Neuropsychopharmacology (2011) 36, 472–487

www.neuropsychopharmacology.org

Regulation of Hippocampal Cannabinoid CB_1 Receptor Actions by Adenosine A_1 Receptors and Chronic Caffeine Administration: Implications for the Effects of Δ^9-Tetrahydrocannabinol on Spatial Memory

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The cannabinoid CB_1 receptor-mediated modulation of γ-aminobutyric acid (GABA) release from inhibitory interneurons is important for the integrity of hippocampal-dependent spatial memory. Although adenosine A_1 receptors have a central role in fine-tuning excitatory transmission in the hippocampus, A_1 receptors localized in GABAergic cells do not directly influence GABA release. CB_1 and A_1 receptors are the main targets for the effects of two of the most heavily consumed psychoactive substances worldwide: Δ^9-tetrahydrocannabinol (THC, a CB_1 receptor agonist) and caffeine (an adenosine receptor antagonist). We first tested the hypothesis that an A_1–CB_1 interaction influences GABA and glutamate release in the hippocampus. We found that A_1 receptor activation attenuated the CB_1-mediated inhibition of GABA and glutamate release and this interaction was manifested at the level of G-protein activation. Using *in vivo* and *in vitro* approaches, we then investigated the functional implications of the adenosine–cannabinoid interplay that may arise following chronic caffeine consumption. Chronic administration of caffeine in mice (intraperitoneally, 3 mg/kg/day, for 15 days, >12 h before trials) led to an A_1-mediated enhancement of the CB_1-dependent acute disruptive effects of THC on a short-term spatial memory task, despite inducing a reduction in cortical and hippocampal CB_1 receptor number and an attenuation of CB_1 coupling with G protein. A_1 receptor levels were increased following chronic caffeine administration. This study shows that A_1 receptors exert a negative modulatory effect on CB_1-mediated inhibition of GABA and glutamate release, and provides the first evidence of chronic caffeine-induced alterations on the cannabinoid system in the cortex and hippocampus, with functional implications in spatial memory. *Neuropsychopharmacology* (2011) **36,** 472–487; doi:10.1038/npp.2010.179; published online 6 October 2010

Keywords: adenosine; cannabinoid; hippocampus; caffeine; THC; memory

INTRODUCTION

The fine tuning of neuronal activity to suit specific cognitive functions is a major task of endogenous neuromodulators, of which adenosine and the endocannabinoids are two important examples. Both modulators are released by neurons and activate G-protein-coupled receptors (GPCRs) that represent some of the most widely and densely expressed GPCRs in the brain (Dunwiddie and Masino, 2001; Herkenham *et al*, 1990). In the hippocampus, the predominant adenosine and cannabinoid receptors are the A_1 and CB_1 receptors, respectively. Several forms of learning, memory, and other cognitive functions require the integrity of the hippocampal circuitry, in which A_1 and CB_1 receptors were shown to have important roles owing to their presynaptic regulation of neurotransmitter release (eg, Ohno and Watanabe, 1996; Wise *et al*, 2009). Moreover, in the hippocampus, these receptors are the main targets for the cognitive effects of two of the most heavily consumed psychoactive substances worldwide: caffeine and Δ^9-tetrahydrocannabinol (THC) (Barone and Roberts, 1996; Leggett, 2006).

Caffeine is present in various dietary sources, such as coffee, tea, and soft drinks, and at moderate doses is an adenosine receptor antagonist with cognitive-enhancing properties (Fredholm *et al*, 1999; Ribeiro and Sebastião,

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Received 3 July 2010; revised 1 September 2010; accepted 1 September 2010

2010). As customary in most coffee consumers, long-term intake of caffeine leads to the development of tolerance to some of its acute effects by mechanisms not yet fully understood, although most studies found an increased number of A_1, but not A_{2A} receptors, in several brain areas (Jacobson *et al*, 1996). Chronic caffeine intake has also been associated with increased behavioral effects of some drugs of abuse, for example, amphetamine and cocaine (Gasior *et al*, 2000; Justinova *et al*, 2009). THC is the main psychoactive constituent of the cannabis plant, which is consumed recreationally or used for medicinal purposes; it mainly activates cannabinoid CB_1 receptors in the central nervous system to produce motor- and cognitive-disrupting effects (see Pertwee, 2008).

Hippocampal CB_1 receptors are primarily found in presynaptic terminals of cholecystokinin (CCK)-expressing γ-aminobutyric acidergic (GABAergic) interneurons from the CA1 and CA3 subfields (Hájos *et al*, 2000; Katona *et al*, 1999). CCK-expressing GABAergic interneurons regulate the temporal coordination in the activity of principal cell assemblies, which is critical for the integrity of hippocampal-dependent memory (Freund and Katona, 2007). Accodingly, it was recently shown that presynaptic CB_1 receptors at GABAergic, but not glutamatergic, neurons are required for THC-induced amnesia (Puighermanal *et al*, 2009). In contrast, A_1 receptors mostly affect excitatory synaptic transmission (Dunwiddie and Fredholm, 1989; Sebastião *et al*, 1990), having no direct influence upon GABAergic transmission in mature hippocampal neurons (Jeong *et al*, 2003; Lambert and Teyler, 1991; Li and Henry, 2000; Yoon and Rothman, 1991) or on GABA release from isolated nerve terminals (Cunha and Ribeiro, 2000). However, A_1 receptors are present in hippocampal GABAergic interneurons (Ochiishi *et al*, 1999), in which they control the actions of vasoactive intestinal peptide (Cunha-Reis *et al*, 2008).

Both A_1 and CB_1 receptors regulate synaptic transmission through activation of G-protein $\alpha_{i/o}$-subunits (Straiker *et al*, 2002), which inhibit adenylyl cyclase, block voltage-gated calcium channels (VGCCs), and activate inwardly rectifying potassium channels (Dunwiddie and Masino, 2001; Howlett, 2005). In the cerebellum, A_1 receptors modulate the motor incoordination effects induced by acute administration of THC or CB_1 receptor agonist CP55,940 (Dar, 2000; Dar and Mustafa, 2002; DeSanty and Dar, 2001). Furthermore, prolonged intracerebellar administration of a CB_1 or A_1 agonist induces cross-tolerance (DeSanty and Dar, 2001), and similar observations were obtained in two subsequent studies (Kouznetsova *et al*, 2002; Selley *et al*, 2004). A more recent study observed that CB_1-mediated inhibition of excitatory synaptic transmission in the hippocampus is modulated by endogenous adenosine, through A_1 receptor activation (Hoffman *et al*, 2010; but see Serpa *et al*, 2009). These previous findings raised the hypothesis that a functional interaction between A_1 and CB_1 receptors in the hippocampus may have cognitive and pathophysiological implications, particularly for the effects of cannabis and caffeine consumption in humans.

This study initially focused upon the possibility that an A_1–CB_1 interaction influences GABA and glutamate release. We found that A_1 receptor activation attenuated the CB_1-mediated inhibition of GABA and glutamate release, and that this interaction is manifested at the level of G-protein activation. We then evaluated the functional consequences of chronic caffeine administration on the memory deficits induced by acute THC administration. Caffeine (intraperitoneally, 3 mg/kg/day, for 15 days, >12 h before trials) increased A_1 receptor levels, and did not by itself cause measurable effects on spatial memory, but led to an A_1-mediated exacerbation of the CB_1-dependent acute effects of THC in a spatial memory task.

MATERIALS AND METHODS

Animals

Adult male 6–8 weeks old Wistar rats (Harlan Interfauna Iberica, Barcelona, Spain) and 12–16 weeks old C57Bl/6J mice (Harlan-Olac, Bicester, UK) were used. Animals were housed in a temperature- and humidity-regulated room with a 12 h dark/light cycle, and free access to food and water. Experiments were performed during the light phase. All experimentation followed the UK Animals (Scientific Procedures) Act, 1986, Portuguese and European Union law concerning animal care. C57Bl/6J mice were used in all experiments involving chronic caffeine administration for logistic advantages and because mice have been extensively used in behavioral studies where the systemic effects of cannabinoids on motor and cognitive function have been assessed (Lichtman *et al*, 2002).

Drugs

4-Amino-[2,3-^{3}H]butyric acid ([^{3}H]GABA), L-[G-^{3}H]glutamic acid ([^{3}H]glutamate), 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX), and [^{3}H]SR141716A were obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). Guanosine 5′-[γ-^{35}S]-thio)triphosphate ([^{35}S]-GTPγS) was from Perkin-Elmer NEN Radiochemicals (Boston, MA, USA). Adenosine deaminase (ADA, EC 3.5.4.4) was from Roche Diagnostics (Indianapolis, IN, USA). THC (>98% purity) was from THC Pharm (Frankfurt, Germany) or Tocris Bioscience (Bristol, UK). $CdCl_2$, caffeine (anhydrous base), GABA, aminooxyacetic acid (AOAA), guanosine diphosphate (GDP), and guanosine 5′-O-[γ-thio]triphosphate (GTPγS) were from Sigma (St Louis, MO, USA). 1-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride (SKF89976A), N^6-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-[4-[(2-aminoethyl) amino]carbonylmethyloxyphenyl] xanthine (XAC), (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate (WIN55,212-2), (3*S*)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone monomethanesulfonate (WIN55,212-3), and *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) were from Tocris Bioscience. (*RS*)-4-amino-3-(4-chlorophenyl)butanoic acid (baclofen) was from Ascent Scientific (Bristol, UK). All other reagents were from Sigma. For *in vitro* experiments, non-water-soluble drugs were dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at −20 °C. The amount of DMSO in solutions was normalized for all conditions in any given experiment, and always <0.02%.

Synaptosomal Preparation

For each experiment, hippocampal synaptosomes were prepared from two rats, or cortico-hippocampal synaptosomes from one mouse. Animals were decapitated under halothane anesthesia and synaptosomes were prepared as described previously (Assaife-Lopes *et al*, 2010), with modifications. Briefly, tissue was dissected in a continuously oxygenated (95% O_2, 5% CO_2) ice-cold artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl 125, KCl 3, NaH_2PO_4 1, $NaHCO_3$ 25, $CaCl_2$ 1.5, $MgSO_4$ 1.2, glucose 10, pH 7.4. Samples were homogenized in ice-cold 0.32 M sucrose solution containing 1 mM EDTA and 10 mM HEPES, pH 7.4. The homogenate was then centrifuged at 3000 *g* for 10 min, and the supernatant obtained was centrifuged at 14 000 *g* for 12 min to obtain a stratified pellet, containing synaptosomes (McMahon *et al*, 1992; Phelan and Gordon-Weeks, 1997).

[^{3}H]Neurotransmitter Release Assays

For [^{3}H]GABA release assays, the pellet was resuspended and synaptosomes were incubated for 20 min, at 37 °C, with [^{3}H]GABA (1.5 μCi/ml, 1.85 nM), and 0.625 μM of unlabelled GABA to decrease specific activity of [^{3}H]GABA to 2.3 μCi/nmol. Incubation and superfusion solutions consisted of oxygenated aCSF containing the GABA transaminase inhibitor AOAA. For [^{3}H]glutamate experiments, the synaptosomal pellet was resuspended in aCSF, which did not contain AOAA, and synaptosomes were incubated for 5 min, at 37°C with 10 μCi/ml [^{3}H]glutamate. Synaptosomes were then layered over GF/C filters (Milipore, MA, USA) on an eight-chamber superfusion (0.8 ml/min) apparatus (Raiteri *et al*, 1974). This constant and rapid flow rate washes out endogenously released substances, thus ensuring drug effect specificity (see Raiteri and Raiteri, 2000). After a 30-min washout period, samples were continuously collected for 36 min, in 2-min fractions. Synaptosomes were stimulated during 2 min with 15 mM K^+ (isomolar substitution of Na^+ with K^+) at the 6th (S_1) and 24th (S_2) minutes of collection time. CB_1 agonists were added to the superfusion medium from the 18th minute onward, to measure effects on S_2. The CB_1 antagonist, AM251, or A_1 and $GABA_B$ receptor ligands were added from the 15th minute of the washout period onward, that is, present during S_1 and S_2 in order to assess their ability to modify the effect of WIN55,212-2 (applied before S_2). Each condition was tested in duplicate, as commonly accepted in this paradigm (eg, Cunha and Ribeiro, 2000). Under similar conditions, the percentage of GABA and glutamate in the K^+-evoked outflow is >90% of the total tritium in the sample (Cunha *et al*, 1997; Lopes *et al*, 2002). Fractional [^{3}H]neurotransmitter release was expressed as the percentage of total radioactivity present in the synaptosomes at each time point (fractional release). The amount of tritium released after each pulse of K^+ (S_1 or S_2) was calculated by integration of the peak area. Effects were calculated by normalizing the S_2/S_1 values of corresponding controls from the same batch of synaptosomes to 0% effect. For example, the effect of WIN55,212-2 (added before S_2), in the presence of CPA (during S_1 and S_2), was calculated using the S_2/S_1 of CPA alone (during S_1 and S_2) as a control, which was obtained from the same experiment and batch of synaptosomes.

Binding Assays

Rat hippocampal or mouse cortico-hippocampal membranes were prepared as described previously (eg, Cunha *et al*, 1999), with modifications. Tissue was homogenized in ice-cold 0.32 M sucrose solution containing 2 mM EGTA, 1 mM DTT, and 50 mM Tris, pH 7.6. The homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant obtained was centrifuged at 14 000 *g* for 12 min. The pellets were resuspended in assay buffer and incubated with 4 U/ml ADA for 30 min at 37 °C, followed by centrifugation at 14 000 *g* for 12 min and resuspension in assay buffer. Assay buffer composition, in mM, for radioligand binding assays was: Tris 50, $MgCl_2$ 2, pH 7.4 and for [^{35}S]GTPγS binding assays it was: Tris 50, $MgCl_2$ 5, NaCl 100, EGTA 0.2, pH 7.4. Protein content was determined by the Bradford method (Bradford, 1976). For [^{3}H]DPCPX binding assays, membranes (40 μg protein) were incubated for 1 h at room temperature in a final incubation volume of 300 μl containing 4 U/ml ADA, and using 2 μM of XAC to measure nonspecific binding. For [^{3}H]SR141716A binding, membranes (50 μg protein) were incubated for 1 h at 30 °C in a final volume of 300 μl containing 1 mg/ml BSA and using 1 μM of AM251 to measure nonspecific binding. For [^{35}S]GTPγS binding assays, membranes (10 μg of protein) were incubated with 0.1 nM [^{35}S]GTPγS and 0.1 nM–10 μM of CB_1 agonist, in the absence or presence of 100 nM CPA or 100 μM baclofen, in assay buffer containing 30 μM GDP, in a total volume of 500 μl, for 30 min at 37 °C. At this GDP concentration, WIN55,212-2 has been shown to induce high-affinity [^{35}S]GTPγS binding (Breivogel *et al*, 1998). Specific binding was calculated by subtracting nonspecific binding obtained by incubation with 10 μM GTPγS. The effect of co-application of CPA or baclofen with CB_1 agonists was calculated by subtracting the increase in [^{35}S]GTPγS induced by CPA or baclofen alone. The reactions were stopped by vacuum filtration through GF/C filters, followed by washing with ice-cold buffer.

In Vivo Drug Administration

Mice were randomly assigned to various groups and habituated to the handling during 5 days before testing began. For chronic treatment with caffeine, animals received caffeine (3 mg/kg/day), or vehicle (saline: 0.9% NaCl), >12 h before trials, for at least 15 days before experimental days, and throughout the course of behavioral testing, in order to avoid withdrawal effects. Total caffeine exposure was for 22–24 days, and euthanization occurred 24 h after last injection. The half-life of caffeine for doses lower than 10 mg/kg ranges from 0.7 to 1.2 h in the rat and mouse (Fredholm *et al*, 1999); therefore, the estimated concentrations of caffeine present in plasma or brain during behavioral testing were negligible. For acute administration, animals received a single dose of vehicle (8% Tween-80 in saline), THC (5 mg/kg), AM251 (3 mg/kg), DPCPX (1 mg/kg), or WIN55,212-2 (1 mg/kg). THC was prepared in Tween-80 as described previously (Pertwee *et al*, 1992); AM251, DPCPX, and WIN55,212-2 were suspended in the

vehicle and carefully sonicated. All drugs were given by intraperitoneal injection in a volume of 2 ml/kg weight. The concentration of Tween-80 used was previously shown not to affect motor activity in mice (Castro *et al*, 1995).

Water Maze Experiments (Trials to Criterion Task)

We performed two separate sets of water maze experiments, in which mice were randomly assigned to four experimental groups of seven to eight subjects (total of 57 animals). The protocol is a version of the Morris water maze test that is sensitive to hippocampal-dependent short-term spatial learning (Chen *et al*, 2000; Daumas *et al*, 2007). To form a stable representation of the environment, mice were first trained to quickly find a hidden platform at a fixed platform location for 5 consecutive days. Subjects then performed several tasks, each consisting of a new platform position. Each animal was given a maximum of eight trials per day, to perform the task until reaching a performance criterion of $\leqslant 7$ s average latency on three consecutive trials. A 15 min intertrial interval was applied, during which animals were allowed to dry under a ceramic heat lamp. Once the criterion was reached, trials stopped and a new task began on the following testing day. Animals first performed 4–5 training tasks in order to learn to optimize their search strategies, and then the effects of acute drug administration were tested in separate tasks, as described in the Results section.

A black infrared-translucent Perspex tank (1 m in diameter) of water (temperature, $22 \pm 1\,^{\circ}C$) was placed over an infrared lightbox (Tracksys, Nottingham, UK) in a room with various visible external cues. A transparent platform was ~ 0.5 cm below the water surface and its position varied between several possible locations, on two concentric circles, according to the original protocol. An infrared-sensitive automated tracking system (*Noldus Ethovision 7.0*, Noldus Information Technology, Wageningen, the Netherlands) monitored all performances.

Statistical Analysis

Statistical significance was tested using paired Student's *t*-test, one- or two-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's *post hoc* tests, as indicated. The two-way ANOVA and *post hoc* tests were performed using the Predictive Analytics Software 18.0 (SPSS, an IBM Company, Chicago, IL). GraphPad Prism 5.0 (GraphPad Prism Software, San Diego, CA, USA) was used for all other statistical tests and nonlinear regression curve fitting. Differences in parameters between binding curves were tested using extra sum-of-squares F test.

RESULTS

A_1 Receptor Activation Attenuates the CB_1 Receptor-Dependent Inhibition of [^{3}H]GABA and [^{3}H]Glutamate Release from Rat Hippocampal Nerve Terminals

In control conditions, pooling data from all experiments performed, the average basal release of [^{3}H]GABA from rat hippocampal synaptosomes was $0.76 \pm 0.02\%$ ($n = 31$, average of first 6 min of collection; Figure 1a) of the total tritium retained by synaptosomes at the same time points. Depolarization of the hippocampal synaptosomes with K^+ (15 mM) for 2 min induced a threefold increase in the [^{3}H]GABA release with an average peak of $2.5 \pm 0.1\%$ during S_1, and $2.3 \pm 0.1\%$ ($n = 31$; Figure 1a) during S_2, giving an average S_2/S_1 of 0.94 ± 0.01. Depolarization by K^+ mainly induced a calcium-dependent release of [^{3}H]GABA, as blockade of VGCCs by Cd^{2+} ($CdCl_2$, 200 μM) inhibited its release by $70 \pm 3.0\%$ ($n = 8$, $p < 0.001$, data not shown). The GABA transporters account for the remaining percentage of [^{3}H]GABA released upon K^+ depolarization, as blockade of GABA transporters with SKF89976a (20 μM) inhibited its release by $34 \pm 1.6\%$ ($n = 8$, $p < 0.001$, data not shown).

To induce a CB_1 receptor-dependent effect on [^{3}H]GABA release, we used the potent cannabinoid receptor agonist WIN55,212-2, which has been previously shown to inhibit evoked [^{3}H]GABA release from hippocampal synaptosomes through a CB_1-specific mechanism, having a maximum CB_1-selective effect at 1 μM (Köfalvi *et al*, 2007). Application of 1 μM WIN55,212-2 6 min before S_2 caused a decrease of basal [^{3}H]GABA outflow and inhibited evoked GABA release (Figure 1a) with an average S_2/S_1 of 0.78 ± 0.01 ($n = 31$) that represents an inhibition of $16.7 \pm 1.4\%$ ($n = 31$), when compared with control S_2/S_1 within each experiment. Blockade of VGCCs by Cd^{2+} ($CdCl_2$, 200 μM) completely abolished the effect of WIN55,212-2 at its maximum CB_1-specific concentration ($n = 5$, $p < 0.01$; Figure 1b). Conversely, blockade of GABA transporters with SKF89976a (20 μM) did not alter the effect of WIN55,212-2 ($n = 5$; Figure 1b), which suggests that the effect of 1 μM WIN55,212-2 upon [^{3}H]GABA release is exerted through the inhibition of Ca^{2+}-dependent exocytotic release. The effect of WIN55,212-2 (0.01–10 μM) on K^+-evoked [^{3}H]GABA release was concentration dependent (Figure 1c). As WIN55,212-2 is known to directly block N-type VGCCs at concentrations above 1 μM (Németh *et al*, 2008; Shen and Thayer, 1998), we tested the specificity of its effect in our preparation using the CB_1 antagonist AM251, as well as WIN55,212-3, an enantiomer of WIN55,212-2 that does not activate the CB_1 receptor, but maintains the Ca^{2+} channel-blocking properties (Shen and Thayer, 1998). AM251 (1 μM) fully blocked the effect of 1 μM, but not of 10 μM of WIN55,212-2 (Figure 1c). Higher concentrations of AM251 were not used to avoid loss of selectivity (see Köfalvi, 2007, 2008). The enantiomer had no significant effect applied at 1 μM, but it inhibited evoked [^{3}H]GABA release by $22.4 \pm 1.4\%$ at 10 μM ($n = 5$, $p < 0.05$; Figure 1c), which indicates that the effect of 1 μM WIN55,212-2 upon [^{3}H]GABA release is CB_1 receptor dependent. It is worth noting that the effect of 10 μM WIN55,212-2 was larger than the effect of 10 μM WIN55,212-3, which indicates that the effect of 10 μM WIN55,212-2 still encompasses a CB_1 receptor-dependent component. The partial CB_1 agonist THC (1 μM) inhibited K^+-evoked [^{3}H]GABA release by $8.9 \pm 0.9\%$ ($n = 8$, $p < 0.05$; Figure 1c).

Consistent with previous observations (Cunha and Ribeiro, 2000), adenosine A_1 receptor activation with the selective agonist CPA (100 nM) before S_2 did not affect K^+-evoked [^{3}H]GABA release ($2.7 \pm 3\%$ of control S_2/S_1, $n = 3$, $p > 0.05$, data not shown). To evaluate the influence of A_1 receptors on the CB_1-mediated inhibition of GABA release, we tested the effect of WIN55,212-2 (applied before S_2) in

the presence of CPA (100 nM, applied throughout the experiment, $S_1 + S_2$). Under these conditions, the effect of WIN55,212-2 at 1 μM ($n = 10$) and at 10 μM ($n = 7$) was significantly attenuated ($p < 0.01$ and < 0.05, respectively; Figure 2a). The average effect of 1 μM WIN55,212-2 was 18 ± 3% ($n = 10$) and CPA attenuated this effect to 12 ± 2% ($p < 0.01$, $n = 10$), which represents a 33 ± 13% decrease in the average effect of WIN55,212-2 alone. To test if the attenuation of the effect of 10 μM WIN55,212-2 caused by CPA occurred through a Ca^{2+} channel-dependent mechanism and not involving CB_1 receptors, we performed the experiments using WIN55,212-3. Application of WIN55, 212-3 (10 μM, S_2) by itself inhibited [^{3}H]GABA release by 22 ± 2%, whereas in the presence of CPA (100 nM), its effect was unaltered (22 ± 2%, $n = 3$; Figure 2a). This indicates that the CPA-induced attenuation of the effect of WIN55, 212-2 was exerted at the CB_1 receptor-dependent component, but not upon the Ca^{2+} channel-dependent mechanisms, affected by high micromolar concentrations of WIN55,212-2. The blockade of A_1 receptors with the antagonist DPCPX (50 nM) did not, on its own, alter the effects of 1 and 10 μM WIN55,212-2, but it fully prevented the CPA-induced attenuation ($p > 0.05$, $n = 7$, paired Student's *t*-test; Figure 2b).

The signaling pathways of CB_1, A_1, and $GABA_B$ are known to converge when co-expressed in cerebellar neurons (Selley *et al*, 2004). Furthermore, both CB_1 and $GABA_B$ receptors are present in inhibitory interneurons (Katona *et al*, 1999; Sloviter *et al*, 1999), couple to the same $G\alpha_{i/o}$-subunits (Straiker *et al*, 2002), and exhibit reciprocal inhibition (Cinar *et al*, 2008) in hippocampal neurons. We therefore evaluated whether the A_1 receptor-dependent attenuation of the effect of WIN55,212-2 was mimicked by the activation of $GABA_B$ receptors. As shown in Figure 2c, the effect of WIN55,212-2 (1 μM) was unchanged ($p > 0.05$, $n = 5$, paired Student's *t*-test) by the presence of the $GABA_B$ receptor agonist baclofen (10 μM). Altogether, these results indicate that the cannabinoid CB_1 receptors in GABAergic nerve terminals are under the modulatory influence of adenosine A_1 receptors, but not $GABA_B$ receptors.

Despite the predominant influence of CB_1 receptors in hippocampal circuitry being exerted through the inhibition of GABA release, CB_1 receptors at glutamatergic presynaptic nerve terminals (Katona *et al*, 2006; Kawamura *et al*, 2006) also inhibit the K^+-evoked release of glutamate (eg, Cannizzaro *et al*, 2006; D'Amico *et al*, 2004; Köfalvi *et al*, 2007). Importantly, the regulation of excitatory synaptic transmission is the most relevant role of the hippocampal A_1 receptors (Dunwiddie and Fredholm, 1989; Sebastião *et al*, 1990). To investigate whether A_1 receptors also

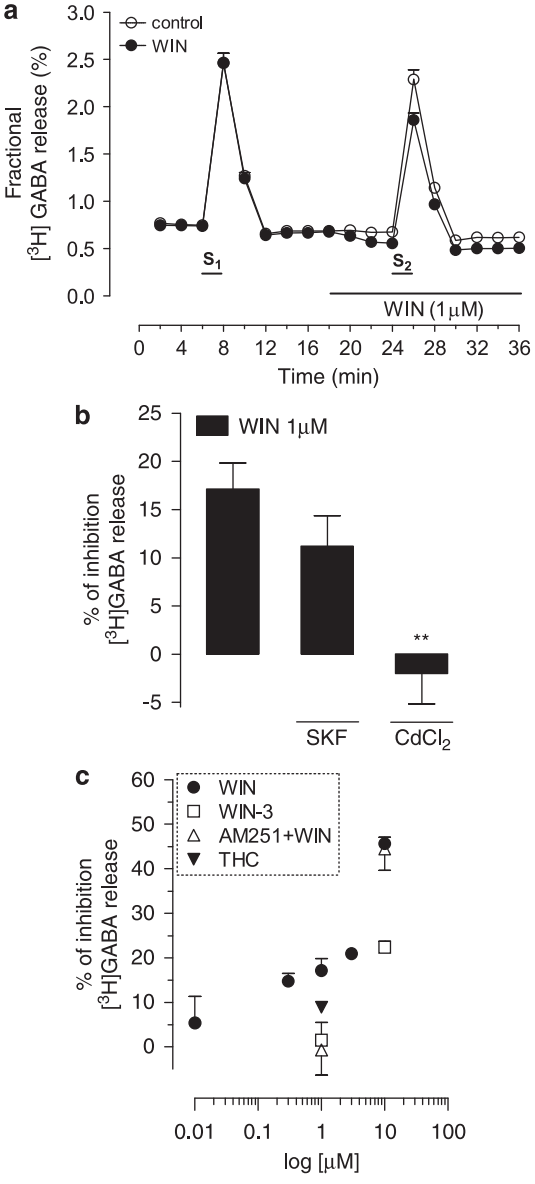

Figure 1 Inhibition of K^+-evoked, Ca^{2+}-dependent release of [^{3}H]GABA from rat hippocampal synaptosomes by WIN55,212-2 (WIN). (a) Fractional release of [^{3}H]GABA evoked by two 15 mM K^+ stimuli of 2-min duration, as indicated (S_1 and S_2); in the test assay, WIN (1 μM) was applied before S_2, as indicated by the horizontal bar. Data represent mean ± standard error of mean (SEM) from 31 experiments performed in duplicate. (b) Percentage inhibition of [^{3}H]GABA release induced by WIN (1 μM) in the absence or in the presence of the calcium channel blocker, $CdCl_2$ (Cd^{2+}, 200 μM), or the GABA transporter inhibitor, SKF89976A (SKF, 20 μM), as indicated below each bar. Note that Cd^{2+} fully blocked the effect of WIN ($n = 5$, $^{**}p < 0.01$, paired Student's *t*-test *vs* effect of WIN alone within the same batch of synaptosomes), whereas SKF did not alter the WIN-induced inhibition ($n = 5$, $p > 0.05$). Data represent mean ± SEM from five experiments, performed in duplicate. (c) Concentration-dependent inhibition of K^+-evoked release of [^{3}H]GABA induced by WIN (0.01–10 μM) in the absence or in the presence of the CB_1 receptor antagonist, AM251 (1 μM); the effect of partial CB_1 receptor agonist, Δ^9-tetrahydrocannabinol (THC) (1 μM), as well as of a WIN enantiomer that is inactive at the CB_1 receptor, WIN55,212-3 (WIN-3; 1–10 μM), is also shown. WIN significantly inhibited [^{3}H]GABA release at all concentrations ($p < 0.01$), except for the lowest concentration tested (0.01 μM, $p > 0.05$); THC (1 μM) also significantly inhibited [^{3}H]GABA release ($p < 0.01$). Note that WIN-3 was devoid of the effect at 1 μM ($p > 0.05$), but not at 10 μM ($p < 0.01$), and that AM251 antagonized the effect of 1 μM, but not of 10 μM WIN, indicating that WIN is CB_1 receptor selective at 1 μM, but not at 10 μM. Each point represents the mean ± SEM of 4–10 independent experiments performed in duplicate, except ($n = 2$) for 0.01 and 3 μM WIN, and 1 μM WIN-3. The S_2/S_1 values from corresponding controls were taken as 0% within each experiment. *P*-values were obtained by a one-way analysis of variance (ANOVA) test with Dunnett *post hoc*, compared with control (0%). SKF, Cd^{2+}, and AM251 were applied 15 min before the start of sample collection and were continuously perfused throughout the experiment, being therefore present during S_1 and S_2 ($S_1 + S_2$); WIN, WIN-3, and THC were added before S_2 (see Materials methods for further details).

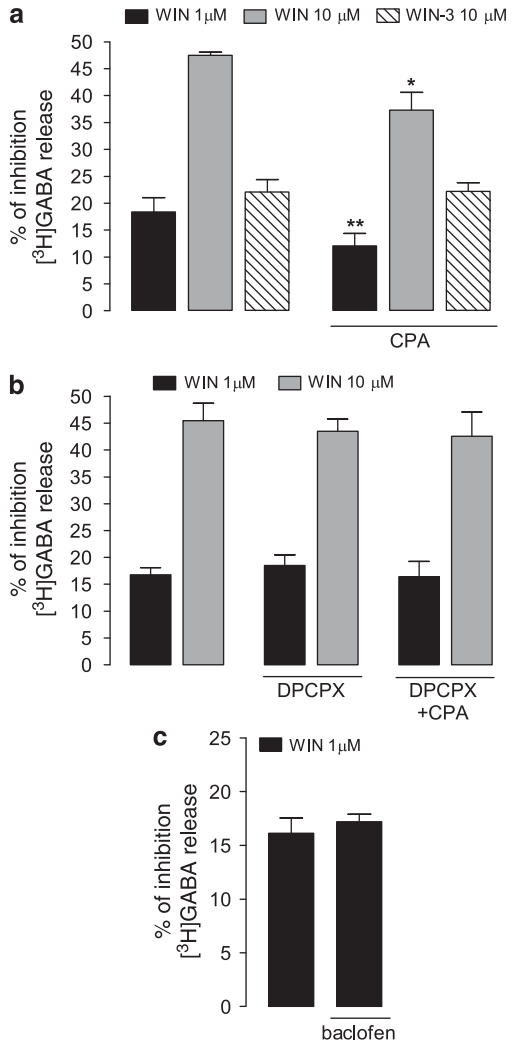

Figure 2 A_1 receptor activation significantly attenuates the CB_1-mediated inhibition of K^+-evoked [^{3}H]GABA release from rat hippocampal synaptosomes. (a) Effects of WIN55,212-2 (WIN, 1 and 10 μM) and of its CB_1 receptor-inactive enantiomer, WIN55,212-3 (WIN, 3, 10 μM), in the absence or in the presence of the selective A_1 receptor agonist, CPA (100 nM), as indicated below each column. Note that CPA significantly attenuated the effect of 1 and 10 μM WIN (**$p<0.01$, *$p<0.05$, respectively, compared with the effect of WIN in the absence of CPA, in the same experiments), whereas the effect of WIN55,212-3 (WIN-3) (10 μM) was unaffected by CPA ($p>0.05$). (b) WIN (1 and 10 μM) was tested in the absence or in the presence of selective A_1 receptor antagonist, DPCPX (50 nM), alone and in combination with CPA (100 nM). (c) WIN (1 μM) was tested in the absence or in the presence of the selective $GABA_B$ receptor agonist, baclofen (10 μM), as indicated below the column. Note that in the presence of DPCPX, CPA did not attenuate the inhibitory effect of WIN ($p>0.05$), and that baclofen did not modify the effect of WIN (1 μM) ($p>0.05$). Bars represent the mean ± standard error of mean (SEM) of 3–10 individual experiments performed in duplicate. The S_2/S_1 values from controls were taken as 0% within each experiment. *P*-values were obtained by paired Student's *t*-test, compared with corresponding controls within the same batch of synaptosomes. WIN and WIN-3 were added before S_2, whereas the other drugs were applied 15 min (CPA or baclofen) or 30 min (DPCPX) before the start of sample collection, being therefore present during S_1 and S_2 (see Materials and methods for further details).

regulate the CB_1-dependent effects upon glutamate release, the influence of A_1 receptor activation on the CB_1-mediated inhibition of K^+-evoked [^{3}H]glutamate release was also tested. The absence of a tonic activation of A_1 receptors by endogenous adenosine was first assessed by using A_1 receptor blocker DPCPX. As Figure 3a shows, WIN55,212-2 (1 μM) inhibited the release of [^{3}H]glutamate by 16 ± 2% and blockade of A_1 receptors with DPCPX (50 nM) did not modify the effect of WIN55,212-2 (15 ± 1%, $p>0.05$, $n=4$). This indicates that endogenous adenosine was effectively washed out by the continuous vertical flow of superfusion medium (see Materials and methods).

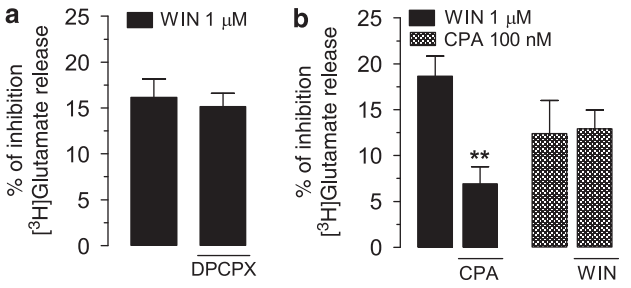

Figure 3 A_1 receptors modulate the CB_1-mediated inhibition of [^{3}H]glutamate. (a) Blockade of A_1 receptors by DPCPX (50 nM) did not modify ($p>0.05$, $n=4$) the effect of WIN55,212-2 (WIN, 1 μM). (b) The inhibition induced by WIN (1 μM) was significantly (**$p<0.01$, $n=5$) attenuated by CPA (100 nM), but the effect of CPA alone (applied before S_2) was not modified ($p>0.05$, $n=4$) when applied in the presence of WIN (1 μM). Bars represent the mean ± standard error of mean of 4–5 individual experiments performed in duplicate. The S_2/S_1 values from controls were taken as 0% within each experiment. *P*-values were obtained by paired Student's *t*-test, compared with corresponding controls within the same batch of synaptosomes.

As shown in Figure 3b, the inhibitory effect of 1 μM WIN55,212-2 upon glutamate release (19 ± 2%) was significantly attenuated to 7 ± 2% ($n=5$, $p<0.01$; Figure 3b) in the presence of CPA. We then evaluated whether the A_1 receptor-mediated inhibition of glutamate release is also under the modulatory control of CB_1 receptors, by comparing the effect of CPA (before S_2) in the absence and in the presence of 1 μM WIN55,212-2. CPA (100 nM) inhibited [^{3}H]glutamate release by 12 ± 4%, and this effect was not modified in the presence of 1 μM WIN55,212-2 (13 ± 2%, $n=4$, $p>0.05$; Figure 3b). These findings further indicate that A_1 receptors negatively modulate the CB_1-mediated effects in the hippocampus and support recent evidence that CB_1-mediated inhibition of excitatory synaptic transmission in the hippocampus is modulated by A_1 receptor activation (Hoffman *et al*, 2010).

A_1 Receptor Activation Attenuates CB_1 Receptor-Induced Stimulation of G Proteins in Rat Hippocampal Membranes

To test whether the adenosine–cannabinoid interaction occurs at the level of G-protein activation, we measured agonist-stimulated [^{35}S]GTPγS binding in hippocampal membranes with the full agonist of CB_1 receptors, WIN55, 212-2, or with the partial agonist THC, in the absence and in the presence of A_1 receptor agonist CPA. The basal [^{35}S]GTPγS binding in the absence of WIN55,212-2 (Figure 4a) or THC (Figure 4b) is represented as 100% in the ordinates, which corresponds to (fmol/mg protein): Figure 4a—149 ± 16 in control (○, $n=7$), 311 ± 50 in the

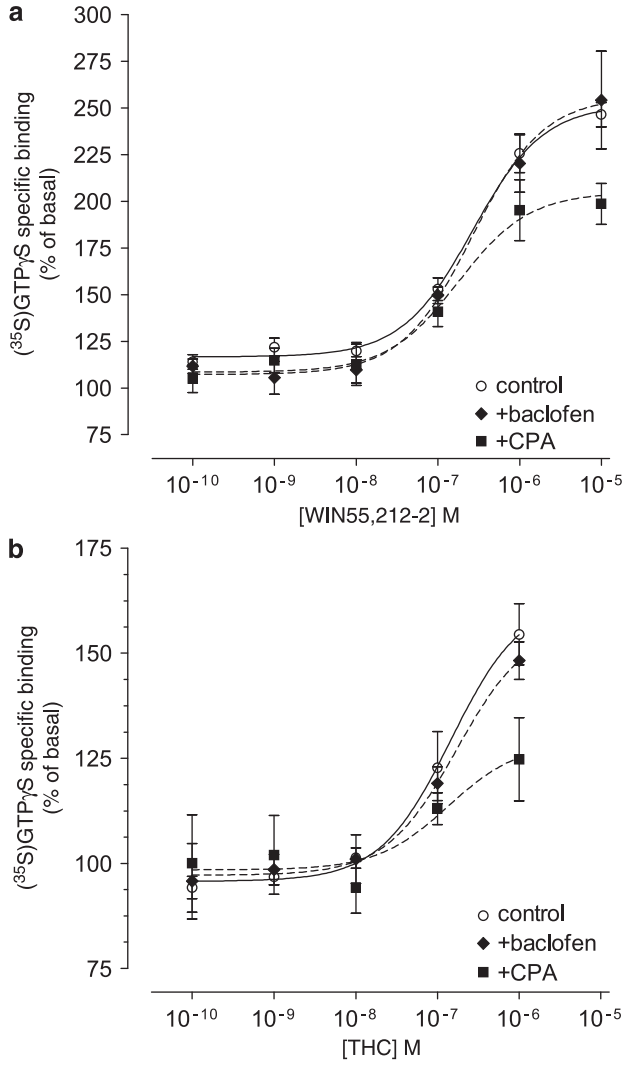

Figure 4 Influence of A_1 or $GABA_B$ receptor activation on CB_1-induced stimulation of G proteins, as assayed by WIN55,212-2 or Δ^9-tetrahydrocannabinol (THC)-induced [^{35}S]GTPγS binding. Rat hippocampal membranes (10 μg protein) were incubated for 30 min at 37 °C with 30 μM GDP, 0.1 nM [^{35}S]GTPγS, and varying concentrations of (a) WIN55,212-2 (0.1 nM–10 μM) or (b) THC (0.1 nM–1 μM), alone (○) or in combination with 100 nM CPA (■), or 100 μM baclofen (◆). E_{max} and log EC_{50} values are shown in Table 1. Data represent mean percentage of basal stimulation ± SEM of $n=7$ (○) and $n=4$ (■, ◆), performed in duplicate. Non-visible error bars are within symbols.

Table 1 E_{max} and Log EC_{50} Values of Agonist-Stimulated [^{35}S]GTPγS Binding in Rat Hippocampal Membranes

	EC_{50} −log, *M*	E_{max} % of stimulation
WIN	−6.589 ± 0.11	251 ± 7
WIN+CPA	−6.752 ± 0.23	204 ± 10[a]
WIN+baclofen	−6.554 ± 0.20	257 ± 12
THC	−6.837 ± 0.20	163 ± 8
THC+CPA	−6.870 ± 0.72	129 ± 13[a]
THC+baclofen	−6.765 ± 0.26	157 ± 9

Note that the A_1 receptor agonist CPA (100 nM) significantly decreased the E_{max} of WIN55,212-2 (WIN) and THC-stimulated [^{35}S]GTPγS binding, but not the EC_{50}. The $GABA_B$ receptor agonist baclofen (100 μM) had no significant influence. Data represent mean values ± SEM ($n=4$) obtained from nonlinear regression analyses of the data shown in Figure 3.
[a]$p<0.05$, compared with appropriate control, calculated using the extra sum-of-squares F test.

presence of CPA (■, $n=4$), and 331 ± 74 in the presence of baclofen (◆, $n=4$); and Figure 4b—98 ± 4 in control (○, $n=4$), 255 ± 25 in the presence of CPA (■, $n=4$), and 161 ± 15 in the presence of baclofen (◆, $n=4$). When applied alone, WIN55,212-2 (0.1 nM–10 μM) concentration dependently stimulated [^{35}S]GTPγS binding, with an EC_{50} ≈255 nM and $E_{max}=251\pm7\%$ ($n=7$; Figure 4a and Table 1). CPA (100 nM) by itself induced a 150 ± 12% net increase from basal [^{35}S]GTPγS binding ($n=4$). Co-application of WIN55,212-2 (0.1 nM–10 μM) with 100 nM CPA (Figure 4a) significantly decreased the E_{max} of WIN55,212-2 to 204 ± 10% ($p<0.001$, $n=4$; Table 1), but not the EC_{50} (≈177 nM; Table 1). This indicates a functional interaction between colocalized CB_1 and A_1 receptors in hippocampal membranes, which impacts on the ability of CB_1 receptors to activate $G\alpha_{i/o}$ proteins. Similarly, the co-application of THC (0.1 nM–1 μM) with 100 nM CPA (Figure 4b) significantly decreased the E_{max} of THC from 163 ± 8% (when applied alone) to 129 ± 13% ($n=4$, $p<0.05$; Table 1), but not the EC_{50}.

To examine if other $G\alpha_{i/o}$-coupled receptors are also capable of interfering with the G-protein coupling of CB_1 receptors, we tested whether combined activation of CB_1 and $GABA_B$ receptors in the hippocampus would also affect the efficacy of WIN55,212-2 in [^{35}S]GTPγS binding. To activate $GABA_B$ receptors, we used 100 μM baclofen, which by itself induced a 118 ± 11% net increase from basal [^{35}S]GTPγS binding ($n=4$), which was not significantly different from the effect of 100 nM CPA. As shown in Figure 4a and b and Table 1, 100 μM baclofen did not affect the WIN55,212-2-induced ($E_{max}=257\pm12\%$, $n=4$) or THC-induced ($E_{max}=157\pm9\%$, $n=4$) stimulation of [^{35}S]GTPγS binding. The reduced efficacy in stimulation of [^{35}S]GTPγS binding by CB_1 with A_1, but not $GABA_B$, suggests that the adenosine A_1 receptors play a specific role in modulating CB_1 signaling in hippocampal presynaptic terminals.

Chronic Caffeine Administration Increases Acute THC-Induced Spatial Memory Deficits in Mice

The evidence that A_1 receptor activation attenuates CB_1 receptor signaling raised the hypothesis that this A_1–CB_1 interplay has a functional impact upon hippocampal-dependent memory. Chronic caffeine consumption is known to induce an increase in adenosine A_1, but not A_{2A} receptors (reviewed by Jacobson *et al*, 1996). Acute systemic THC administration induces CB_1-dependent deficits in working memory (Wise *et al*, 2009). Therefore, we used a hippocampal-dependent, short-term spatial memory testing protocol (Chen *et al*, 2000; see Materials and methods) to study the effects of chronic caffeine administration on the memory deficits induced by an acute systemic

THC injection in mice. Two separate sets of experiments were performed, in which caffeine (3 mg/kg/day), or vehicle, was administered >12 h before trials, and for at least 15 days before the first test with THC.

During the training phase, all subjects learned to perform efficiently at all test parameters, in both sets of experiments (see Figures 5a–c and 6a–c). The number of trials, the total latency, and total pathlength needed to reach the criterion decreased progressively from task to task reaching a plateau in the last training task. A repeated measures two-way ANOVA on these parameters revealed a significant overall learning effect. For 'pathlength' (representative parameter of memory performance), significance values were: $F(4, 100) = 10.2$, $p < 0.0001$ (Figure 5c); and $F(3, 72) = 13.5$, $p < 0.0001$ (Figure 6c). The average swim speed (control parameter for motor activity) was constant throughout the training tasks (Figure 5d: $F(4, 100) = 0.4$, $p = 0.8$; Figure 6d: $F(3, 72) = 1$, $p = 0.4$), with no significant differences between groups (Figure 5d: $F(3, 25) = 1.8$, $p = 0.2$; Figure 6d: $F(3, 24) = 0.2$, $p = 0.9$). In both sets of experiments, there were no differences between groups and no 'group × task' interaction in any parameter during the training period ($p > 0.05$ for all parameters). Thus, chronic caffeine administration by itself did not affect memory performance or motor activity.

For the first set of experiments, the effect of THC (5 mg/kg), or vehicle, given at task 6 (30 min before first trial), as well as the modification of this effect by AM251 (3 mg/kg), given at task 7 (15 min before THC, or vehicle), are displayed in Figure 5a–d. After completion of task 6, each subject rested for 1 day to allow for the metabolic clearance of THC.

There was a significant effect of THC on 'trials to criterion' ($F(1, 25) = 4.24$, $p = 0.05$), 'latency' ($F(1, 25) = 6.98$, $p = 0.01$), and 'pathlength' ($F(1, 25) = 7.18$, $p = 0.01$), but no significant effect on 'average speed' ($F(1, 25) < 0.001$, $p = 0.98$). There were no effects of chronic caffeine treatment on all parameters, but a marginally significant 'chronic caffeine × THC' interaction on 'pathlength' ($F(1,25) = 3.11$, $p = 0.09$). Although acute THC injection (*vs* vehicle) did not induce significant effects in the control (vehicle-treated) group at any parameter, the effect of THC was exacerbated in the chronic caffeine group, on 'latency' ($p = 0.03$, Tukey's *post hoc*; Figure 5b) and 'pathlength' ($p = 0.02$, Tukey's *post hoc*; Figure 5c).

When mice received AM251 pretreatment, there were no significant effects of any treatment group on all parameters, indicating that the effects of THC were dependent on the activation of CB_1 receptors. These results show that chronic caffeine exacerbates the CB_1-dependent actions of THC in a short-term spatial memory task.

For the second set of experiments, the effect of THC (5 mg/kg), or vehicle, given at task 5 (30 min before first trial), the modification of this effect by DPCPX (1 mg/kg), given at task 7 (15 min before THC, or vehicle), as well as the effect of WIN55,212-2 (1 mg/kg), or vehicle, given at task 8 (30 min before first trial), are displayed in Figure 6a–d. After completion of each test task, subjects rested for one

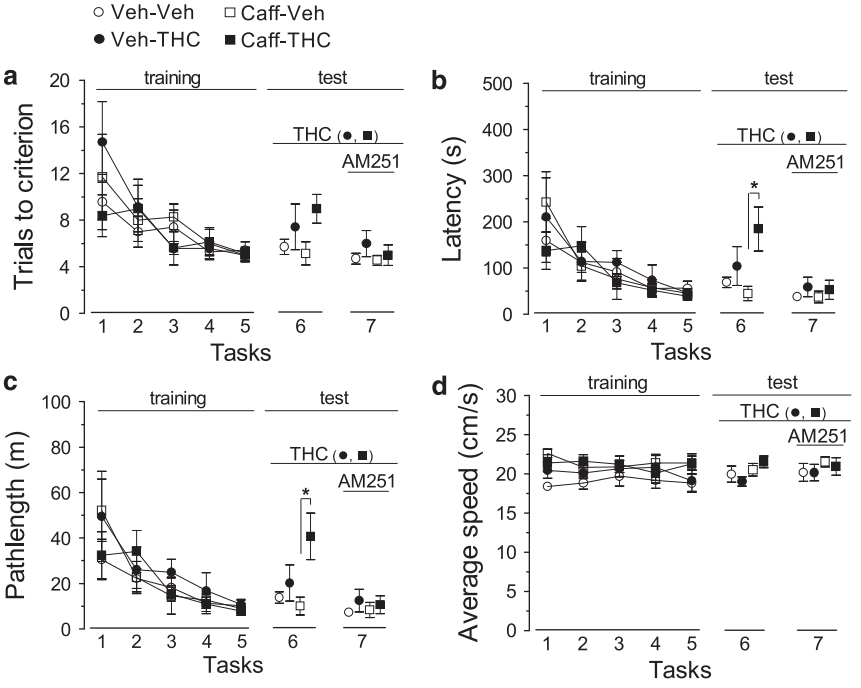

Figure 5 Influence of chronic caffeine administration upon the Δ^9-tetrahydrocannabinol (THC)-induced short-term spatial memory deficits in mice. Caffeine was given daily (3 mg/kg, >12 h before trials), for 15 days before testing the effect of THC (see Methods). (a–d) Mice familiarized with the escape strategies during the first 5 tasks (training) and all groups showed improved performance in (a) the number of trials to reach criterion, (b) the escape latency, (c) swim pathlength, whereas (d) average swim speed remained constant. THC (5 mg/kg), or vehicle, was then tested in the absence (task 6) and in the presence (task 7) of AM251 (3 mg/kg). Subjects rested for 1 day *off drug* after task 6, to allow full metabolization of THC. For clarity of comparison between groups, symbols were nudged at tasks 6 and 7. Note that chronic caffeine exacerbated the spatial memory deficits induced by acute THC, and this effect of THC was fully prevented by previous administration of AM251. All data represent mean ± standard error of mean (SEM) of $n = 7$–8. *$p < 0.05$, two-way analysis of variance, followed by Tukey's *post hoc* test (see text for more details).

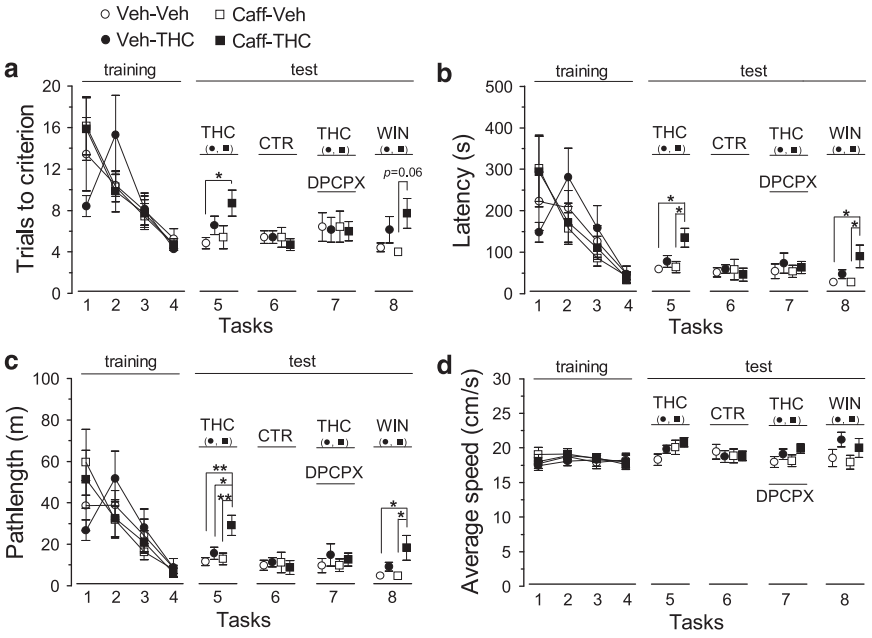

Figure 6 Influence of chronic caffeine administration, and involvement of the adenosine A_1 receptors, upon the acute effects of Δ^9-tetrahydrocannabinol (THC) and WIN55,212-2 on mice tested in a short-term spatial memory task. Caffeine was given daily (3 mg/kg, >12 h before trials), for 15 days before testing the effect of THC (see Methods). (a–d) Mice familiarized with the escape strategies during the first 4 tasks (training) and all groups showed improved performance in (a) the number of trials to reach criterion, (b) the escape latency, and (c) swim pathlength, whereas (d) average swim speed remained constant. THC (5 mg/kg), or vehicle, was tested in the absence (task 5) and in the presence (task 7) of DPCPX (1 mg/kg). A control (CTR) test in the absence of acute drugs was performed at task 6 to measure whether performance levels returned to baseline values. Subjects rested for one day *off drug* after each test task, to allow full metabolization of THC. The effect of WIN55,212-2 (WIN, 1 mg/kg), or vehicle, was tested at task 8. For clarity of comparison between groups, symbols were nudged in tasks 5–8. Note that chronic caffeine exacerbated the spatial memory deficits induced by acute THC and WIN. The effect of THC was prevented by the previous administration of DPCPX. All data represent mean ± standard error of mean (SEM) of $n=7$. $^*p<0.05$, $^{**}p<0.01$, two-way analysis of variance, followed by Tukey's *post hoc* tests (see text for more details).

day *off-drug* to allow for metabolic clearance of THC. Task 6 was a control test in which no acute drug was given, to measure whether performance levels returned to baseline values 48 h after acute THC administration.

Consistent with the first set of experiments, there was a significant effect of THC on 'trials to criterion' ($F(1,24)=6.34$, $p=0.02$), 'latency' ($F(1,24)=8.16$, $p=0.01$), and 'pathlength' ($F(1,24)=9.19$, $p=0.01$), but no significant effect on 'average speed' ($F(1,24)=2.09$, $p=0.16$). On 'pathlength' there was also a significant effect of chronic caffeine treatment ($F(1,24)=4.82$, $p=0.04$), and a marginally significant 'chronic caffeine × THC' interaction ($F(1,24)=3.29$, $p=0.08$). Although acute THC injection (*vs* vehicle) did not induce significant effects in the control (vehicle-treated) group at any parameter, the effect of THC was exacerbated in the chronic caffeine group, on 'latency' ($p=0.02$, Tukey's *post hoc*; Figure 6b) and 'pathlength' ($p=0.01$, Tukey's *post hoc*; Figure 6c). There were also significant differences between THC on the chronic caffeine group *vs* THC in the vehicle group on 'pathlength' ($p=0.04$), and between THC on the chronic caffeine group *vs* the vehicle control group on 'trials to criterion' ($p=0.05$), 'latency' ($p=0.01$), and 'pathlength' ($p=0.006$).

When mice were tested 48 h after the last THC injection, there were no significant effects of any treatment group on all parameters, indicating that the effects of THC were not prevailing after this period. Importantly, when mice received a pretreatment of DPCPX, there were also no significant effects of any treatment group on all parameters, indicating that the effects of THC were reversed by the blockade of A_1 receptors.

Finally, there was a significant effect of WIN55,212-2 on 'trials to criterion' ($F(1,24)=7.64$, $p=0.01$), 'latency' ($F(1,24)=7.50$, $p=0.01$), and 'pathlength' ($F(1,24)=7.74$, $p=0.01$), but no significant effect on 'average speed' ($F(1,24)=4.03$, $p=0.06$). The acute injection of WIN55, 212-2 (*vs* vehicle) did not induce significant effects in the control (vehicle-treated) group at any parameter, but its effects were exacerbated in the chronic caffeine group, on 'latency' ($p=0.03$, Tukey's *post hoc*; Figure 6b) and 'pathlength' ($p=0.03$, Tukey's *post hoc*; Figure 6c). There were also significant differences between WIN55,212-2 on the chronic caffeine group *vs* the vehicle control group on 'latency' ($p=0.03$) and 'pathlength' ($p=0.03$). The lower effect of WIN55,212-2, compared with that of THC, could be owing to pharmacokinetic differences, as the penetration of WIN55,212-2 in the brain following intraperitoneal injection is much lower than that of THC (Petitet *et al*, 1999). Higher doses of WIN55,212-2 were not used to avoid nonspecific effects (Varvel and Lichtman, 2002). These findings show a significant chronic caffeine-induced, and A_1 receptor-mediated, exacerbation of the CB_1-dependent effects on short-term spatial memory.

Chronic Caffeine and A_1 Receptor Number

To quantify the influence of chronic caffeine administration upon A_1 receptor number and affinity in cortico-hippo-

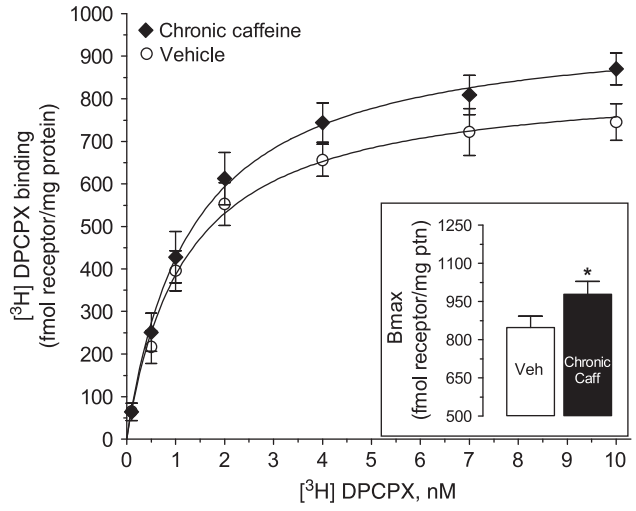

Figure 7 Saturation analysis of specific [^{3}H]DPCPX binding (0.1–10 nM) to cortico-hippocampal membranes (40 μg protein) from chronic caffeine- (3 mg/kg/day, for 22 days, ♦) and vehicle- (○) treated mice. Inset: B_{max} obtained from nonlinear regression analysis. Nonspecific binding was determined at all [^{3}H]DPCPX concentrations by the addition of 2 μM XAC. All points represent mean ± standard error of mean (SEM) of $n = 6$, and each saturation experiment was performed in duplicate. *$p < 0.05$, *vs* control, calculated using the extra sum-of-squares F test.

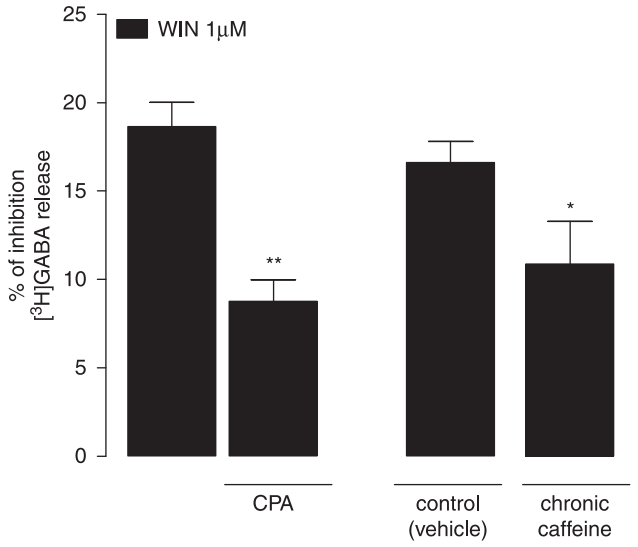

Figure 8 Influence of the adenosine A_1 receptor agonist, CPA (100 nM), and of chronic caffeine administration on the CB_1-mediated inhibition of K^+-evoked [^{3}H]GABA release from mouse cortico-hippocampal synaptosomes. WIN55,212-2 (WIN, 1 μM) was tested in the absence and in the presence of CPA on synaptosomes prepared from untreated mice, as well as on synaptosomes prepared from chronic caffeine- (3 mg/kg/day, for 22 days) or vehicle-treated mice, as indicated below each column (see legend to Figure 1 for details). Note that the effect of WIN was significantly attenuated by CPA as well as by chronic caffeine consumption. Bars represent mean ± SEM of four experiments, performed in duplicate. **$p < 0.01$, compared with the effect of WIN alone; *$p < 0.05$, compared with the effect of WIN in vehicle-treated mice (paired Student's *t*-test).

campal membranes, [^{3}H]DPCPX (0.1–10 nM) binding assays were performed (Figure 7). In vehicle-treated mice, the total number of specific binding sites obtained by nonlinear regression analysis (B_{max}) was 848 ± 44 fmol/mg of protein, whereas the equilibrium dissociation constant (K_D) was 1.20 ± 0.21 nM. In the chronic caffeine group, the B_{max} was increased to 980 ± 50 fmol/mg of protein ($p < 0.05$, $n = 6$, *vs* vehicle group), but K_D (1.31 ± 0.23 nM) was not significantly ($p > 0.05$) affected. Thus, animals under chronic caffeine had ~16% higher density of A_1 receptor without changes in affinity.

Chronic Caffeine and CB_1 Receptor Signaling in Mouse Cortico-Hippocampal Tissue

We first tested the consistency of our *in vitro* results between rats and mice by testing the effect of CPA (100 nM) on the WIN55,212-2-mediated inhibition of K^+-evoked [^{3}H]GABA release from cortico-hippocampal synaptosomes prepared from untreated mice (Figure 8). Consistent to previous observations in rats, the effect of 1 μM WIN55,212-2 alone was 19 ± 1%, and it was significantly attenuated to 9 ± 1% in the presence of CPA ($p < 0.01$, $n = 4$, paired Student's *t*-test; Figure 8). We then analyzed the influence of chronic caffeine administration upon the CB_1 receptor-mediated inhibition of K^+-evoked [^{3}H]GABA release (Figure 8). In control (vehicle-treated) mice, 1 μM WIN55,212-2 inhibited [^{3}H]GABA release by 17 ± 1%, whereas in the chronic caffeine group, the effect of WIN55,212-2 was significantly reduced to 11 ± 2% ($p < 0.05$, $n = 4$, paired Student's *t*-test; Figure 8).

As Figure 9 and Table 2 show, in cortico-hippocampal membranes of vehicle-treated mice, WIN55,212-2 stimulated [^{35}S]GTPγS binding (% of basal) with an EC_{50} ≈989 nM and $E_{max} = 321 \pm 11\%$ ($n = 5$; Figure 9a), whereas THC had an EC_{50} ≈41 nM and $E_{max} = 167 \pm 11\%$ ($n = 4$; Figure 9c). Chronic caffeine administration did not affect the EC_{50} of WIN55,212-2 or THC, but it significantly reduced the E_{max} of WIN55,212-2 to 269 ± 8%, and of THC to 135 ± 8% ($p < 0.05$, $n = 4$–5, extra sum-of-squares F test; Figure 9a and c). As observed in rats (Figure 4a), the co-application of 100 nM CPA in control mice significantly decreased the E_{max} of WIN55,212-2 (254 ± 13%, $p < 0.05$, $n = 5$, extra sum-of-squares F test; Figure 9b and Table 2), but not the EC_{50}. This reduction in WIN55,212-2-stimulated [^{35}S]GTPγS binding, caused by CPA in membranes from control animals, was of similar magnitude as the decrease observed in the chronic caffeine-treated group in the absence of CPA (Table 2). In the chronic caffeine group, CPA did not induce a further decrease in the efficacy of WIN55,212-2 to stimulate G-protein activation (E_{max} = 252 ± 8%; Figure 9a and b and Table 2), which may suggest that chronic caffeine treatment and A_1 receptor activation do not have additive effects upon the modification of CB_1 receptor signaling.

The basal [^{35}S]GTPγS binding in the absence of WIN55,212-2 (Figure 9a and b) or THC (Figure 9c) is represented as 100% in the ordinates, which corresponds to (fmol/mg protein): Figure 9a and b—143 ± 10 (○, $n = 5$), 154 ± 15 (♦, $n = 5$), 324 ± 13 (△, $n = 5$), and 327 ± 25 (▼, $n = 5$); Figure 9c—53 ± 9 (○, $n = 4$) and 67 ± 10 (♦, $n = 4$). CPA (100 nM), by itself, enhanced [^{35}S]GTPγS binding by 122 ± 9% (over twofold net increase from basal binding) in membranes prepared from vehicle-treated subjects, and by 111 ± 7% in the chronic caffeine group ($p > 0.05$, $n = 5$, Student's *t*-test, data not shown); hence, the ability of A_1 receptors to activate G proteins is unaltered

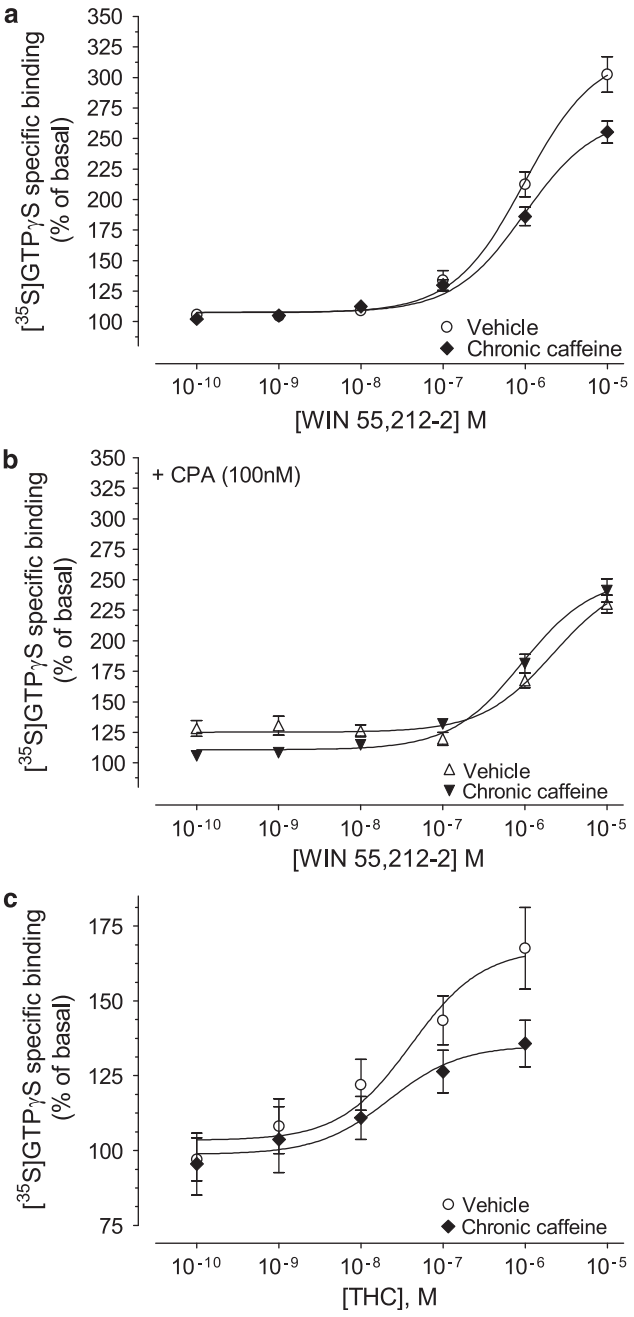

Figure 9 Influence of chronic caffeine administration on CB_1-induced stimulation of G proteins, as assayed by WIN55,212-2- or Δ^9-tetrahydrocannabinol (THC)-induced [^{35}S]GTPγS binding. Cortico-hippocampal membranes (10 μg protein) from chronic caffeine- (◆,▼) and vehicle- (○, △) treated mice were incubated for 30 min at 37 °C with 30 μM GDP, 0.1 nM [^{35}S]GTPγS, and varying concentrations of WIN (0.1 nM–10 μM) in the absence (a) or in the presence of 100 nM CPA (b), or varying concentrations of THC (0.1 nM–1 μM) (c). E_{max} and log EC_{50} values are shown in Table 2. Data represent mean percentage of basal stimulation ± standard error of mean (SEM) of $n = 5$ (a, b) and $n = 4$ (c), performed in duplicate. Non-visible error bars are within symbols.

in chronic caffeine-treated mice. Accordingly, there were no statistically significant differences in the bottom of the nonlinear regression binding curves between chronic caffeine- and vehicle-treated animals, in the presence of CPA ($p > 0.05$, $n = 5$, extra sum-of-squares F test; Figure 9b).

Table 2 E_{max} and Log EC_{50} Values of WIN55,212-2 and THC-stimulated [^{35}S]GTPγS Binding in Mouse Cortico-hippocampal Membranes

	EC_{50}	E_{max}
	−log, *M*	**% of stimulation**
Vehicle		
WIN	−6.005 ± 0.09	321 ± 11
WIN+CPA	−5.654 ± 0.16	254 ± 13[a]
THC	−7.389 ± 0.37	167 ± 11
Chronic caffeine		
WIN	−6.006 ± 0.08	269 ± 8[a]
WIN+CPA	−6.035 ± 0.10	252 ± 8[a]
THC	−7.660 ± 0.56	135 ± 8[a]

Note that the E_{max}, but not the EC_{50}, of WIN55,212-2 (WIN) and THC-stimulated [^{35}S]GTPγS binding was significantly decreased in the chronic caffeine group (3 mg/kg/day, for 22 days), compared with control (vehicle-treated) mice. The A_1 receptor agonist, CPA (100 nM), reduced the E_{max} of WIN55,212-2 in control mice, but did not further decrease the E_{max} of WIN55,212-2 in chronic caffeine-treated mice. The EC_{50} of WIN in either vehicle or chronic caffeine groups was not significantly affected by CPA ($p > 0.05$, extra sum-of-squares F test). Data represent mean values ± SEM ($n = 4$–5) obtained from nonlinear regression analyses of the data shown in Figure 8.

[a]$p < 0.05$, versus corresponding control, calculated using the extra sum-of-squares F test.

Chronic Caffeine and CB_1 Receptor Number

The effects of chronic caffeine administration upon CB_1 signaling *in vitro* suggested that CB_1 receptor number and/or affinity are decreased in these mice. We then directly analyzed the effect of chronic caffeine administration in CB_1 receptor number and affinity by performing [^{3}H]SR141716A (0.1–4 nM) saturation binding assays in tissue collected from mice used in the behavioral experiments. The nonlinear regression analysis of [^{3}H]SR141716A binding to cortical membranes of vehicle-treated mice showed a $B_{max} = 1425 \pm 123$ fmol/mg of protein and a $K_D = 1.4 \pm 0.3$ nM ($n = 10$; Figure 10a). In hippocampal membranes, the B_{max} of [^{3}H]SR141716A was 1322 ± 97 fmol/mg of protein and the $K_D = 1.0 \pm 0.2$ nM ($n = 5$; Figure 10b). In the chronic caffeine group, the B_{max} of [^{3}H]SR141716A binding was lower ($p < 0.05$, compared with vehicle group), and this reduction was observed both in cortical membranes ($B_{max} = 1151 \pm 65$ fmol/mg of protein, $n = 10$) and hippocampal membranes ($B_{max} = 1089 \pm 57$ fmol/mg of protein, $n = 5$). There were no significant differences in affinity, as in the chronic caffeine group the K_D values for [^{3}H]SR141716A binding were 1.0 ± 0.2 nM in the cortical and 0.9 ± 0.1 nM in the hippocampal membranes.

DISCUSSION

This study shows that adenosine A_1 receptors located in GABAergic and glutamatergic nerve terminals of the hippocampus exert a negative modulatory effect on the cannabinoid CB_1 receptor-mediated inhibition of GABA

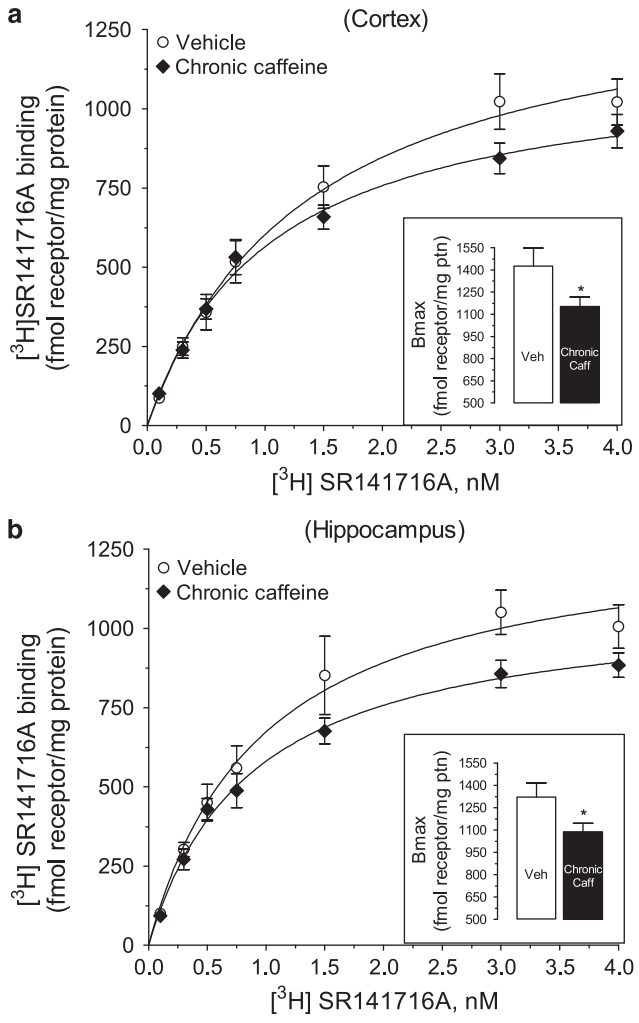

Figure 10 Saturation analysis of specific [^{3}H]SR141716A binding (0.1–4 nM) to (a) cortical and (b) hippocampal membranes (50 μg protein) from chronic caffeine- (3 mg/kg/day, for 22 days) and vehicle-treated mice. Insets: B_{max} values, obtained from nonlinear regression analysis. Nonspecific binding was determined at all [^{3}H]SR141716A concentrations by the addition of 1 μM AM251. All points represent mean ± standard error of mean (SEM) of 5–10 experiments, each performed in duplicate. *$p < 0.05$, *vs* control, calculated using the extra sum-of-squares F test.

and glutamate release. CB_1-mediated G-protein activation is also impaired by A_1 receptor activation. In addition, chronic administration of caffeine leads to an A_1 receptor-mediated enhancement of the CB_1-dependent effects of THC upon short-term spatial memory, despite a reduction in CB_1 receptor number and signaling. This provides first evidence for chronic caffeine-induced alterations in cannabinoid actions in the cortex and hippocampus.

The CB_1–A_1 receptor cross-talk might occur at the G-protein level, as A_1 receptor activation with CPA reduced the efficacy of CB_1 receptor agonists to stimulate [^{35}S]GTPγS binding in the hippocampus. This is in accordance with a previous observation that simultaneous application of CB_1 and A_1 agonists produces less than additive stimulation of [^{35}S]GTPγS binding in cerebellar membranes (Selley *et al*, 2004). Similarly to the A_1 receptors, $GABA_B$ receptors couple to $G\alpha_{i/o}$ proteins and are expressed in the same interneuron populations as CB_1 receptors (Neu *et al*, 2007; Sloviter *et al*, 1999). However, CB_1 receptor-mediated signaling, assessed either as inhibition of [^{3}H]GABA release or stimulation of [^{35}S]GTPγS binding, was unaffected by $GABA_B$ receptor activation, which indicates that the modulation of CB_1 receptor signaling by A_1 receptors is not shared by all $G\alpha_{i/o}$-coupled receptors.

The CB_1 receptor agonist WIN55,212-2 inhibited calcium-dependent [^{3}H]GABA release with a maximum specific effect at 1 μM, in agreement with previous studies (Katona *et al*, 2000; Köfalvi *et al*, 2007). There are clear differences in the magnitude of the reported effects of WIN55,212-2 in studies using different methodologies. For example, we and others (Köfalvi *et al*, 2007) observed that 1 μM WIN55,212-2 induces 15–20% inhibition of K^+-evoked [^{3}H]GABA release from rat hippocampal synaptosomes, whereas several reports show that the same concentration of WIN55,212-2, by activating presynaptic CB_1 receptors, inhibits GABAergic inhibitory postsynaptic currents (IPSCs) in rat hippocampal slices by ~50% (eg, Hájos *et al*, 2000; Hoffman and Lupica, 2000; Wilson and Nicoll, 2001). These differences are likely owing to a combination of factors. The main reason possibly lies in the fact that [^{3}H]GABA release assays provide a quantitative measurement of the amount of GABA released from the whole population of GABAergic nerve terminals at the hippocampus, whereas patch-clamp techniques provide a quantification of endogenous GABA release by measuring the post-synaptic responses of a single hippocampal pyramidal neuron. In addition, differences are likely owing to the type of stimulus used (electrical *vs* high K^+), the time and length of WIN55,212-2 application, and to an amplifying effect of multiple afferents upon IPSC measurements. The effect of 1 μM WIN55,212-2 on the release of [^{3}H]GABA from rat hippocampal slices (Katona *et al*, 1999) is also larger than in synaptosomes. Again, a longer exposure time (6 *vs* 18 min) to WIN55,212-2 and/or the amplification by intrinsic circuits in the slices is a likely explanation for these differences.

The CB_1 receptor-mediated modulation of GABA release from hippocampal CCK-positive interneurons, which express large quantities of CB_1 receptors, is a critical mechanism for spatial and episodic memory, as these interneurons regulate the temporal coordination of principal cell assemblies (Hájos *et al*, 2000; Robbe and Buzsáki, 2009; Robbe *et al*, 2006). However, the CCK-expressing interneuron populations mostly receive input from glutamatergic neurons (see Freund and Buzsáki, 1996; Freund and Katona, 2007), which also express CB_1 (Katona *et al*, 2006; Kawamura *et al*, 2006) and A_1 (Ochiishi *et al*, 1999) receptors. We found that A_1 receptor activation also attenuates CB_1 receptor-mediated inhibition of glutamate release from hippocampal synaptosomes. It was recently reported that endogenous adenosine, by activating A_1 receptors, regulates CB_1-mediated inhibition of glutamatergic synaptic transmission (Hoffman *et al*, 2010; but see Serpa *et al*, 2009). Thus, A_1 and CB_1 receptors also interact at glutamatergic neurons, which indicates that the inhibitory effect of A_1 receptor activation upon the CB_1-dependent stimulation of [^{35}S]GTPγS binding might be derived from an A_1–CB_1 receptor interaction at both GABAergic and glutamatergic neurons. Interestingly,

WIN55,212-2 did not attenuate the inhibitory action of CPA upon glutamate release, suggesting that the modulatory action of A_1 receptors upon CB_1 receptors is not reciprocal.

The relevance of the GABAergic circuitry for the CB_1 receptor-mediated influences upon memory function became firmly established after the demonstration that intraperitoneal THC administration disrupts hippocampal-dependent memory through the activation of CB_1 receptors (Wise *et al*, 2009) in GABAergic, but not glutamatergic, neurons (Puighermanal *et al*, 2009). We now show that chronic administration of a moderate dose of caffeine leads to increased levels of A_1 receptors in the cortico-hippocampal membranes, and to an A_1 receptor-mediated increase of the disruptive effects of acute THC in a hippocampal-dependent short-term spatial memory task. This finding points toward a significant functional relevance of the cross-talk between A_1 and CB_1 receptors in the hippocampus. Interestingly, the motor impairments induced by THC are enhanced by acute activation of A_1 receptors (Dar, 2000). In contrast, acute administration of caffeine antagonizes THC-induced changes in cortico-hippocampal EEG wave recordings (Consroe *et al*, 1976). Several studies show that chronic exposure to adenosine receptor antagonists causes similar actions to acute agonist exposure (see Jacobson *et al*, 1996; Von Lubitz *et al*, 1993), whereas acute administration of caffeine is expected to have opposite effects to acute agonist exposure. The timing of caffeine administration and the presence of caffeine in the blood during testing must also be taken into account when comparing data from different studies. The behavioral tests now reported were performed in the absence of relevant plasma concentrations of caffeine (>12 h after caffeine injection), which was given 2 h after the last behavioral trial, to prevent effects on memory consolidation (Angelucci *et al*, 2002). It is therefore not surprising that acute caffeine administration prevents THC-induced effects (Consroe *et al*, 1976), whereas chronic caffeine exposure exacerbates the memory disruption induced by CB_1 receptor agonists (present work). In a recent study, chronic administration of a high dose of caffeine (210 mg/kg/day) in rats was shown to potentiate CB_1-dependent effects at striatal GABAergic, but not glutamatergic, synapses (Rossi *et al*, 2009). However, it is difficult to draw a comparison with this study, given the differences in the experimental approach, namely the dose of caffeine used, which is not adenosine receptor-selective and is more than about 70 times higher than the equivalent daily human intake. Exposure to high doses of caffeine ($\sim$100 mg/kg/day) leads to altered brain levels of several receptors (Shi *et al*, 1993, 1994), inhibit phosphodiesterases, and may even block $GABA_A$ receptors, among others (see Daly and Fredholm, 1998).

The conclusion that A_1 receptors are involved in the chronic caffeine-induced exacerbation of the effects of THC presently reported is reinforced by the finding that A_1 receptor blockade with DPCPX fully prevented the effects of THC in the chronic caffeine group. DPCPX was administered at a dose that occupies A_1 receptors (Baumgold *et al*, 1992; Hooper *et al*, 1996) while not affecting motor activity (present work; Von Lubitz *et al*, 1993). In addition, DPCPX by itself had no effects in the absence of THC, which suggests that A_1 receptors do not directly influence short-term spatial memory. Furthermore, acute application of DPCPX did not influence the action of THC in vehicle-treated animals, which further supports previous evidence (see above) that chronic and acute blockade of A_1 receptors have different functional consequences. The effects of DPCPX also exclude the involvement of A_{2A} receptors, which are known to modulate the actions of CB_1 receptors in the striatum (Carriba *et al*, 2007; Tebano *et al*, 2009). A_1 and A_{2A} receptors have similar affinities for caffeine (Fredholm *et al*, 1994), yet the expression of A_{2A} receptors in the hippocampus and cortex is much lower than that of A_1 receptors (reviewed by Ribeiro *et al*, 2002). Furthermore, chronic caffeine exposure does not alter the expression of A_{2A} receptors (Jacobson *et al*, 1996).

The increase in A_1 receptor expression caused by moderate doses of chronically administered caffeine results from prevention of tonic adenosine-mediated receptor downregulation (see Fredholm *et al*, 1999). The dose of caffeine we have administered to mice is equivalent to the estimated US average human daily caffeine consumption (Barone and Roberts, 1996) and, in addition to the expected increase in A_1 receptor levels, it also caused a decrease of cortical and hippocampal CB_1 receptors. Accordingly, in chronic caffeine-treated mice there was a reduction in the CB_1 receptor-mediated inhibition of GABA release and stimulation of G-protein activation. As tonic activation of A_1 receptors was prevented through elimination of endogenous adenosine by vertical superfusion in the [^{3}H]GABA release assays, and by ADA in the [^{35}S]GTPγS binding assays, it is unlikely that chronic caffeine-induced A_1 receptor upregulation could be responsible for reduction of CB_1-dependent actions in the *in vitro* assays. Most probably, chronic caffeine intake, by inducing an imbalance in adenosinergic signaling, disturbs the A_1–CB_1 cross-talk, which reflects in CB_1 receptor downregulation. Independently of the exact mechanisms involved, it is clear that CB_1 receptors are affected after chronic caffeine exposure.

Given that chronic caffeine decreases CB_1 and increases A_1 receptor levels, and that activation of A_1 receptors inhibited the CB_1-mediated actions in the *in vitro* assays, it was somewhat surprising that the memory impairment caused by the CB_1 receptor agonists was exacerbated by chronic caffeine intake. It is therefore evident that changes observed *in vitro* do not necessarily reflect, in a linear way, the effects upon the integrated hippocampal circuitry *in vivo*. An imbalance in GABAergic transmission resulting from the chronic caffeine-induced alterations of A_1 and CB_1 levels may have occurred, leading to some adaptive changes in the pyramidal cells and/or in the parvalbumin-expressing (PV) GABAergic neurons, which do not express CB_1 receptors (Katona *et al*, 1999). Interestingly, the blockade of GABAergic transmission was shown to reverse the cognitive effects of acute THC *in vivo* (Varvel *et al*, 2005). A critical imbalance in the temporal coordination of pyramidal cell firing could have become evident when THC was administered, if there was an enhanced sensitivity to the fast spiking activity of PV cells; hence, leading to increased inhibition of pyramidal cell firing.

In summary, this work highlights two relevant factors influencing cannabinoid CB_1 signaling in the hippocampus: the activity of A_1 receptors and the chronic consumption of caffeine. This A_1–CB_1 receptor interaction therefore points toward the possibility that the pathophysiological or

therapeutically relevant actions operated by CB_1 receptors can be significantly affected by interference with A_1 receptor activity, as is the case of chronic caffeine intake.

ACKNOWLEDGEMENTS

We thank Dr Stephanie Daumas and Dr Bruno da Silva (University of Edinburgh) for helpful assistance in the experimental design of the behavioral tests. We acknowledge the Institute of Physiology at the Faculty of Medicine (University of Lisbon) for the animal house facility, Prof. Alexandre de Mendonça (University of Lisbon) and Dr Attila Kofalvi (University of Coimbra) for useful scientific discussions, and Dr João Maroco (Instituto Superior de Psicologia Aplicada, ISPA) for valuable advice with statistical analysis. This work was supported by grants from Fundação para a Ciência e Tecnologia (FCT, VCS: SFRH/BD/21359/2005 and NAL: SFRH/BD/21374/2005), and the European Union (European Cooperation in Science and Technology (COST) COST B30 concerted action, Neural Regeneration and Plasticity (NEREPLAS)).

DISCLOSURE

The authors declare that, except for income received from primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal holdings that could be perceived as constituting a potential conflict of interest.

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