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RESEARCH PAPER

The cannabinoid CB_1 receptor antagonists rimonabant (SR141716) and AM251 directly potentiate $GABA_A$ receptors

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BACKGROUND AND PURPOSE
Rimonabant (SR141716) and the structurally related AM251 are widely used in pharmacological experiments as selective cannabinoid receptor CB_1 antagonists / inverse agonists. Concentrations of 0.5–10 μM are usually applied in *in vitro* experiments. We intended to show that these drugs did not act at $GABA_A$ receptors but found a significant positive allosteric modulation instead.

EXPERIMENTAL APPROACH
Recombinant $GABA_A$ receptors were expressed in *Xenopus* oocytes. Receptors were exposed to AM251 or rimonabant in the absence and presence of GABA. Standard electrophysiological techniques were used to monitor the elicited ionic currents.

KEY RESULTS
AM251 dose-dependently potentiated responses to 0.5 μM GABA at the recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor with an EC_{50} below 1 μM and a maximal potentiation of about eightfold. The Hill coefficient indicated that more than one binding site for AM251 was located in this receptor. Rimonabant had a lower affinity, but a fourfold higher efficacy. AM251 potentiated also currents mediated by $\alpha_1\beta_2$, $\alpha_x\beta_2\gamma_2$ (x = 2,3,5,6), $\alpha_1\beta_3\gamma_2$ and $\alpha_4\beta_2\delta$ $GABA_A$ receptors, but not those mediated by $\alpha_1\beta_1\gamma_2$. Interestingly, the CB_1 receptor antagonists LY320135 and O-2050 did not significantly affect $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor-mediated currents at concentrations of 1 μM.

CONCLUSIONS AND IMPLICATIONS
This study identified rimonabant and AM251 as positive allosteric modulators of $GABA_A$ receptors. Thus, potential GABAergic effects of commonly used concentrations of these compounds should be considered in *in vitro* experiments, especially at extrasynaptic sites where GABA concentrations are low.

LINKED ARTICLES
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Abbreviations
CB_1, cannabinoid receptor type 1; $GABA_A$ receptor, γ-aminobutyric acid type A receptor; IPSC, inhibitory post-synaptic current

Introduction

In the CNS, endocannabinoids are synthesized post-synaptically in neurons, reach the pre-synaptic terminal in a yet poorly understood manner and act at G-protein-coupled cannabinoid CB_1 receptors (nomenclature follows Alexander *et al.*, 2011) to inhibit the release of excitatory and inhibitory neurotransmitters (see Hashimotodani *et al.*, 2007; Katona and Freund, 2008). The central action of endocannabinoids may be modulated by inhibition of biosynthesis or

degradation (Petrosino and Di Marzo, 2010; Piomelli, 2005) or by specifically blocking their action at CB_1 receptors.

Antagonism of the CB_1 receptor has a broad spectrum of pharmacological effects, including body weight reduction and improved metabolic profile (Kunos, 2007; Di Marzo, 2008; Kunos *et al.*, 2008; Kunos *et al.*, 2009), modulation of peripheral inflammatory processes (Karsak *et al.*, 2007; Leonti *et al.*, 2010), modulation of emotion (Lutz, 2009) and anti-craving effects (Solinas *et al.*, 2008). The CB_1 receptor inverse agonist rimonabant from Sanofi-Aventis was the first cannabinoid anorectic anti-obesity drug (Fernandez and Allison, 2004), but its approval in Europe was officially withdrawn by the European Medicines Agency in January 2009 because of central side effects. The iodinated rimonabant analogue AM251 was synthesized by the group of Makriyannis (Gatley *et al.*, 1997). The CB_1 receptor inverse agonist LY320135, which was developed by Eli Lilly (Felder *et al.*, 1998), and the neutral CB_1 receptor antagonist O-2050 (Gardner and Mallet, 2006), have also been reported to have selectivity towards CB_1 receptors over CB_2 receptors (see Pertwee *et al.*, 2010).

To date, rimonabant and AM251 are regarded as selective and specific CB_1 receptors inverse agonists characterized by K_i values <10 nM (reviewed by Pertwee *et al.*, 2010). Both compounds are frequently used in *in vitro* experiments. A PubMed search with the term 'AM251' shows that this compound was mentioned in 427 publications either in the title or in the abstract. In brain slice experiments, the compounds are typically applied in a concentration range of 0.5–10 μM. As rimonabant was the first clinical CB_1 receptor antagonist developed, its chemical scaffold was extensively profiled for off-target effects (Fong *et al.*, 2009). Because such profiling is usually carried out with radioligand assays, any significant functional effect at $GABA_A$ receptors may have been overlooked.

The $GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the CNS and have been estimated to be expressed at about 30% of all synapses. Each receptor is composed of five subunits that are arranged pseudosymmetrically around the central chloride ion selective channel (Macdonald and Olsen, 1994; Rabow *et al.*, 1995). The subunits are selected from a total of 19 different but homologous subunits, indicating an as yet poorly understood diversity of $GABA_A$ receptors. The pharmacological properties of a $GABA_A$ receptor depend on subunit composition (Sieghart and Sperk, 2002) and arrangement (Minier and Sigel, 2004).

Here we report the unexpected direct effects of low concentrations of rimonabant and AM251 on different recombinant $GABA_A$ receptors, indicating that these compounds do not act solely at CB receptors. This observation may lead to reinterpretation of several scientific results.

Methods

AM251 and rimonabant

AM251 was obtained from Tocris and rimonabant (SR141716) from Fluka (Buchs, Switzerland). The drugs were dissolved at a concentration of 2.5 mM in DMSO and stored at –20°C. Immediately before the experiment the stock solutions were diluted in the experimental medium. The maximal final DMSO concentration was 0.4% (v/v). In control experiments, DMSO up to a concentration of 0.6% (v/v) did not affect the amplitude of the current response to GABA. Figure 1 compares the structures of the four CB antagonists used. While AM251 and rimonabant differ only by a halogen substituent, LY320135 and O-2050 belong to different structural classes.

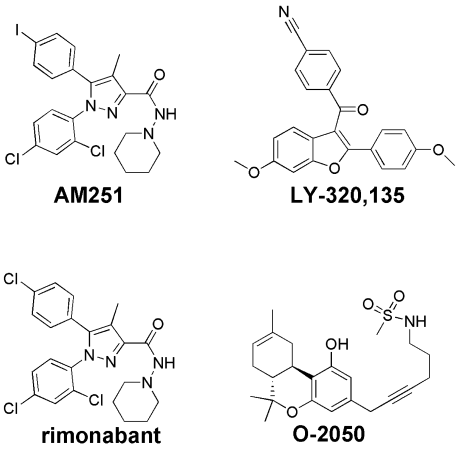

Figure 1
Chemical structure of the compounds studied.

Expression in Xenopus oocytes

Capped cRNAs were synthesized (Ambion, Austin, TX, USA) from the linearized vectors containing the cDNAs coding for the different rat subunits respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (United States Biologicals, Cleveland, OH, USA). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, the dried pellet dissolved in water and stored at –80°C. cRNA mixtures were prepared from these stock solutions and stored at –80°C.

Xenopus laevis oocytes were prepared, injected and defolliculated as described previously (Sigel, 1987; Sigel and Minier, 2005). They were injected with 50 nL of the cRNA solution containing rat α_1, β_2 and γ_2 subunits at a concentration of 10 nM : 10 nM : 50 nM (Boileau *et al.*, 2002), and then incubated in modified Barth's solution (10 mM HEPES, pH 7.5, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.34 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 100 units·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin) at 18°C for at least 24 h before the measurement.

Functional characterization in Xenopus oocytes

Currents were measured using a specially built two-electrode voltage clamp amplifier in combination with a XY-recorder (90% response time 0.1 s) or digitized at 100 Hz using a MacLab/200 (AD Instruments). Tests with a model oocyte were performed to ensure linearity in the larger current range.

The response was linear up to 20 μA. Electrophysiological experiments were performed at a holding potential of –80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM Na-HEPES (pH 7.4). The perfusion solution (6 mL·min^{-1}) was applied through a glass capillary with an inner diameter of 1.35 mm, the mouth of which was placed about 0.4 mm from the surface of the oocyte (Baur and Sigel, 2007). The experiments were performed after the currents induced by GABA had become constant. Concentration response curves for GABA or AM251 were fitted with the equation $I(c) = I_{max}/[1 + (EC_{50}/c)^n]$, where c is the concentration of GABA or AM251, EC_{50} the concentration of GABA eliciting half maximal current amplitude, I_{max} is the maximal current amplitude, I the current amplitude and n the Hill coefficient. Allosteric potentiation by the CB_1 receptor antagonists was measured at a GABA concentration eliciting 2–5% of the maximal GABA current amplitude. GABA was applied for 20 s alone or in combination with allosteric compound. Relative current potentiation by the different compounds was determined as $[I_{(compound + GABA)}/I_{GABA} - 1] \times 100\%$. The perfusion system was cleaned between drug applications, by washing with 100% DMSO to avoid contamination. Care was taken to ensure full removal of DMSO after this cleaning procedure.

Results

Low concentrations of the cannabinoid receptor antagonist AM251 potentiate $GABA_A$ receptors

Recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were expressed in *Xenopus* oocytes and currents induced by GABA measured. Figure 2A shows two applications of 0.5 μM GABA followed by combined application of the same concentration of GABA with 0.3 μM AM251. To our surprise, in the presence of such a small concentration of AM251 the current amplitude was enhanced more than threefold. Figure 2B shows averaged concentration response curves to AM251 and rimonabant. The curve for AM251 was characterized by an EC_{50} of 0.40 ± 0.13 μM and a maximal potentiation of 881 ± 167% (n = 4). The large Hill coefficient amounting to 1.5 ± 0.1 is indicative of the presence of more than one binding site in such a receptor. The curve for rimonabant was characterized by an EC_{50} of 7.3 ± 0.5 μM, a maximal potentiation of 3381 ± 165% (n = 4) and a Hill coefficient of 1.5 ± 0.1. In the absence of GABA, 3 μM AM251 or 3 μM rimonabant elicited small currents by itself, of about 1% and 2% of the maximal current amplitude elicited by GABA, respectively (not shown).

We then tested the effects of AM251 on the GABA concentration response curve of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. Increasing concentrations of GABA were applied to oocytes in the absence and presence of 1 μM AM251 (Figure 3). The curves were characterized by an EC_{50} of 15.4 ± 0.8 μM (n = 7) and 5.5 ± 0.4 μM (n = 4) and a Hill coefficient of 1.3 ± 0.1 and 1.0 ± 0.1 in the absence and presence of AM251 respectively. A GABA application without AM251 was carried out during the concentration response curves with AM251. This allowed an estimate of the relative maximal current amplitudes and indicated a 26 ± 15% (n = 4) increase by AM251.

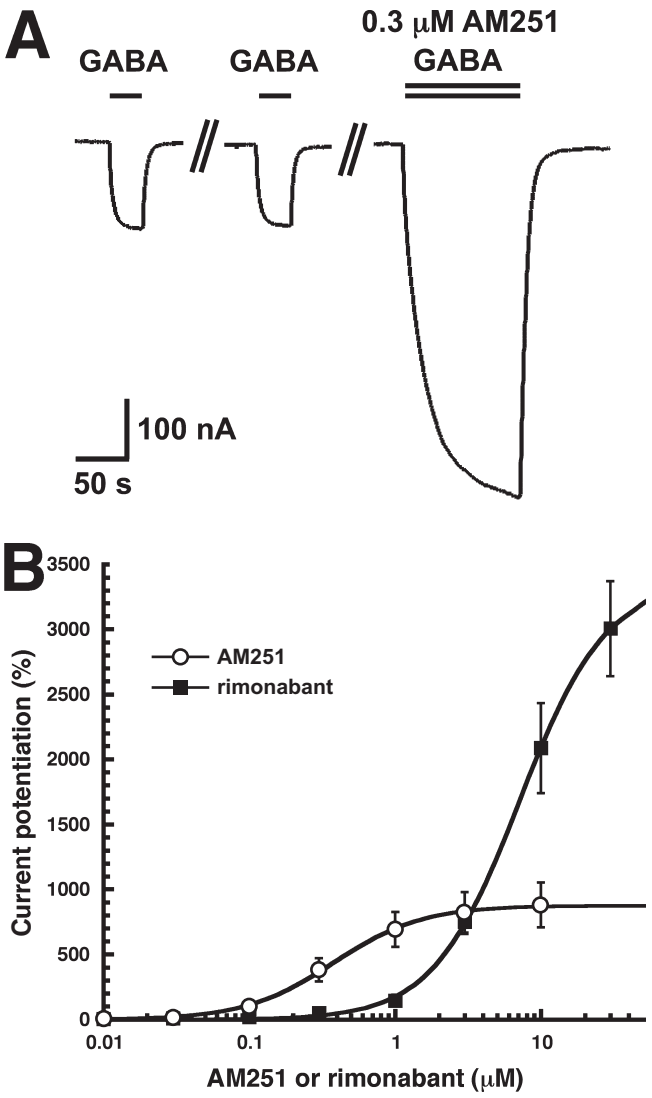

Figure 2

AM251 affects $GABA_A$ receptor currents. (A) Low concentrations of AM251 strongly potentiate currents mediated by recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. Currents were measured in *Xenopus* oocytes expressing recombinant receptors. Two applications of 0.5 μM GABA were followed by a combined application of the same concentration of GABA with 0.3 μM AM251. (B) Concentration-response curves of the potentiation by AM251 and by rimonabant. Either no AM251 or increasing concentrations of AM251 or rimonabant were co-applied with 0.5 μM GABA. Individual curves were first normalized to the observed maximal current amplitude and subsequently averaged. Mean ± SEM of experiments carried out with four oocytes from two batches of oocytes are shown.

Site of action

Figure 4 shows that exclusion of γ_2 subunits from $GABA_A$ receptors reduced the potentiation by AM251. Replacement of the α_1 subunit in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors by α_2 did not significantly affect potentiation while replacement by α_3, α_5 or α_6 led to a decrease in potentiation. Replacement of the β_2 subunit in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors by β_1 lead to an almost complete loss of potentiation by AM251, while replacement

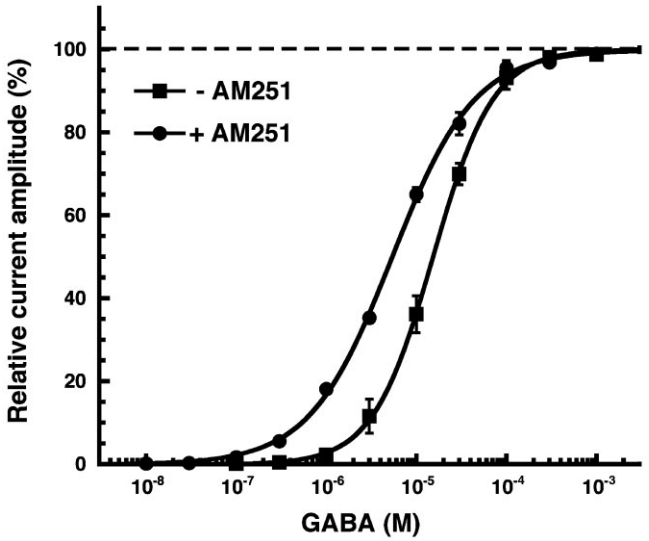

Figure 3

GABA concentration response curve in the absence and presence of 1 μM AM251. Increasing concentrations of GABA were applied in the absence and presence of 1 μM AM251. Individual curves obtained in the absence of AM251 were first normalized to the observed maximal current amplitude and subsequently averaged. Curves obtained in the presence of AM251 were treated in the same way. As such a procedure does not detect effects of AM251 on maximal current amplitudes, additional experiments were performed as described in the Results. Mean ± SEM of experiments carried out with at least four oocytes from two batches of oocytes are shown.

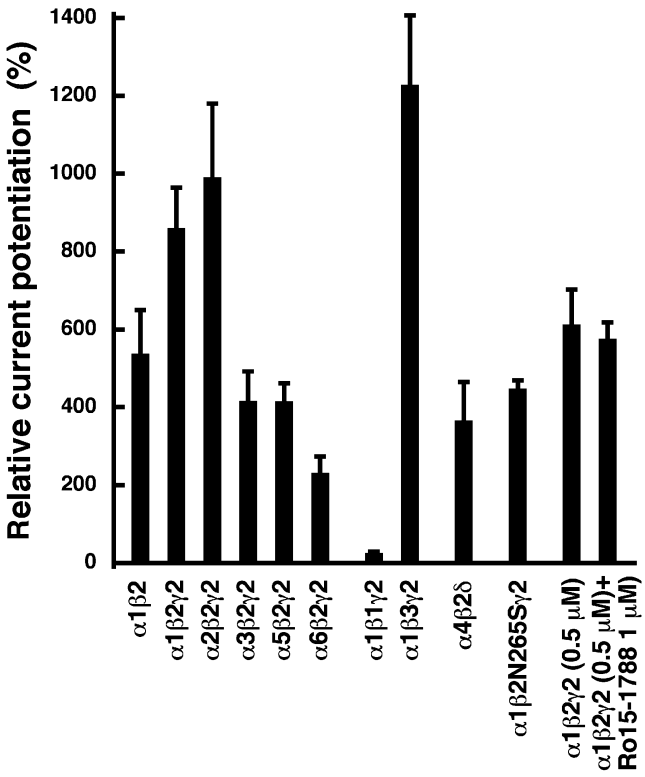

Figure 4

Subunit dependence and sensitivity to Ro15-1788 and to a point mutation of the potentiation by AM251. Currents were measured in *Xenopus* oocytes expressing recombinant $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_2\gamma_2$, $\alpha_6\beta_2\gamma_2$, $\alpha_1\beta_1\gamma_2$, $\alpha_1\beta_3\gamma_2$ and $\alpha_4\beta_2\delta$ $GABA_A$ receptors. Two applications of 0.5 μM GABA were followed by a combined application of the same concentration of GABA with 3 μM AM251. Mean ± SEM of experiments carried out with at least four oocytes are shown. Potentiation by 0.5 μM AM251 was tested as above and subsequently we tried to counteract potentiation by 1 μM Ro15-1788. Potentiation by 3 μM AM251 was also tested in point mutated $\alpha_1\beta_2$N265Sγ_2 receptors.

by β_3 tended to increase the potentiation.The $\alpha_4\beta_2\delta$ $GABA_A$ receptors were also modulated.

We were interested to see if AM251 acts at the same sites as the benzodiazepines or loreclezole. We found that 1 μM of the benzodiazepine antagonist Ro15-1788 did not counteract potentiation of the current by AM251 (Figure 4). In the point mutated receptor $\alpha_1\beta_2$N265Sγ_2 $GABA_A$, where loreclezole has little effect, AM251 still potentiated the current response to GABA to about 50% of the wild-type receptor (Figure 4). These findings indicate that AM251 acts at neither of the mentioned sites.

Experiments with pentobarbital and the neurosteroid tetrahydrodeoxycorticosterone (THDOC) indicated that AM251 did not compete with these two ligands. Potentiation using the same oocytes for three subsequent measurements was 550 ± 77% (n = 4) for 5 μM pentobarbital, 908 ± 215% (n = 4) for 0.5 μM AM251 and 2003 ± 308% (n = 4) for the combined application of 5 μM pentobarbital and 0.5 μM AM251. Potentiation using the same oocytes for the three subsequent measurements was 483 ± 59% (n = 4) for 0.5 μM THDOC, 631 ± 80% (n = 4) for 0.5 μM AM251 and 1233 ± 125% (n = 4) for the combined application of 0.5 μM THDOC and 0.5 μM AM251.

The CB_1 receptor antagonists LY320135 and O-2050

In AM251 the *p*-chloro group of rimonabant attached to a phenyl substituent is replaced with a *p*-iodo group. Both compounds robustly stimulated currents mediated by GABA (Figure 5). Two additional CB_1 antagonists with an entirely different structure (Figure 1) were also examined. As shown in Figure 5 and 1 μM LY320135 and 1 μM O-2050 had no significant effect on the current elicited by GABA. A total of 10 μM LY320135 and 10 μM O-2050 potentiated these currents only by 19 ± 11% (n = 4) and 36 ± 11% (n = 4) respectively.

Discussion

AM251 is generally regarded as a selective and specific CB_1 receptor ligand, while rimonabant has previously been shown to target and antagonize the transient receptor potential (TRP)V1 channels at μM concentrations (Gibson *et al.*, 2008). Both compounds also bind at nM concentrations to the orphan GPCR GPR55 and act as agonists with yet unknown functional consequences (see Ross, 2009). Here we

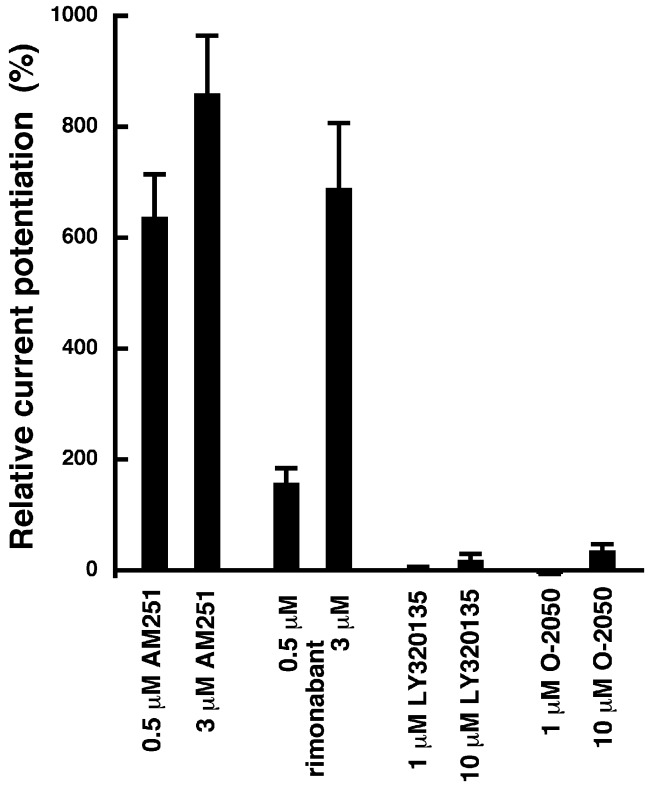

Figure 5

Effect of rimonabant, LY320135 and O-2050. The experiments were carried out as described under Figure 1A. Mean ± SEM of experiments carried out with 4–11 oocytes from at least two batches of oocytes are shown.

show that, *in vitro*, rimonabant and AM251 allosterically potentiate all but the β_1 subunit containing $GABA_A$ receptors at nM concentrations. It is worth noting that also the putatively extrasynaptic $\alpha_4\beta_2\delta$ $GABA_A$ receptors are subject to modulation. Interestingly, β_3 subunit containing receptors, which are expressed in the spinal cord (Seo *et al.*, 2010) and potentially associated with pain perception, are more strongly potentiated by rimonabant and AM251. The site of action is not identical to that of either the benzodiazepines, lorecezole, pentobarbital or neurosteroids. As the β_1 subunit containing $GABA_A$ receptors were not modulated, the site is probably located in the β subunit.

Our experiments were performed in *Xenopus* oocytes. In brain slices, results are influenced by inhibition of GABA release caused by CB_1 receptor activation by endocannabinoids produced in the post-synaptic cell upon depolarization. Indeed, AM251 and rimonabant lead to a CB_1 receptor-mediated increase in GABA tone (Kim and Alger, 2010; Menzies *et al.*, 2010). Chelation of Ca^{2+} in the post-synaptic cell to prevent production of endocannabinoids has been reported to suppress to a large extent this inhibition of GABA release as determined by the amplitude of inhibitory post-synaptic currents (IPSCs). In the presence of a Ca^{2+} chelator, rimonabant induced only a small increase in the amplitude of IPSCs (Kim and Alger, 2010). This observation does not contradict our results as they predict little direct stimulation of current mediated by $GABA_A$ receptors elicited by high concentrations of GABA, like those expected to be present in the synaptic cleft at the time of peak amplitudes of IPSCs. Our results would instead predict that the predominantly extrasynaptic $GABA_A$ receptors where concentrations of GABA should be low, would be most likely to be affected by AM251 or rimonabant. In support of our data, Mendiguren and Pineda (2009) have reported that AM251 and rimonabant decreased the firing rate of 5-HT dorsal raphe nucleus neurons in rat brain slices and that this effect was inhibited by the $GABA_A$ receptor antagonist picrotoxin.

We also studied CB_1 receptor antagonists differing widely in structure (Figure 1). While LY320135 and low concentrations of O-2050 had no significant effect on the current elicited by GABA, rimonabant and AM251 showed strong potentiation of $GABA_A$ receptors. The fact that these two molecules, rimonabant and AM251, differ very little in structure (Figure 1), but differ about fourfold in the maximal current stimulation is surprising. The difference between these two compounds consists only of a halide substituent that is most probably located in a receptor region that upon interaction with the two ligands assumes different conformations, differing in their ability to stabilize the open channel state.

We are not aware of any report on sedative or anxiolytic properties of rimonabant or AM251 up to 10 $mg \cdot kg^{-1}$ i.p., which clearly suggests that either the drugs do not reach the brain in sufficient amounts, or that the effects on CB_1 receptors dominate the *in vivo* pharmacology. There is very little information on levels of these antagonists in brain after peripheral administration. In one study, mice were injected i.p. with 0.3 $mg \cdot kg^{-1}$ rimonabant (Barna *et al.*, 2009), corresponding to approximately the single oral dose of 20 mg recommended for human treatment. In this study, brain levels of about 0.25 $nmol \cdot g^{-1}$ tissue were reported. If a random distribution in the tissue is assumed, this corresponds to a concentration of 0.25 μM. At this concentration, we would predict some behavioural effects through interaction of rimonabant at $GABA_A$ receptors, unless the CB_1 receptor-mediated pharmacology predominates. However, the above estimate of the free concentration may be too high as the hydrophobic drug binds to lipids.

We conclude that all the *in vitro* studies with brain slices or neuronal tissues that use either rimonabant or AM251 as selective inhibitors of CB_1 receptors at concentrations higher than 0.03 μM AM251 or 0.1 μM rimonabant will need to take into account a potential direct modulation of $GABA_A$ receptors.

Acknowledgements

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Conflicts of interest

None.

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