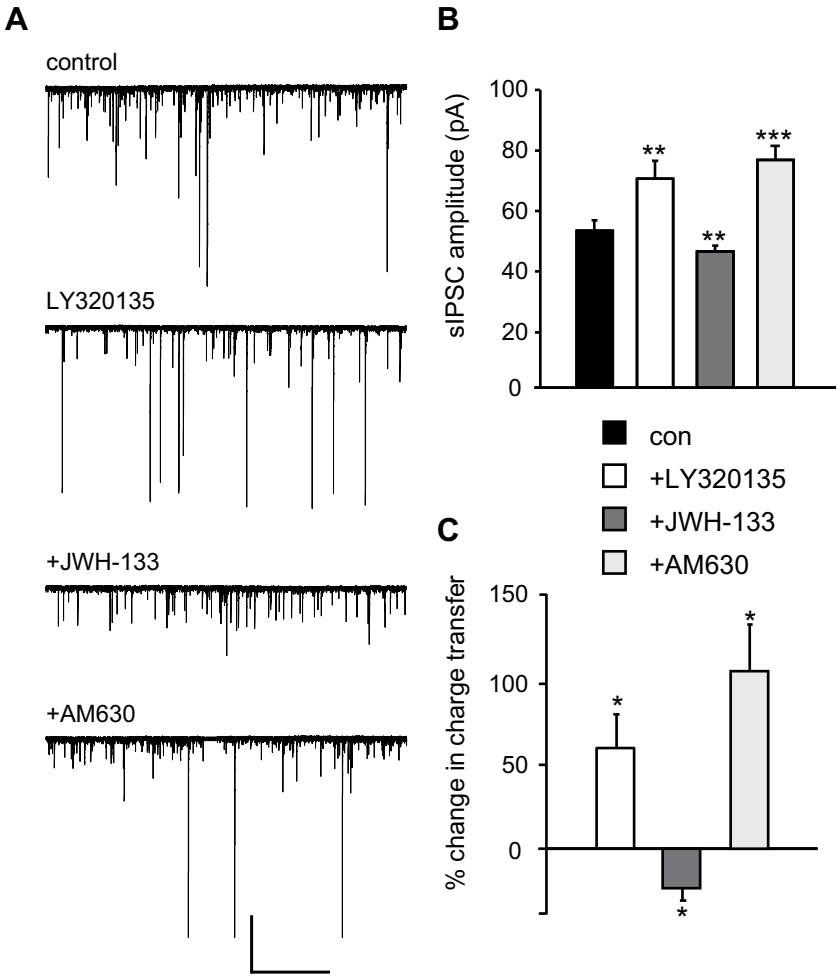

Fig. 5. A CB2R-selective agonist, JWH-133, mimics the effects of 2-AG in layer II of the mEC. A, Representative data showing the effect of cannabinoid ligands on sIPSCs recorded in layer II of the mEC. sIPSC amplitude was increased by initial application of the CB1R inverse agonist LY320135 (500 μM), but this did not prevent reduction in sIPSC amplitude by JWH-133 (50 nM). Subsequent application of the CB2R-selective inverse agonist AM-630 (50 nM) reversed the effects of JWH-133 on sIPSC amplitude. Scale bar 200 pA × 2 s. B, Summary bar graph showing sIPSC amplitude during each period of drug application. C, Summary bar graph showing normalised inhibitory charge transfer during each period of drug application.

and 500 nM in 5 recordings from different slices (Fig. 9C and D). We found no significant difference ($P = 0.7$, ANOVA, $n = 5$) in mean mIPSC amplitude ($n = 2000$ mIPSCs) between control (34.3 ± 0.51 pA) and either 100 nM JWH-133 (33.5 ± 0.6 pA) or 500 nM JWH-133 (35.9 ± 0.6 pA). Similarly, no significant differences ($P = 0.34$, ANOVA, $n = 5$) were found for IEI values (control 113.1 ± 3.5 ms versus 123.6 ± 4.7 ms in 100 nM JWH-133 and 124.2 ± 4.2 ms in 500 nM JWH-133). We observed no effects on rise or decay times in these experiments (mean rise times 95.4 ± 2.7% and 97.5 ± 2.6%) of control in 100 nM and 500 nM JWH-133 respectively ($n = 5$, ANOVA NS), mean decay times 98.8 ± 1.9% and 101.3 ± 1.9% of control in 100 nM and 500 nM JWH-133 respectively. Taken together, these data indicate that there is no effect of CB2R agonists and antagonists on action potential independent GABA release, suggesting that any CB2R-mediated change in decay times is restricted purely to AP-dependent sIPSCs.

4. Discussion

Our data indicate that CB2R-like response can be found in both layers II and V of the mEC, using a variety of specific and selective ligands, and as such, this is the first report of CB2R-mediated effects on inhibitory synaptic function in the CNS.

When we applied a non-specific CBR agonist or a selective agonist at CB2R in the presence of LY320135, we observed suppression of GABAergic inhibitory signalling, even whilst CB1R were blocked. Application of 2-AG, in the presence of the CB1R antagonist LY320135 should have had little or no effect on layer II or V sIPSCs and inhibitory charge transfer. In the current experiments, application of 2-AG to the bath in addition to LY320135 decreased inhibitory charge transfer in both layer II and V of the mEC. As 2-AG was added at a concentration just above the K_i for CB1Rs, and LY320135 was present at 3× its K_i value, it seems unlikely that 2-AG was out-competing LY320135 at the CB1R itself. We hypothesised that 2-AG was acting at CB2 type receptors, and this seems to be supported by our data showing that in both layers II and V, the CB2R-specific agonist JWH-133 mimicked the effects of 2-AG, and in both cases, this was reversed by the CB2R-specific antagonist AM-630. When we reversed the experiments, using AM-630 alone, we saw significant increases in GABAergic activity. Furthermore, when AM-630 was applied at 100 nM in layers II and V, subsequent co-application of JWH-133 (50 nM) failed to suppress GABAergic signalling. The fact that the effects of JWH-133 could be blocked by prior application of AM-630 suggests that the two cannabinoid ligands are acting at the same site, rather than having non-specific effects. In addition to these data, a structurally unrelated, selective

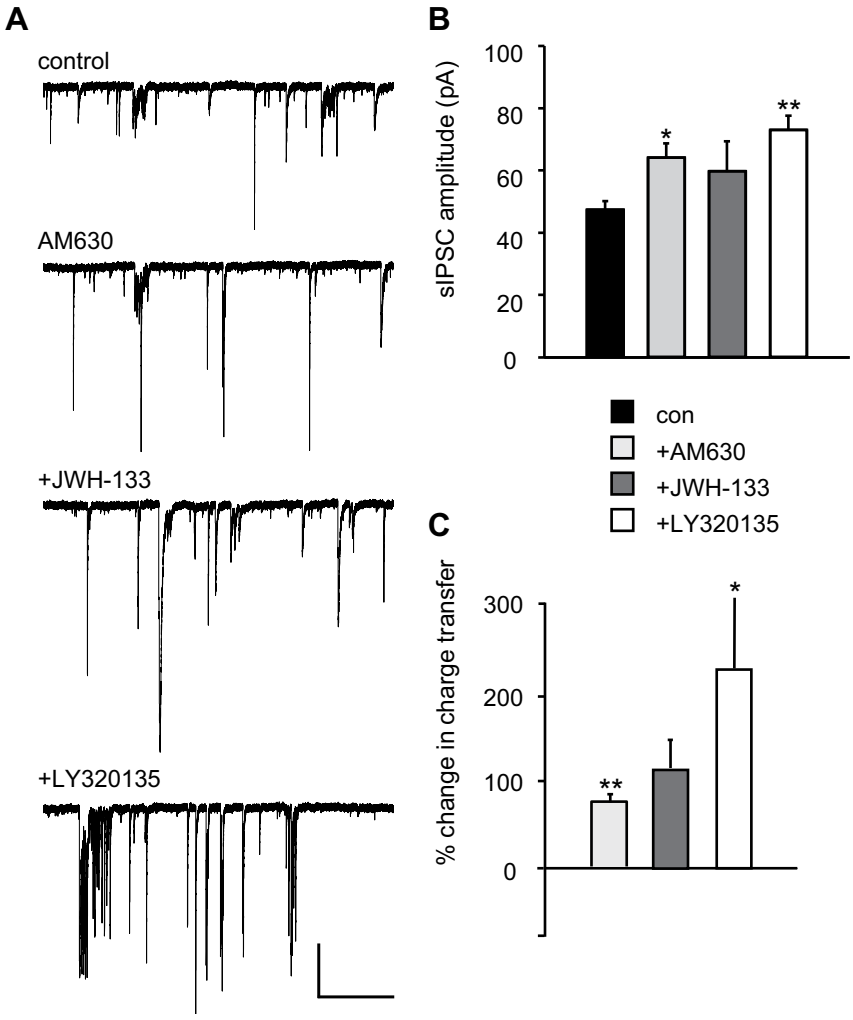

Fig. 6. Pre-application of AM-630 blocks the effects of JWH-133, but not LY320135 in layer V of the mEC. A, Representative data showing the effect of cannabinoid ligands on sIPSCs recorded in layer V of the mEC. sIPSC amplitude was increased by initial application of the CB2R inverse agonist AM-630 (50 nM), and this blocked the reduction in sIPSC amplitude by JWH-133 (50 nM). Subsequent application of the CB1R selective inverse agonist LY320135 (500 μM) increased sIPSC amplitude. Scale bar 500 pA × 2 s. B, Summary bar graph showing sIPSC amplitude during each period of drug application. C, Summary bar graph showing normalised inhibitory charge transfer during each period of drug application.

CB2R antagonist/inverse agonist, JTE-907, also increased GABAergic signalling in layers II and V. The specificity of AM-630 and JTE-907 for CB2R has yet to be comprehensively investigated at the vast array of native receptor types in rat brain, and so it is possible, though unlikely, that the two structurally unrelated drugs have similar effects on GABAergic inhibition through non-cannabinoid receptor mechanisms. However, to date no studies appear to have identified non-specific effects at the nanomolar concentrations used here. It is also notable that JTE-907 is most potent and selective for rat CB2R, at K_i 0.38 nM as compared to human (K_i 35 nM) or mouse (K_i 1.55 nM) CB2R expressed on CHO cells (Iwamura et al., 2001). In the case of AM-630, antagonist properties at brain CBR have clearly been demonstrated (Hosohata et al., 1997). Previously, Hajos et al. (2001) showed that the CB1R antagonist SR141716A blocked the effects of WIN 55,212-2 while Hentges et al. (2005) showed that the CB_1R antagonist AM-251 blocked the effects of continuously released endocannabinoids in hypothalamic propiomelanocortin (POMC) neurons, and Kreitzer and Regehr (2001) showed AM-251 blocked DSI. These data indicate that in some situations, CB1R antagonists can completely block the effects of exogenous and endogenous cannabinoid receptor activation. It is possible that in our study, the effects of 2-AG and JWH-133 were due to actions at non-receptor sites. 2-AG, along with other endogenous cannabinoids such as AEA, has been shown to have a range of non-CBR specific effects. For example, in addition to interacting with CB1R and CB2Rs it has been shown endogenous cannabinoids can modulate properties of voltage-gated ion channels such as calcium, sodium and potassium channels. In addition to this, endogenous cannabinoids have also been shown to interact with ligand-gated ion channels such as nicotinic acetylcholine receptors, glycine receptors and ionotropic glutamate receptors (for a review of non-CBR effects of the endogenous cannabinoids see Oz, 2006). However, non-CBR effects have not been clearly demonstrated for synthetic cannabinoids, and given that the effects we have described are repeatable using structurally dissimilar agents, and are reversible using specific antagonists, it seems unlikely that non-receptor effects can account fully for our data.

Apart from non-specific affects of endogenous cannabinoids another potential site of action for 2-AG is a putative CB3 cannabinoid receptor. Hajos et al. (2001) and Breivogel et al. (2001) have reported evidence in the brain for a non-CB1, non-CB2 cannabinoid receptor using CB1R knockout mice and immunocytochemistry for

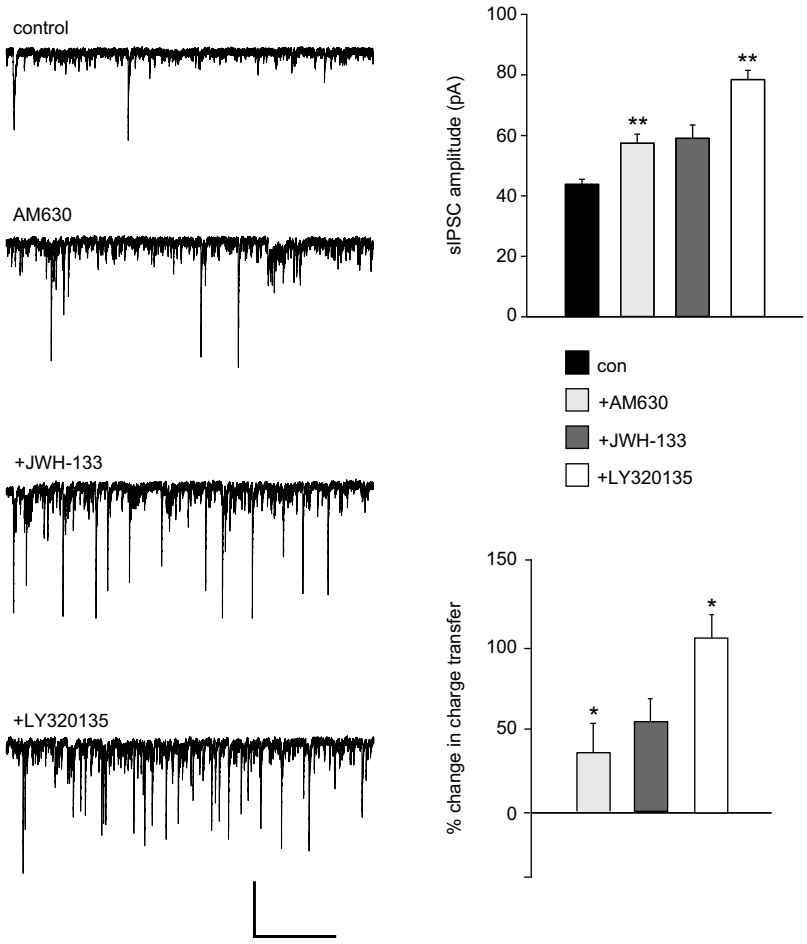

Fig. 7. Pre-application of AM-630 blocks the effects of JWH-133, but not LY320135 in layer II of the mEC. A, Representative data showing the effect of cannabinoid ligands on sIPSCs recorded in layer V of the mEC. sIPSC amplitude was increased by initial application of the CB2R inverse agonist AM-630 (50 nM), and this blocked the reduction in sIPSC amplitude by JWH-133 (50 nM). Subsequent application of the CB1R selective inverse agonist LY320135 (500 μM) increased sIPSC amplitude. Scale bar layer V 100 pA × 2 s; layer II 400 pA × 2 s. B, Summary bar graph showing sIPSC amplitude during each period of drug application. C, Summary bar graph showing normalised inhibitory charge transfer during each period of drug application.

CBRs. More recently, the orphan receptor GPR55 has been proposed as a candidate novel cannabinoid receptor (Baker et al., 2006; Ryberg et al., 2007). Interestingly while GPR55 showed binding and responses to some cannabinoids it did not respond to WIN 55,212-2 and when the response of GPR55 to AM-251 was tested, it was found to behave as an agonist. Hence while Ryberg et al. (2007) have indicated that GPR55 is novel cannabinoid receptor, it is unlikely to be responsible for the effects we see in these experiments, although GPR55 does respond to 2-AG there is no evidence to suggest that it is also capable of being activated by the CB2R-specific ligands JWH-133 and AM-630, or that it has other pharmacological properties in common with the CB2R. Further evidence to support the argument that the ligands used in these experiments were acting at the CB2 receptor is the low concentrations of the receptor-specific ligands used, most notably 500 pM-1 nM JTE-907, which has 2760-fold selectivity for rat CB2R over rat CB1R (K_i value of 1050 nM at CB1R versus 0.38 nM at CB2R, Iwamura et al., 2001).

The presence of CB2Rs within the immune system is well documented with CB2R mRNA being found in spleen and bone marrow; CB2Rs are also expressed by many immune specific cells (see Cabral and Dove Pettit, 1998 for review). However, the presence of CB2R in the CNS has been subject to debate. While various immunohistochemical and autoradiographical studies (such as the work of Tsou et al., 1998; Glass et al., 1997) have shown the presence of CB1Rs in the CNS, no such studies showing CB2Rs in the CNS existed, moreover researchers such as Schatz et al. (1996) and Griffin et al. (1999) have been unable to show the presence of CB2Rs in the CNS, and thus it was concluded that these receptors were not expressed in neuronal tissue. In more recent years the development of more specific CB2R antibodies has led to the identification of functional CB2R on neurons of the brain stem (Van Sickle et al., 2005). In 2006, Gong et al. produced immunohistochemical evidence for CB2R expression in the rat brain, using a combination of RT-PCR and immunohistochemical techniques. These researchers showed that not only is CB2R mRNA in the rat brain but that CB2Rs are expressed on neuron cell bodies and processes through out the brain. Onaivi et al. (2006), showed that *in vivo* the CB2R agonist JWH-015 caused a decrease in locomotor activity in mice, suggesting a functional role for CB2Rs in CNS. CB2Rs have also been shown to be expressed on microglia and astroglia cells in Down's syndrome (Núñez et al., 2008) and in disease states such as Alzheimer's disease (Benito et al., 2003) where glial cells associated with neuritic plaques express CB2Rs. CB2Rs have also been shown to be expressed in certain types of brain tumour (Miklaszewska et al., 2007). Furthermore, the recent report by Suárez et al. (2008) has demonstrated the full complement of the known elements of the endocannabinoid system exists in

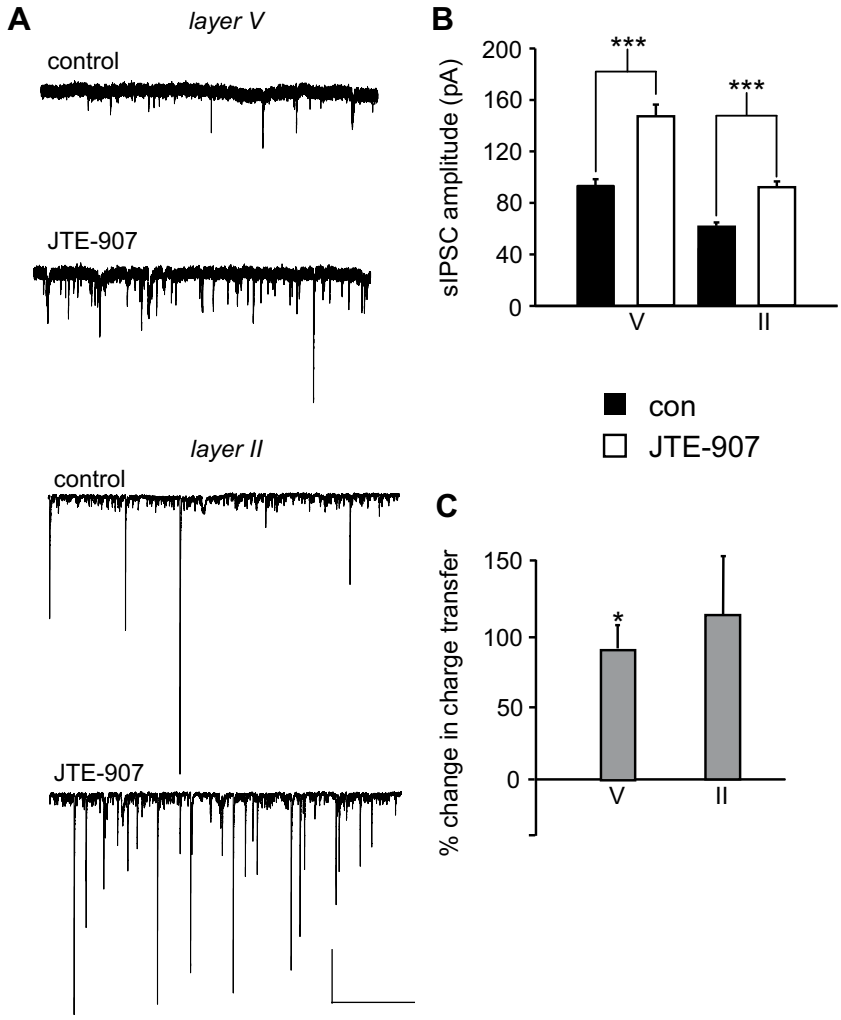

Fig. 8. JTE-907, a CB2R inverse agonist structurally unrelated to AM-630, enhances sIPSC amplitude in layers II and V of the mEC at picomolar concentrations. A, Representative data showing the effect of JTE-907 on sIPSCs recorded in layers II and V of the mEC. sIPSC amplitude was increased by JTE-907. B, Summary bar graph showing sIPSC amplitude during each period of drug application. C, Summary bar graph showing normalised inhibitory charge transfer during each period of drug application.

cerebellum, CB2R, and the enzymes involved in synthesis (DAGLα, DAGLβ, NAPE-PLD) and degradation (FAAH and MAGL) of endocannabinoids. The strong evidence provided by Suárez et al. (2008) also described specific distribution of, for example, CB2R in Pinceau formations and parallel fibres, and used Western blot analysis to detect CB1R and CB2R protein. Most recently, Brusco et al. (2008) have provided clear ultrastructural evidence that CB2R are present at hippocampal synapses, and have suggested that they are located postsynaptically. Finally, there is clear evidence that the endocannabinoid 2-AG is produced in the CNS in response to neuronal activity (Maejima et al., 2005) and it has been argued that 2-AG is the main endogenous ligand for the CB2R (Sugiura et al., 1999, 2000).

It is pertinent to explore why CB2R responses may not have been observed in previous experiments using CBR ligands in CNS. In this regard, it is notable that the most often used agonist, WIN 55,212-2, has poor selectivity for CB1R (K_i at rat CB1R = 0.14 ± 0.07 μM and at CB2R = 1.30 ± 0.33 μM; Iwamura et al., 2001). Similarly, whilst some CB1R antagonists have almost no activity at peripheral CB2R (e.g. SR141716A; Rinaldi-Carmona et al., 1995), other commonly used agents such as AM-251 are active at CB2R (e.g. New and Wong, 2003).

It is relevant to consider why two subtypes of CBR might be expressed in CNS and how this may related to function. Clearly, there is abundant evidence that CB1R are present at postsynaptic sites in CNS and that they mediate transient, presynaptic suppression of inhibition via depolarisation-induced suppression of inhibition (DSI; Llano et al., 1991; Pitler and Alger, 1992; Wilson and Nicoll, 2001; Freund et al., 2003). Recently, a subset of neurons that express CB1R and cholecystokinin (CCK) have been suggested to act, through DSI, to differentiate subgroups of pyramidal cells into neuronal assemblies which are then entrained by fast-spiking inhibitory cells ('sparse coding', Klausberger et al., 2005). It is possible that CB2R may be present on non-CB1R bearing neurons, and thus may play a similar, but complementary role to CB1R in modulating neurotransmitter release and hence network activity. It would seem likely that CB2R are located postsynaptically, as has been shown in hippocampus (Brusco et al., 2008). However, given the highly lipophilic and diffusible nature of CB ligands, effects might be expected at both pre- and postsynaptic loci close to the site of release. In support of a postsynaptic location for the effects of CB2R, our data concerning decay time constants showed that CB2R inverse agonists AM-630 and JTE-907 reliably increased tau by 10–15% in both drug naive slices and those in which CB1R ligands had previously been applied. However, when we applied AM-630 in the presence of TTX, we observed no change in frequency or amplitude of mIPSCs, suggesting that any postsynaptic effects on receptors are unlikely to

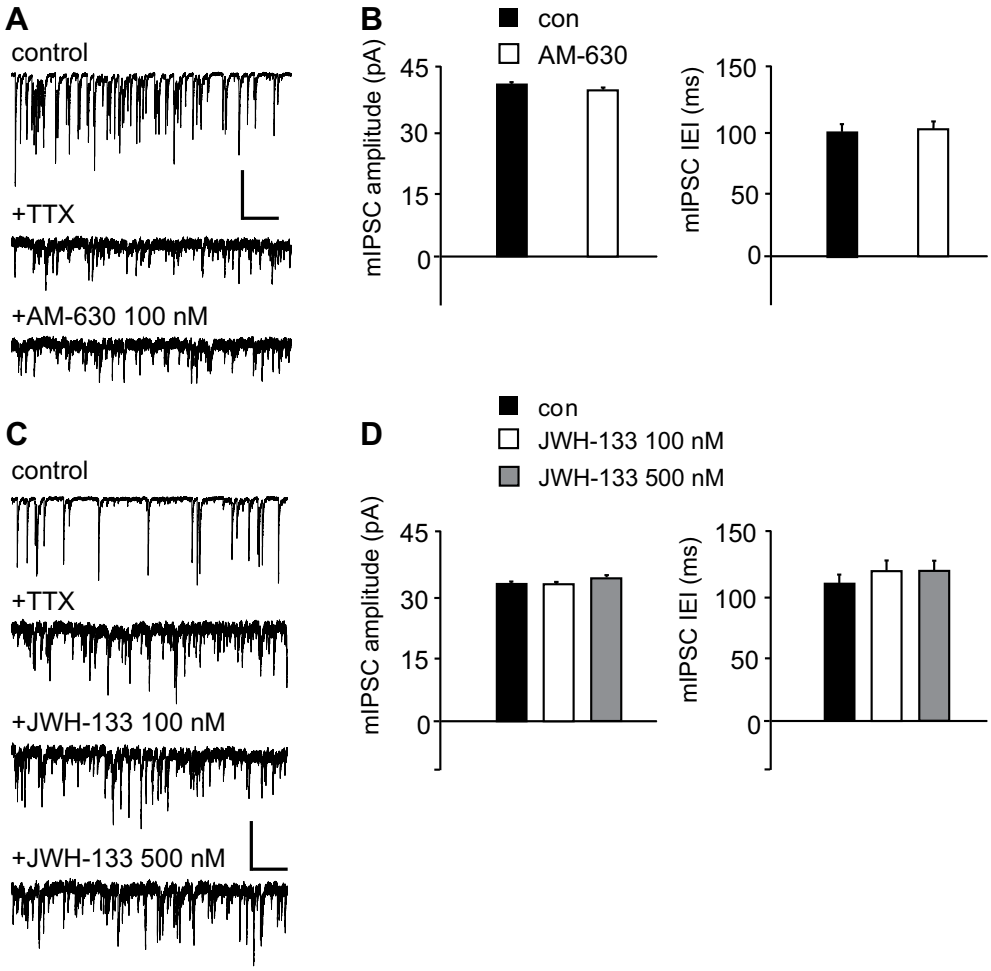

Fig. 9. Neither AM-630 nor JWH-133 affects mIPSCs parameters in layer II of mEC. A, Representative data showing the effect of AM-630 on mIPSCs recorded in layer II of the mEC. Scale bars 400 pA × 1 s upper panel and 50 pA × 1 s lower panels. B, Summary bar graphs showing sIPSC amplitude and IEI prior to and following drug application. C, Representative data showing the effect of JWH-133 on mIPSCs recorded in layer II of the mEC. Scale bars 500 pA × 1 s upper panel and 70 pA × 1s lower panels. D, Summary bar graphs showing sIPSC amplitude and IEI prior to and following drug application.

account for the large changes we observed in sIPSC amplitude. Since it was possible that TTX may have reduced any endogenous cannabinoid release and therefore precluded any observable antagonist effect on mIPSCs, we applied an agonist at CB2R. Application of the selective CB2R agonist JWH-133, even at high concentration, had no effect on mIPSC kinetics, amplitude or IEI.

Clearly, the mIPSC data indicate that there is no effect of CB2R ligands at the level of postsynaptic receptors, since changes in decay times would be expected similar to those seen with sIPSCs. Hence, the data favour a presynaptic locus for CB2R effects. This interpretation might appear to be confounded by the data indicating no effect of CB2R ligands on the frequency of mIPSCs, which is normally taken to be a corollary of presynaptic action. However, it is likely that the effects of CB2R are restricted to predominantly AP-dependent, Ca^{2+}-driven release, as is the case for CB1R. Lack of effects of CB2R ligands on mIPSC frequency does not rule out a presynaptic locus since this might be expected if AP-independent release was not Ca^{2+}-dependent. In support of this latter scenario, Yamasaki et al. (2006) have shown in cerebellum that miniature PSCs elicited by a Ca^{2+} rise, but not those which are Ca^{2+}-independent, are sensitive to cannabinoids. Finally, our own and recent studies by others have strongly indicated that AP-dependent and AP-independent, background neurotransmitter release draw vesicles from different pools (Woodhall et al., 2007; Fredj and Burrone, 2009) and so it is problematic to conflate the two forms of release. In this regard, it is pertinent to note that changes in decay times are not a reliable indicator of postsynaptic action, since elements such as snapin regulate rise and decay times through purely presynaptic effects on vesicle fusion synchrony (Pan et al., 2009). Hence, when these factors and our data are considered with the anatomical studies described above, it seems likely that CB2R may be exerting most of their effects, like CB1R, via regulation of action potential dependent GABA release.

Finally, the role of AM-630 and JTE-907 in increasing GABAergic inhibition in mEC may suggest that CB2R are tonically active at some synapses. This interpretation is consistent with the concept that many G-protein coupled receptors show constitutive activity, and this has been demonstrated in terms of a constitutively active CB1R (Mato et al., 2002), and of constitutively released endocannabinoids (Hentges et al., 2005), although demonstration of constitutively active native receptors in the total absence of ligand is complex (see Savinainen et al., 2005).

References

Alger, B.E., Pitler, T.A., Wagner, J.J., Martin, L.A., Morishita, W., Kirov, S.A., Lenz, R.A., 1996. Retrograde signalling in depolarization-induced suppression of inhibition in rat hippocampal CA1 cells. J. Physiol. 496, 197–209.

Baker, D., Pryce, G., Davies, W.L., Hiley, C.R., 2006. In silico patent searching reveals a new cannabinoid receptor. Trends Pharmacol. Sci. 27, 1–3.

Beltramo, M., Bernardini, N., Bertorelli, R., Campanella, M., Nicolussi, E., Fredduzzi, S., Reggiani, A., 2006. CB2 receptor-mediated antihyperalgesia: possible direct involvement of neural mechanisms. Eur. J. Neurosci. 23 (6), 1530–1538.

Benito, B., Núñex, E., Tolón, R.M., Carrier, E.J., Rábano, A., Hillard, C.J., Romero, J., 2003. Cannabinoid CB2 receptors and fatty acid hydrolase are selectively over expressed in neuritic plaque associated glia in Alzheimer's disease brains. J. Neurosci. 3, 11136–11141.

Berretta, N., Jones, R.S., 1996. A comparison of spontaneous EPSCs in layer II and layer IV-V neurons of the rat entorhinal cortex in vitro. J. Neurophysiol. 76 (2), 1089–1100.

Breivogel, C.S., Griffin, G., Di Marzo, V., Martin, B.R., 2001. Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. Mol. Pharmacol. 60, 155–163.

Brusco, A., Tagliaferro, P., Saez, T., Onaivi, E.S., 2008. Postsynaptic localization of CB2 cannabinoid receptors in the rat hippocampus. Synapse 62 (12), 944–949.

Cabral, G.A., Dove Pettit, D.A., 1998. Drugs and immunity: cannabinoids and their role in decreased resistance to infectious disease. J. Neuroimmunol. 83, 116–123.

Chan, P.K., Chan, S.C., Yung, W.H., 1998. Presynaptic inhibition of GABAergic inputs to rat substantia nigra pars reticulata neurones by a cannabinoid agonist. Neuroreport 9 (4), 671–675.

Devane, W.A., Dysarz 3rd, F.A., Johnson, M.R., Melvin, L.S., Howlett, A.C., 1988. Determination and characterization of a cannabinoid receptor in rat brain. Mol. Pharmacol. 34 (5), 605–613.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258 (5090), 1946–1949.

Felder, C.C., Joyce, K.E., Briley, E.M., Glass, M., Mackie, K.P., Fahey, K.J., Cullinan, G.J., Hunden, D.C., Johnson, D.W., Chaney, M.O., Koppel, G.A., Brownstein, M., 1998. LY320135, a novel cannabinoid CB1 receptor antagonist, unmasks coupling of the CB1 receptor to stimulation of cAMP accumulation. J. Pharmacol. Exp. Ther. 284 (1), 291–297.

Freund, T.F., Katona, I., Piomelli, D., 2003. Role of endogenous cannabinoids in synaptic signaling. Physiol. Rev. 83, 1017–1066.

Fredj, N.B., Burrone, J., 2009. A resting pool of vesicles is responsible for spontaneous vesicle fusion at the synapse. Nat. Neurosci. 12 (6), 751–758.

Glass, M., Dragunow, M., Faull, R.L.M., 1997. Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain. Neuroscience 77, 299–318.

Gong, J.P., Onaivi, E.S., Ishiguro, H., Liu, Q.R., Tagliaferro, P.A., Brusco, A., Uhl, G.R., 2006. Cannabinoid CB2 receptors: immunhistochemical localization in rat brain. Brain Res. 1071, 10–23.

Griffin, G., Wray, E.J., Tao, Q., McAllister, D., Rorrer, W.K., Aung, M., Martin, B.R., Abood, M.E., 1999. Evaluation of the cannabinoid CB_2 receptor-selective antagonist, SR144528: further evidence for cannabinoid CB_2 receptor absence in the rat central nervous system. Eur. J. Pharmacol. 377, 117–125.

Hájos, N., Katona, I., Naiem, S.S., MacKie, K., Ledent, C., Mody, I., Freund, T.F., 2000. Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. Eur. J. Neurosci. 12 (9), 3239–3249.

Hajos, N., Ledent, C., Freund, T.F., 2001. Novel cannabinoid sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. Neuroscience 106, 1–4.

Hentges, S.T., Low, M.J., Williams, J.T., 2005. Differential regulation of synaptic inputs by constitutively released endocannabinoids and exogenous cannabinoids. J. Neurosci. 25 (42), 9746–9751.

Hollrigel, G.S., Soltesz, I., 1997. Slow kinetics of miniature IPSCs during early postnatal development in granule cells of the dentate gyrus. J. Neurosci. 17 (13), 5119–5128.

Hosohata, Y., Quock, R.M., Hosohata, K., Makriyannis, A., Consroe, P., Roeske, W.R., Yamamura, H.I., 1997. AM630 antagonism of cannabinoid-stimulated [35S]GTP gamma S binding in the mouse brain. Eur. J. Pharmacol. 321 (1), R1–R3.

Huffman, J.W., Liddle, J., Yu, S., Aung, M.M., Abood, M.E., Wiley, J.L., Martin, B.R., 1999. 3-(1′,1′-Dimethylbutyl)-1-deoxy-delta8-THC and related compounds: synthesis of selective ligands for the CB2 receptor. Bioorg. Med. Chem. 7 (12), 2905–2914.

Iwamura, H., Suzuki, H., Ueda, Y., Kaya, T., Inaba, T., 2001. In vitro and in vivo pharmacological characterization of JTE-907, a novel selective ligand for the CB_2 receptor. J. Pharmacol. Exp. Ther. 296, 420–425.

Klausberger, T., Marton, L.F., O'Neill, J., Huck, J.H.J., Dalezios, Y., Fuentealba, P., Suen, W.Y., Papp, E., Kaneko, T., Watanabe, M., Csicsvari, J., Somogyi, P., 2005. Complementary roles of cholecystokinin-and parvalbumin-expressing GABAergic neurons in hippocampal network oscillations. J. Neurosci. 25, 9782–9793.

Kreitzer, A.C., Regehr, W.G., 2001. Cerebellar depolarization-induced suppression of inhibition is mediated by endogenous cannabinoids. J. Neurosci. 21 (20), RC174.

Liu, P., Bilkey, D.K., Darlington, C.L., Smith, P.F., 2003. Cannabinoid CB1 receptor protein expression in the rat hippocampus and entorhinal, perirhinal, postrhinal and temporal cortices: regional variations and age-related changes. Brain Res. 979 (1–2), 235–239.

Llano, I., Leresche, N., Marty, A., 1991. Calcium entry increases the sensitivity of cerebellar Purkinje cells to applied GABA and decreases inhibitory synaptic currents. Neuron 6, 565–574.

Lynn, A.B., Herkenham, M., 1994. Localization of cannabinoid receptors and nonsaturable high-density cannabinoid binding sites in peripheral tissues of the rat: implications for receptor-mediated immune modulation by cannabinoids. J. Pharmacol. Exp. Ther. 268 (3), 1612–1623.

Maejima, T., Oka, S., Hashimotodani, Y., Ohno-Shosaku, T., Aiba, A., Wu, D., Waku, K., Sugiura, T., Kano, M., 2005. Synaptically driven endocannabinoid release requires Ca^{2+}-assisted metabotropic glutamate receptor subtype 1 to phospholipase C β4 signaling cascade in the cerebellum. J. Neurosci. 25, 6826–6835.

Marriott, K.S., Huffman, J.W., 2008. Recent advances in the development of selective ligands for the cannabinoid CB(2) receptor. Curr. Top. Med. Chem. 8 (3), 187–204.

Mato, S., Pazos, A., Valdizán, E.M., 2002. Cannabinoid receptor antagonism and inverse agonism in response to SR141716A on cAMP production in human and rat brain. Eur. J. Pharmacol. 443, 43–46.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.

Miklaszewska, A.E., Gaubrusiewilz, K., Kaminska, B., Konarska, L., 2007. Distinctive pattern of cannabinoid receptor type II (CB2) expression in adult and paediatric brain tumours. Brain Res. 1137, 161–169.

Moldrich, G., Wenger, T., 2000. Localization of the CB1 cannabinoid receptor in the rat brain. An immunohistochemical study. Peptides 21 (11), 1735–1742.

Morgan, N.H., Stanford, I.M., Woodhall, G.L., 2008. Modulation of network oscillatory activity and GABAergic synaptic transmission by CB1 cannabinoid receptors in the rat medial entorhinal cortex. Neural Plast. 2008, 808564.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., et al., 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem. Pharmacol. 50 (1), 83–90.

Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365 (6441), 61–65.

New, D.C., Wong, Y.H., 2003. BML-190 and AM251 act as inverse agonists at the human cannabinoid CB2 receptor: signalling via cAMP and inositol phosphates. FEBS Lett. 536 (1–3), 157–160.

Núñez, E., Benito, C., Tolón, R.M., Hillard, C.J., Griffin, W.S.T., Romero, J., 2008. Glial expression of cannabinoid CB2 receptors and fatty acid amide hydrolase are beta amyloid-linked events in Down's syndrome. Neuroscience 151, 104–110.

Onaivi, E.S., Ishiguro, H., Gong, J.P., Patel, S., Perchuk, A., Meozzi, P.A., Myers, L., Mora, Z., Tagliaferro, P., Gardener, E., Bruscho, A., Akinshola, B.E., Liu, Q.R., Hope, B., Iwasaki, S., Arinami, T., Teasenfitz, L., Uhl, G.R., 2006. Discovery of the presence and functional expression of cannabinoid CB_2 receptors in brain. Ann. NY Acad. Sci. 1074, 514–536.

Oz, M., 2006. Receptor-independent effects of endocannabinoids on ion channels. Curr. Pharm. Des. 12 (2), 227–239.

Pan, P.Y., Tian, J.H., Sheng, Z.H., 2009. Snapin facilitates the synchronization of synaptic vesicle fusion. Neuron 61 (3), 412–424.

Pattij, T., Wiskerke, J., Schoffelmeer, A.N., 2008. Cannabinoid modulation of executive functions. Eur. J. Pharmacol. 585 (2–3), 458–463.

Pertwee, R.G., 2000. Cannabinoid receptor ligands: clinical and neuropharmacological considerations, relevant to future drug discovery and development. Expert Opin. Investig. Drugs 9 (7), 1553–1571.

Pitler, T.A., Alger, B.E., 1992. Postsynaptic spike firing reduces synaptic GABAA responses in hippocampal pyramidal cells. J. Neurosci. 12 (10), 4122–4132.

Rinaldi-Carmona, M., Barth, F., Héaulme, M., Alonso, R., Shire, D., Congy, C., Soubrié, P., Brelière, J.C., Le Fur, G., 1995. Biochemical and pharmacological characterisation of SR141716A, the first potent and selective brain cannabinoid receptor antagonist. Life Sci. 56 (23–24), 1941–1947.

Rosell, S., Agurell, S., 1975. Effects of 7-hydroxy-delta-6-tetrahydrocannabinol and some related cannabinoids on the guinea pig isolated ileum. Acta Physiol. Scand. 94 (1), 142–144.

Ross, R.A., Coutts, A.A., McFarlane, S.M., Anavi-Goffer, S., Irving, A.J., Pertwee, R.G., MacEwan, D.J., Scott, R.H., 2001. Actions of cannabinoid receptor ligands on rat cultured sensory neurones: implications for antinociception. Neuropharmacology 40 (2), 221–232.

Ryberg, E., Larsson, N., Sjögren, S., Leonova, J., Elebring, T., Nilsson, K., Drmota, T., Greasley, P.J., 2007. The orphan receptor GPR55 is a novel cannabinoid receptor. Br. J. Pharm. 152, 1092–1101.

Savinainen, J.R., Kokkola, T., Salo, O.M., Poso, A., Järvinen, T., Laitinen, J.T., 2005. Identification of WIN55212-3 as a competitive neutral antagonist of the human cannabinoid CB2 receptor. Br. J. Pharmacol. 145 (5), 636–645.

Schatz, A.R., Lee, M., Condie, R.B., Pulaski, J.T., Kaminski, N.E., 1996. Cannabinoid receptors CB_1 and CB_2: a characterization of expression and adenylate cyclase modulation with in the immune system. Toxicol. Appl. Pharmacol. 142, 278–287.

Ständer, S., Schmelz, M., Metze, D., Luger, T., Rukwied, R., 2005. Distribution of cannabinoid receptor 1 (CB1) and 2 (CB2) on sensory nerve fibers and adnexal structures in human skin. J. Dermatol. Sci. 38 (3), 177–188.

Suárez, J., Bermúdez-Silva, F.J., Mackie, K., Ledent, C., Zimmer, A., Cravatt, B.F., de Fonseca, F.R., 2008. Immunohistochemical description of the endogenous cannabinoid system in the rat cerebellum and functionally related nuclei. J. Comp. Neurol. 509 (4), 400–421.

Sugiura, T., Kodaka, T., Nakane, S., Miyashita, T., Kondo, S., Suhara, Y., Takayama, H., Waku, K., Seki, C., Baba, N., Ishima, Y., 1999. Evidence that the cannabinoid CB1 receptor is a 2-arachidonoylglycerol receptor. Structure–activity relationship of 2-arachidonylglycerol, ether-linked analogues and related compounds. J. Biol. Chem. 274, 2794–2801.

Sugiura, T., Kondo, S., Kishimoto, S., Miyashita, T., Nakane, S., KodakaSuhara, Y., Takayama, H., Waku, K., 2000. Evidence that 2 arachidonoylglycerol but not

N-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB_2 receptor. Comparison of the agonist activities of various cannabinoid receptor ligands in HL-60 cells. J. Biol. Chem. 275, 605–612.

Szabo, B., Dörner, L., Pfreundtner, C., Nörenberg, W., Starke, K., 1998. Inhibition of GABAergic inhibitory postsynaptic currents by cannabinoids in rat corpus striatum. Neuroscience 85 (2), 395–403.

Tsou, K., Brown, S., Sañudo-Peña, M.C., Mackie, K., Walker, J.M., 1998. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83 (2), 393–411.

Van Sickle, M.D., Duncan, M., Kingsley, P.J., Mouihate, A., Urbani, P., Mackie, K., Stella, N., Makriyannis, A., Piomelli, D., Davison, J.S., Marnett, L.J., Di Marzo, V., Pittman, Q.J., Patel, K.D., Sharkey, K.A., 2005. Identification and functional characterization of brainstem cannabinoid CB2 receptors. Science 310 (5746), 329–332.

Viveros, M.P., Marco, E.M., Llorente, R., López-Gallardo, M., 2007. Endocannabinoid system and synaptic plasticity: implications for emotional responses. Neural Plast. 2007, 52908.

Wilson, R.I., Nicoll, R.A., 2001. Endogenous cannabinoids mediate retrograde signaling at hippocampal synapses. Nature 410, 588–592.

Wilson, R.I., Kunos, G., Nicoll, R.A., 2001. Presynaptic specificity of endocannabinoid signaling in the hippocampus. Neuron 31 (3), 453–462.

Woodhall, G., Evans, D.I., Cunningham, M.O., Jones, R.S., 2001. NR2B-containing NMDA autoreceptors at synapses on entorhinal cortical neurons. J. Neurophysiol. 86 (4), 1644–1651.

Woodhall, G.L., Bailey, S.J., Thompson, S.E., Evans, D.I.P., Jones, R.S.G., 2005. Fundamental differences in spontaneous synaptic inhibition between deep and superficial layers of the rat entorhinal cortex. Hippocampus 15, 232–245.

Woodhall, G.L., Ayman, G., Jones, R.S., 2007. Differential control of two forms of glutamate release by group III metabotropic glutamate receptors at rat entorhinal synapses. Neuroscience 148 (1), 7–21.

Yamasaki, M., Hashimoto, K., Kano, M., 2006. Miniature synaptic events elicited by presynaptic Ca^{2+} rise are selectively suppressed by cannabinoid receptor activation in cerebellar Purkinje cells. J. Neurosci. 26 (1), 86–95.