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## Title

Regulation of hippocampal cannabinoid CB<sub>1</sub> receptor actions by adenosine A<sub>1</sub> receptors and chronic caffeine administration: implications for the effects of  $\Delta^9$ -tetrahydrocannabinol on spatial memory

**Running Title:** A<sub>1</sub>-CB<sub>1</sub> receptor interplay in the hippocampus

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**Abstract**

The cannabinoid CB<sub>1</sub> receptor-mediated modulation of  $\gamma$ -aminobutyric acid (GABA) release from inhibitory interneurons is important for the integrity of hippocampal-dependent spatial memory. Although adenosine A<sub>1</sub> receptors have a central role in fine-tuning excitatory transmission in the hippocampus, A<sub>1</sub> receptors localized in GABAergic cells do not directly influence GABA release. CB<sub>1</sub> and A<sub>1</sub> receptors are the main targets for the effects of two of the most heavily consumed psychoactive substances worldwide:  $\Delta^9$ -tetrahydrocannabinol (THC, a CB<sub>1</sub> receptor agonist) and caffeine (an adenosine receptor antagonist). We first tested the hypothesis that an A<sub>1</sub>-CB<sub>1</sub> interaction influences GABA and glutamate release in the hippocampus. We found that A<sub>1</sub> receptor activation attenuated the CB<sub>1</sub>-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 (i.p., 3 mg/kg/day, for 15 days, >12h before trials) led to an A<sub>1</sub>-mediated enhancement of the CB<sub>1</sub>-dependent acute disruptive effects of THC on a short-term spatial memory task, despite inducing a reduction in cortical and hippocampal CB<sub>1</sub> receptor number and an attenuation of CB<sub>1</sub> coupling with G-protein. A<sub>1</sub> receptor levels were increased following chronic caffeine administration. This study demonstrates that A<sub>1</sub> receptors exert a negative modulatory effect on CB<sub>1</sub>-mediated inhibition of GABA release, and provides the first evidence of chronic caffeine-induced alterations on the cannabinoid system in cortex and hippocampus, with functional implications in spatial memory.

**Key words:** 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<sub>1</sub> and CB<sub>1</sub> receptors, respectively. Several forms of learning, memory and other cognitive functions require the integrity of the hippocampal circuitry, where A<sub>1</sub> and CB<sub>1</sub> receptors were shown to have important roles due to their presynaptic regulation of neurotransmitter release (e.g. 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  $\Delta^9$ -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, 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<sub>1</sub>, but not A<sub>2A</sub> 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, e.g. 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<sub>1</sub> receptors in the central nervous system (CNS) to produce motor and cognitive disrupting effects (see Pertwee, 2008).

Hippocampal CB<sub>1</sub> receptors are primarily found in presynaptic terminals of cholecystokinin (CCK)-expressing 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). Accordingly, it was recently demonstrated that presynaptic CB<sub>1</sub> receptors at GABAergic, but not glutamatergic, neurons are required for THC-induced amnesia (Puighermanal *et al*, 2009). In contrast, A<sub>1</sub> 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<sub>1</sub> receptors are present in hippocampal GABAergic interneurons (Ochiishi *et al*, 1999), where they control the actions of vasoactive intestinal peptide (Cunha-Reis *et al*, 2008).

Both A<sub>1</sub> and CB<sub>1</sub> 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<sub>1</sub> receptors modulate the motor incoordination effects induced by acute administration of THC or CB<sub>1</sub> receptor

agonist CP55,940 (Dar, 2000; Dar and Mustafa, 2002; DeSanty and Dar, 2001). Furthermore, prolonged intracerebellar administration of a CB<sub>1</sub> or A<sub>1</sub> 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<sub>1</sub>-mediated inhibition of excitatory synaptic transmission in the hippocampus is modulated by endogenous adenosine, through A<sub>1</sub> receptor activation (Hoffman *et al*, 2010; but see Serpa *et al*, 2009). These previous findings raised the hypothesis that a functional interaction between A<sub>1</sub> and CB<sub>1</sub> receptors in the hippocampus may have cognitive and pathophysiological implications, particularly for the effects of cannabis and caffeine consumption in humans.

The present study initially focused upon the possibility that an A<sub>1</sub>-CB<sub>1</sub> interaction influences GABA and glutamate release. We found that A<sub>1</sub> receptor activation attenuated the CB<sub>1</sub>-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 (i.p., 3mg/kg/day, for 15 days, >12h before trials) increased A<sub>1</sub> receptor levels, and did not by itself cause measurable effects on spatial memory, but led to an A<sub>1</sub>-mediated exacerbation of the CB<sub>1</sub>-dependent acute effects of THC in a spatial memory task.

## Methods

### Animals

Adult male 6-8 weeks old Wistar rats (Harlan Interfauna Iberica, Spain), and 12-16 weeks old C57Bl/6J mice (Harlan-Olac, UK) were used. Animals were housed in a temperature and humidity regulated room with a 12h 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-<sup>3</sup>H]butyric acid ([<sup>3</sup>H]GABA), L-[G-<sup>3</sup>H]glutamic acid ([<sup>3</sup>H]glutamate), 1,3-[<sup>3</sup>H]-dipropyl-8-cyclopentylxanthine ([<sup>3</sup>H]-DPCPX), and [<sup>3</sup>H]SR141716A were obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). Guanosine 5'-( $\gamma$ -[<sup>35</sup>S]-thio) Triphosphate, ([<sup>35</sup>S]-GTP $\gamma$ S) was from Perkin Elmer NEN Radiochemicals (Boston, MA, USA). Adenosine deaminase (ADA, EC 3.5.4.4) was from Roche Diagnostics (Indianapolis, IN. USA).  $\Delta^9$ -Tetrahydrocannabinol (THC, >98% purity) was from THC Pharm (Frankfurt, Germany), or Tocris Bioscience (Bristol, UK). CdCl<sub>2</sub>, caffeine (anhydrous base), GABA, aminooxyacetic acid (AOAA), guanosine diphosphate (GDP), guanosine 5'-O-[gamma-thio]triphosphate (GTP $\gamma$ S) were from Sigma (St Louis, MO,



USA). 1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride (SKF89976A), N<sup>6</sup>-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-[4-[(2-Aminoethyl) amino]carbonylmethoxyphenyl] 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), [(3S)-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-1H-pyrazole-3-carboxamide (AM251), were from Tocris Bioscience (Bristol, UK). ((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 2 rats, or cortico-hippocampal synaptosomes from 1 mouse. Animals were decapitated under halothane anesthesia and synaptosomes were prepared as previously described (Assaife-Lopes *et al*, 2010), with modifications. Briefly, tissue was dissected in a continuously oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) ice-cold artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl 125, KCl 3, NaH<sub>2</sub>PO<sub>4</sub> 1, NaHCO<sub>3</sub> 25, CaCl<sub>2</sub> 1.5, MgSO<sub>4</sub> 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 14000 *g* for 12 min to obtain a stratified pellet, containing synaptosomes (McMahon *et al*, 1992; Phelan and Gordon-Weeks, 1997).

### **[<sup>3</sup>H]-neurotransmitter release assays**

For [<sup>3</sup>H]GABA release assays, the pellet was resuspended and synaptosomes were incubated for 20 min, at 37°C, with [<sup>3</sup>H]GABA (1.5 μCi/ml, 1.85 nM), and 0.625 μM of unlabelled GABA to decrease specific activity of [<sup>3</sup>H]GABA to 2.3 μCi/nmol. Incubation and superfusion solutions consisted of oxygenated aCSF containing the GABA transaminase inhibitor AOAA. For [<sup>3</sup>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 [<sup>3</sup>H]glutamate. Synaptosomes were then layered over GF/C filters (Milipore, MA, USA) on an 8-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<sup>+</sup> (isomolar substitution of Na<sup>+</sup> with K<sup>+</sup>) at the 6<sup>th</sup> (S<sub>1</sub>) and 24<sup>th</sup> (S<sub>2</sub>) minutes of collection time. CB<sub>1</sub> agonists were added to the superfusion medium from the 18<sup>th</sup> minute onward, to measure effects on S<sub>2</sub>. The CB<sub>1</sub> antagonist, AM251, or A<sub>1</sub> and GABA<sub>B</sub> receptor ligands were added from the 15<sup>th</sup> minute of the washout period onward, i.e. present during S<sub>1</sub> and S<sub>2</sub> in order to assess their ability to modify the effect of WIN55,212-2 (applied before S<sub>2</sub>). Each condition was tested in duplicate, as commonly accepted in

this paradigm (e.g. 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 [ $^3H$ ]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 previously described (e.g. 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 14000 *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 14000 *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. For [ $^{35}S$ ]-GTP $\gamma$ S binding assays: Tris 50,  $MgCl_2$  5, NaCl 100, EGTA 0.2, pH 7.4. Protein content was determined by the Bradford method (Bradford, 1976). For

[<sup>3</sup>H]DPCPX binding assays, membranes (40 µg protein) were incubated for 1h at room temperature in a final incubation volume of 300 µl containing 4 U/ml ADA, and using 2 µM of XAC to measure non-specific binding. For [<sup>3</sup>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 non-specific binding. For [<sup>35</sup>S]-GTPγS binding assays, membranes (10 µg of protein) were incubated with 0.1 nM [<sup>35</sup>S]-GTPγS and 0.1 nM-10 µM of WIN55,212-2, in 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 [<sup>35</sup>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<sub>1</sub> agonists was calculated by subtracting the increase in [<sup>35</sup>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 prior to 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 previously described (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 (i.p.) 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 2 separate sets of water maze experiments in which mice were randomly assigned to 4 experimental groups of 7 to 8 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 8 trials per day, to perform the task until reaching a performance criterion of  $\leq 7$  s average latency on three consecutive trials. A 15 min inter-trial 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, 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,  $22\pm 1^\circ\text{C}$ ) was placed over an infrared lightbox (Tracksys, Nottingham, UK) in a room with various visible external cues. A transparent platform was  $\sim 0.5$  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, Netherlands) monitored all performances .

### **Statistical analysis**

Statistical significance was tested using paired Student's t-test, one-way 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 Inc., an IBM Company, Chicago, IL). GraphPad Prism 5.0 (GraphPad Prism Software Inc., San Diego, CA) 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<sub>1</sub> receptor activation attenuates the CB<sub>1</sub> receptor-dependent inhibition of [<sup>3</sup>H]GABA and [<sup>3</sup>H]glutamate release from rat hippocampal nerve terminals**

In control conditions, pooling data from all experiments performed, the average basal release of [<sup>3</sup>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 3-fold increase in the [<sup>3</sup>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  $0.94 \pm 0.01$ . Depolarization by  $K^+$  mainly induced a calcium-dependent release of [<sup>3</sup>H]GABA since blockade of voltage-gated  $Ca^{2+}$  channels (VGCCs) by  $Cd^{2+}$  ( $CdCl_2$ , 200  $\mu$ M) 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 [<sup>3</sup>H]GABA released upon  $K^+$  depolarization, since blockade of GABA transporters with SKF89976a (20  $\mu$ M) inhibited its release by  $34 \pm 1.6\%$  ( $n=8$ ,  $p < 0.001$ , data not shown).

To induce a CB<sub>1</sub> receptor-dependent effect on [<sup>3</sup>H]GABA release, we used the potent cannabinoid receptor agonist WIN55,212-2, which has been previously shown to inhibit evoked [<sup>3</sup>H]GABA release from hippocampal synaptosomes through a CB<sub>1</sub> specific mechanism, having a maximum CB<sub>1</sub> selective effect at 1  $\mu$ M (Köfalvi *et al*, 2007). Application of 1  $\mu$ M WIN55,212-2 six minutes before  $S_2$ , caused a decrease of basal [<sup>3</sup>H]GABA outflow and inhibited evoked GABA release (Figure 1a) with an average  $S_2/S_1$  of  $0.78 \pm 0.01$  ( $n=31$ ) that represents an inhibition of  $16.7 \pm 1.4\%$  ( $n=31$ ),

when compared to control  $S_2/S_1$  within each experiment. Blockade of VGCCs by  $Cd^{2+}$  ( $CdCl_2$ , 200  $\mu M$ ) 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  $\mu M$ ) did not alter the effect of WIN55,212-2 ( $n=5$ , Figure 1b), which suggests that the effect of 1  $\mu M$  WIN55,212-2 upon  $[^3H]GABA$  release is exerted through the inhibition of  $Ca^{2+}$ -dependent exocytotic release. The effect of WIN55,212-2 (0.01-10  $\mu M$ ) on  $K^+$ -evoked  $[^3H]GABA$  release was concentration-dependent (Figure 1c). Since WIN55,212-2 is known to directly block N-type VGCCs at concentrations above 1  $\mu M$  (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  $\mu M$ ) fully blocked the effect of 1  $\mu M$  but not of 10  $\mu M$  of WIN55,212-2 (Figure 1c). Higher concentrations of AM251 were not used to avoid loss of selectivity (see Köfalvi, 2008; Köfalvi *et al*, 2007). The enantiomer had no significant effect applied at 1  $\mu M$  but it inhibited evoked  $[^3H]GABA$  release by  $22.4\pm 1.4\%$  at 10  $\mu M$  ( $n=5$ ,  $p<0.05$ , Figure 1c), which indicates that the effect of 1  $\mu M$  WIN55,212-2 upon  $[^3H]GABA$  release is  $CB_1$  receptor-dependent. It is noteworthy that the effect of 10  $\mu M$  WIN55,212-2 was larger than the effect of 10  $\mu M$  WIN55,212-3, which indicates that the effect of 10  $\mu M$  WIN55,212-2 still encompasses a  $CB_1$  receptor-dependent component. The partial  $CB_1$  agonist  $\Delta^9$ -Tetrahydrocannabinol (THC, 1  $\mu M$ ) inhibited  $K^+$ -evoked  $[^3H]GABA$  release by  $8.9\pm 0.9\%$  ( $n=8$ ,  $p<0.05$ , Figure 1c).



Consistent with previous observations (Cunha and Ribeiro, 2000), adenosine A<sub>1</sub> receptor activation with the selective agonist CPA (100 nM) before S<sub>2</sub>, did not affect K<sup>+</sup>-evoked [<sup>3</sup>H]GABA release (2.7±3% of control S<sub>2</sub>/S<sub>1</sub>, n=3, *p*>0.05, data not shown). To evaluate the influence of A<sub>1</sub> receptors on the CB<sub>1</sub>-mediated inhibition of GABA release, we tested the effect of WIN55,212-2 (applied before S<sub>2</sub>) in the presence of CPA (100 nM, applied throughout the experiment, S<sub>1</sub>+S<sub>2</sub>). 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 *p*<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<sup>2+</sup> channel-dependent mechanism and not involving CB<sub>1</sub> receptors, we performed the experiments using WIN55,212-3. Application of WIN55,212-3 (10 μM, S<sub>2</sub>) by itself inhibited [<sup>3</sup>H]GABA release by 22±2%, while in 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<sub>1</sub> receptor-dependent component, but not upon the Ca<sup>2+</sup> channel-dependent mechanisms, affected by high micromolar concentrations of WIN55,212-2. The blockade of A<sub>1</sub> receptors with the antagonist DPCPX (50 nM) did not, on its own, alter the effects of 1 μM 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<sub>1</sub>, A<sub>1</sub> and GABA<sub>B</sub> are known to converge when co-expressed in cerebellar neurons (Selley *et al*, 2004). Furthermore, both CB<sub>1</sub> and GABA<sub>B</sub>

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 activation of  $GABA_B$  receptors. As shown in Figure 2c, the effect of WIN55,212-2 (1  $\mu$ M) was unchanged ( $p > 0.05$ ,  $n = 5$ , paired Student's t-test) by the presence of the  $GABA_B$  receptor agonist baclofen (10  $\mu$ M). 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 (e.g. 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 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  $\mu$ M) inhibited the release of [ $^3$ H]glutamate by  $16 \pm 2\%$  and blockade of  $A_1$  receptors with DPCPX (50 nM) did not modify the effect of WIN55,212-2 ( $15 \pm 1\%$ ,  $p > 0.05$ ,  $n = 4$ ). This

indicates that endogenous adenosine was effectively washed out by the continuous vertical flow of superfusion medium (see Methods).

As shown in Figure 3b, the inhibitory effect of 1  $\mu$ M WIN55,212-2 upon glutamate release ( $19\pm 2\%$ ) was significantly attenuated to  $7\pm 2\%$  ( $n=5$ ,  $p<0.01$ , 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  $\mu$ M WIN55,212-2. CPA (100 nM) inhibited [ $^3$ H]glutamate release by  $12\pm 4\%$  and this effect was not modified in the presence of 1  $\mu$ M WIN55,212-2 ( $13\pm 2\%$ ,  $n=4$ ,  $p>0.05$ , 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]-GTP $\gamma$ S binding in hippocampal membranes with the full agonist of  $CB_1$  receptors, WIN55,212-2, or with the partial agonist THC, in absence and in the presence of  $A_1$  receptor agonist CPA. The basal [ $^{35}$ S]GTP $\gamma$ S 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:  $149 \pm 16$  in control ( $\circ$ ,  $n=7$ ),  $311 \pm 50$  in presence of CPA ( $\blacksquare$ ,  $n=4$ ), and  $331 \pm 74$  in presence of baclofen ( $\blacklozenge$ ,  $n=4$ ); Figure 4b:  $98 \pm 4$  in control ( $\circ$ ,  $n=4$ ),  $255 \pm 25$  in presence of CPA ( $\blacksquare$ ,  $n=4$ ), and  $161 \pm 15$  in presence of baclofen ( $\blacklozenge$ ,  $n=4$ ). When applied alone, WIN55,212-2 (0.1 nM-10  $\mu$ M) concentration-dependently stimulated [ $^{35}$ S]-GTP $\gamma$ S binding, with an  $EC_{50} \approx 255$  nM and  $E_{max}=251 \pm 7\%$  (Figure 4a, Table 1,  $n=7$ ). CPA (100 nM) by itself induced a  $150 \pm 12\%$  net increase from basal [ $^{35}$ S]-GTP $\gamma$ S binding ( $n=4$ ). Co-application of WIN55,212-2 (0.1 nM-10  $\mu$ M) with 100 nM CPA (Figure 4a) significantly decreased the  $E_{max}$  of WIN55,212-2 to  $204 \pm 10\%$  (Table 1,  $p < 0.001$ ,  $n=4$ ), but not the  $EC_{50}$  ( $\approx 177$  nM, Table 1). This indicates a functional interaction between co-localized 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  $\mu$ M) with 100 nM CPA (Figure 4b) significantly decreased the  $E_{max}$  of THC from  $163 \pm 8\%$  (when applied alone) to  $129 \pm 13\%$  (Table 1,  $n=4$ ,  $p < 0.05$ ), 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]-GTP $\gamma$ S binding. To activate GABA $_B$  receptors we used 100  $\mu$ M baclofen, which by itself induced a  $118 \pm 11\%$  net increase from basal [ $^{35}$ S]-GTP $\gamma$ S binding ( $n=4$ ), that was not significantly different from the effect of 100 nM CPA. As shown in Figure 4a,b and Table 1, 100  $\mu$ M baclofen did not affect the WIN55,212-2-induced ( $E_{max}=257 \pm 12\%$ ,  $n=4$ ) or THC-induced ( $E_{max}=157 \pm 9\%$ ,  $n=4$ ) stimulation of [ $^{35}$ S]-GTP $\gamma$ S binding. The reduced efficacy in stimulation of [ $^{35}$ S]-GTP $\gamma$ S binding by CB $_1$  with A $_1$  but not GABA $_B$  suggests

that the adenosine A<sub>1</sub> receptors play a specific role in modulating CB<sub>1</sub> signaling in hippocampal presynaptic terminals.

### **Chronic caffeine administration increases acute THC-induced spatial memory deficits in mice**

The evidence that A<sub>1</sub> receptor activation attenuates CB<sub>1</sub> receptor signaling raised the hypothesis that this A<sub>1</sub>-CB<sub>1</sub> interplay has a functional impact upon hippocampal-dependent memory. Chronic caffeine consumption is known to induce an increase in adenosine A<sub>1</sub>, but not A<sub>2A</sub> receptors (reviewed by Jacobson *et al*, 1996). Acute systemic THC administration induces CB<sub>1</sub>-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 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 Figure 5a-c, 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 2-way analysis of variance (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 $\times$ 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 one 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 $\times$ THC' interaction on 'pathlength' [ $F(1,25)=3.11$ ,  $p=0.09$ ]. Whilst 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 *post hoc*, Figure 5b) and 'pathlength' ( $p=0.02$ , Tukey *post hoc*, Figure 5c).

When mice received AM251 pre-treatment, 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<sub>1</sub> receptors. These results show that chronic caffeine exacerbates the CB<sub>1</sub>-dependent actions of THC in a short-term spatial memory task.

For the second set of experiments, the effect of THC (5 mg/kg, i.p.), or vehicle, given at task 5 (30 min before first trial), the modification of this effect by DPCPX (1 mg/kg, i.p.), given at task 7 (15 min before THC, or vehicle), as well as the effect of WIN55,212-2 (1 mg/kg, i.p.), 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 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 hours 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  $\times$  THC' interaction [ $F(1,24)=3.29$ ,  $p=0.08$ ]. Whilst 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 *post hoc*, Figure 6b) and 'pathlength' ( $p=0.01$ , Tukey *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 pre-treatment 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 *post hoc*, Figure 6b) and 'pathlength' ( $p=0.03$ , Tukey *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 to that of THC, could be due to pharmacokinetic differences, since the penetration of WIN55,212-2 in the brain following i.p. injection is much lower than that of THC (Petitet *et al*, 1999). Higher doses of WIN55,212-2 were not used to avoid non-specific 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<sub>1</sub> receptor number**

To quantify the influence of chronic caffeine administration upon A<sub>1</sub> receptor number and affinity in cortico-hippocampal membranes, [<sup>3</sup>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<sub>max</sub>) was 848±44 fmol/mg of protein, while the equilibrium dissociation constant (K<sub>D</sub>) was 1.20±0.21 nM. In the chronic caffeine group, the B<sub>max</sub> was increased to 980±50 fmol/mg of protein (p<0.05, n=6, vs vehicle group) but K<sub>D</sub> (1.31±0.23 nM) was not significantly (p>0.05) affected. Thus, animals under chronic caffeine had ~16% higher density of A<sub>1</sub> receptor without changes in affinity.

### **Chronic caffeine and CB<sub>1</sub> 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<sup>+</sup>-evoked [<sup>3</sup>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<sub>1</sub> receptor-mediated inhibition of K<sup>+</sup>-evoked [<sup>3</sup>H]GABA release (Figure 8). In control (vehicle-treated) mice, 1 μM WIN55,212-2 inhibited [<sup>3</sup>H]GABA release by 17±1%, while 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 [<sup>35</sup>S]-GTP $\gamma$ S binding (% of basal) with an EC<sub>50</sub>  $\approx$ 989 nM and E<sub>max</sub>=321 $\pm$ 11% (n=5, Figure 9a), while THC had an EC<sub>50</sub>  $\approx$ 41 nM and E<sub>max</sub>=167 $\pm$ 11% (n=4, Figure 9c). Chronic caffeine administration did not affect the EC<sub>50</sub> of WIN55,212-2 or THC, but it significantly reduced the E<sub>max</sub> of WIN55,212-2 to 269 $\pm$ 8%, and of THC to 135 $\pm$ 8% ( $p$ <0.05, n=4-5, extra sum-of-squares F test, Figure 9a, c). As observed in rats (Figure 4a), the co-application of 100 nM CPA in control mice significantly decreased the E<sub>max</sub> of WIN55,212-2 (254 $\pm$ 13%,  $p$ <0.05, n=5, extra sum-of-squares F test, Figure 9b, Table 2), but not the EC<sub>50</sub>. This reduction in WIN55,212-2-stimulated [<sup>35</sup>S]-GTP $\gamma$ S 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<sub>max</sub>=252 $\pm$ 8%, Figure 9a,b, Table 2), which may suggest that chronic caffeine treatment and A<sub>1</sub> receptor activation do not have additive effects upon the modification of CB<sub>1</sub> receptor signaling.

The basal [<sup>35</sup>S]GTP $\gamma$ S binding in the absence of WIN55,212-2 (Figure 9a,b) or THC (Figure 9c) is represented as 100% in the ordinates, which corresponds to (fmol/mg protein): Figure 9a,b: 143  $\pm$  10 (O, n=5), 154  $\pm$  15 (◆, n=5), 324  $\pm$  13 ( $\Delta$ , n=5), and 327  $\pm$  25 ( $\blacktriangledown$ , n=5); Figure 9c: 53  $\pm$  9 (O, n=4), 67  $\pm$  10 (◆, n=4). CPA (100 nM), by itself, enhanced [<sup>35</sup>S]-GTP $\gamma$ S binding by 122 $\pm$ 9% (over 2 fold net increase from basal binding) in membranes prepared from vehicle-treated subjects, and by 111 $\pm$ 7% in the chronic caffeine group ( $p$ >0.05, n=5, Student's t-test, data not shown), hence the ability of A<sub>1</sub>

receptors to activate G-proteins is unaltered in chronic caffeine treated mice. Accordingly, there were no statistically significant differences in the bottom of the non-linear 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).

### **Chronic caffeine and CB<sub>1</sub> receptor number**

The effects of chronic caffeine administration upon CB<sub>1</sub> signaling *in vitro* suggested that CB<sub>1</sub> receptor number and/or affinity are decreased in these mice. We then directly analyzed the effect of chronic caffeine administration in CB<sub>1</sub> receptor number and affinity by performing [<sup>3</sup>H]SR141716A (0.1-4nM) saturation binding assays in tissue collected from mice used in the behavioral experiments. The non-linear regression analysis of [<sup>3</sup>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 [<sup>3</sup>H]SR141716A was  $1322 \pm 97$  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 [<sup>3</sup>H]SR141716A binding was lower ( $p < 0.05$ , compared to 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 [<sup>3</sup>H]SR141716A binding were  $1.0 \pm 0.2$  nM in cortical, and  $0.9 \pm 0.1$  nM in hippocampal membranes.

## Discussion

The present study demonstrates 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 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 since  $A_1$  receptor activation with CPA reduced the efficacy of  $CB_1$  receptor agonists to stimulate [ $^{35}$ S]-GTP $\gamma$ S 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]-GTP $\gamma$ S 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]-GTP $\gamma$ S 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  $\mu$ M, 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  $\mu$ M WIN55,212-2 induces 15-20% inhibition of K<sup>+</sup>-evoked [<sup>3</sup>H]GABA release from rat hippocampal synaptosomes, while several reports show that the same concentration of WIN55,212-2, by activating presynaptic CB<sub>1</sub> receptors, inhibits GABAergic inhibitory postsynaptic currents (IPSCs) in rat hippocampal slices by ~50% (e.g. Hájos *et al*, 2000; Hoffman and Lupica, 2000; Wilson and Nicoll, 2001). These differences are likely due to a combination of factors. The main reason possibly lies in the fact that [<sup>3</sup>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, while 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 due to the type of stimulus used (electrical vs. high K<sup>+</sup>), the time and length of WIN55,212-2 application, and to an amplifying effect of multiple afferents upon IPSC measurements. The effect of 1  $\mu$ M WIN55,212-2 on the release of [<sup>3</sup>H]GABA from rat hippocampal slices (Katona *et al*, 1999) is also larger than in synaptosomes. Again, a longer exposure time (6 min 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<sub>1</sub> receptor-mediated modulation of GABA release from hippocampal CCK-positive interneurons, which express large quantities of CB<sub>1</sub> 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<sub>1</sub> (Katona *et al*, 2006; Kawamura *et al*, 2006) and A<sub>1</sub> (Ochiishi *et al*, 1999) receptors. We found that A<sub>1</sub> receptor activation also attenuates CB<sub>1</sub> receptor-mediated inhibition of glutamate release from hippocampal synaptosomes. It was recently reported that endogenous adenosine, by activating A<sub>1</sub> receptors, regulates CB<sub>1</sub>-mediated inhibition of glutamatergic synaptic transmission (Hoffman *et al*, 2010; but see Serpa *et al*, 2009). Thus, A<sub>1</sub> and CB<sub>1</sub> receptors also interact at glutamatergic neurons, which indicates that the inhibitory effect of A<sub>1</sub> receptor activation upon the CB<sub>1</sub>-dependent stimulation of [<sup>35</sup>S]-GTPγS binding might be derived from an A<sub>1</sub>-CB<sub>1</sub> 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<sub>1</sub> receptors upon CB<sub>1</sub> receptors is not reciprocal.

The relevance of the GABAergic circuitry for the CB<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> receptors in the cortico-hippocampal membranes, and to an A<sub>1</sub> receptor-mediated increase of the disruptive effects of acute THC in a hippocampal-dependent short-term spatial memory task. This finding points towards a significant functional relevance of the cross-talk between A<sub>1</sub>

and CB<sub>1</sub> receptors in the hippocampus. Interestingly, the motor impairments induced by THC are enhanced by acute activation of A<sub>1</sub> 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), while 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<sub>1</sub> 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<sub>1</sub>-dependent effects at striatal GABAergic, but not glutamatergic, synapses (Rossi *et al*, 2009). However, it is difficult to draw a comparison with the present 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 (~100 mg/kg/day) leads to altered brain levels of several receptors (Shi *et al*, 1993, 1994), inhibit

phosphodiesterases and may even block GABA<sub>A</sub> receptors, among others (see Daly and Fredholm, 1998).

The conclusion that A<sub>1</sub> receptors are involved in the chronic caffeine-induced exacerbation of the effects of THC presently reported is reinforced by the finding that A<sub>1</sub> receptor blockade with DPCPX fully prevented the effects of THC in the chronic caffeine group. DPCPX was administered at a dose that occupies A<sub>1</sub> receptors (Baumgold *et al*, 1992; Hooper *et al*, 1996) while not affecting motor activity (present data and (Von Lubitz *et al*, 1993). In addition, DPCPX by itself had no effects in the absence of THC, which suggests that A<sub>1</sub> 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<sub>1</sub> receptors have different functional consequences. The effects of DPCPX also exclude the involvement of A<sub>2A</sub> receptors, which are known to modulate the actions of CB<sub>1</sub> receptors in the striatum (Carriba *et al*, 2007; Tebano *et al*, 2009). A<sub>1</sub> and A<sub>2A</sub> receptors have similar affinities for caffeine (Fredholm *et al*, 1994), yet the expression of A<sub>2A</sub> receptors in the hippocampus and cortex is much lower than that of A<sub>1</sub> receptors (reviewed by Ribeiro *et al*, 2002). Furthermore, chronic caffeine exposure does not alter the expression of A<sub>2A</sub> receptors (Jacobson *et al*, 1996).

The increase in A<sub>1</sub> receptor expression caused by moderate doses of chronically administered caffeine results from prevention of tonic adenosine-mediated receptor down-regulation (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<sub>1</sub> receptor



levels, it also caused a decrease of cortical and hippocampal CB<sub>1</sub> receptors. Accordingly, in chronic caffeine-treated mice there was a reduction in the CB<sub>1</sub> receptor-mediated inhibition of GABA release and stimulation of G-protein activation. Since tonic activation of A<sub>1</sub> receptors was prevented through elimination of endogenous adenosine by vertical superfusion in the [<sup>3</sup>H]GABA release assays, and by ADA in the [<sup>35</sup>S]-GTPγS binding assays, it is unlikely that chronic caffeine-induced A<sub>1</sub> receptor up-regulation could be responsible for reduction of CB<sub>1</sub> dependent actions in the *in vitro* assays. Most probably, chronic caffeine intake, by inducing an imbalance in adenosinergic signaling, disturbs the A<sub>1</sub>-CB<sub>1</sub> cross-talk, which reflects in CB<sub>1</sub> receptor down-regulation. Independently of the exact mechanisms involved, it is clear that CB<sub>1</sub> receptors are affected after chronic caffeine exposure.

Given that chronic caffeine decreases CB<sub>1</sub> and increases A<sub>1</sub> receptor levels, and that activation of A<sub>1</sub> receptors inhibited the CB<sub>1</sub>-mediated actions in the *in vitro* assays, it was somewhat surprising that the memory impairment caused by the CB<sub>1</sub> 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<sub>1</sub> and CB<sub>1</sub> 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<sub>1</sub> 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, the present work highlights two relevant factors influencing cannabinoid CB<sub>1</sub> signaling in the hippocampus: the activity of A<sub>1</sub> receptors, and the chronic consumption of caffeine. This A<sub>1</sub>-CB<sub>1</sub> receptor interaction therefore points towards the possibility that the pathophysiological or therapeutically relevant actions operated by CB<sub>1</sub> receptors can be significantly affected by interference with A<sub>1</sub> receptor activity, as is the case of chronic caffeine intake.

**Disclosure/Conflict of Interest**

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 three 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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**Tables:****Table 1.**  $E_{\max}$  and  $\log EC_{50}$  values of agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in rat hippocampal membranes.

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

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]GTP $\gamma$ S binding, but not the  $EC_{50}$ ; The  $GABA_B$  receptor agonist baclofen (100  $\mu$ M) had no significant influence. Data represent mean values  $\pm$  s.e.m (n=4) obtained from nonlinear regression analyses of the data shown in Figure 3. \* $p$ <0.05, compared to appropriate control, calculated using the extra sum-of-squares F test.

**Table 2.**  $E_{max}$  and  $\log EC_{50}$  values of WIN55,212-2 and THC-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in mouse cortico-hippocampal membranes.

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

Note that the  $E_{max}$ , but not the  $EC_{50}$ , of WIN55,212-2 (WIN) and THC-stimulated [ $^{35}$ S]GTP $\gamma$ S binding was significantly decreased in the chronic caffeine group (3 mg/kg/day, for 22 days), compared to control (vehicle treated) mice. The  $A_1$  receptor agonist, CPA (100nM) 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  $\pm$  s.e.m (n=4-5) obtained from nonlinear regression analyses of the data shown in Figure 8.  $*p < 0.05$ , versus corresponding control, calculated using the extra sum-of-squares F test.

**Figure legends:**

**Figure 1.** Inhibition of  $K^+$ -evoked,  $Ca^{2+}$ -dependent release of  $[^3H]GABA$  from rat hippocampal synaptosomes by WIN55,212-2 (WIN). **(a)** Fractional release of  $[^3H]GABA$  evoked by two 15 mM  $K^+$  stimuli of 2 min duration, as indicated ( $S_1$  and  $S_2$ ); in the test assay, WIN (1  $\mu M$ ) was applied before  $S_2$ , as indicated by the horizontal bar. Data represent mean  $\pm$  s.e.m. from 31 experiments performed in duplicate. **(b)** Percentage inhibition of  $[^3H]GABA$  release induced by WIN (1  $\mu M$ ) in the absence or in the presence of the calcium channel blocker,  $CdCl_2$  ( $Cd^{2+}$ , 200  $\mu M$ ), or the GABA transporter inhibitor, SKF89976A (SKF, 20  $\mu M$ ), 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  $\pm$  s.e.m. from 5 experiments, performed in duplicate. **(c)** Concentration-dependent inhibition of  $K^+$ -evoked release of  $[^3H]GABA$  induced by WIN (0.01–10  $\mu M$ ) in the absence or in the presence of the  $CB_1$  receptor antagonist, AM251 (1  $\mu M$ ); the effect of partial  $CB_1$  receptor agonist, THC (1  $\mu M$ ), as well as of a WIN enantiomer that is inactive at the  $CB_1$  receptor, WIN55,212-3 (WIN-3; 1-10  $\mu M$ ), is also shown. WIN significantly inhibited  $[^3H]GABA$  release at all concentrations ( $p<0.01$ ), except for the lowest concentration tested (0.01  $\mu M$ ,  $p>0.05$ ); THC (1  $\mu M$ ) also significantly inhibited  $[^3H]GABA$  release ( $p<0.01$ ). Note that WIN-3 was devoid of effect at 1  $\mu M$  ( $p>0.05$ ), but not at 10  $\mu M$  ( $p<0.01$ ), and that AM251 antagonized the effect of 1  $\mu M$  but not of 10  $\mu M$  WIN, indicating that WIN is  $CB_1$  receptor-selective at 1  $\mu M$ , but not at 10  $\mu M$ . Each point represents the mean  $\pm$  s.e.m. of 4 to 10 independent experiments performed in duplicate, except ( $n=2$ ) for 0.01  $\mu M$  and

3  $\mu\text{M}$  WIN, and 1  $\mu\text{M}$  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 ANOVA test with Dunnett *post hoc*, compared to control (0%). SKF,  $\text{Cd}^{2+}$  and AM251 were applied 15 min before 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 Methods for further details).

**Figure 2.**  $A_1$  receptor activation significantly attenuates the  $\text{CB}_1$ -mediated inhibition of  $\text{K}^+$ -evoked [ $^3\text{H}$ ]GABA release from rat hippocampal synaptosomes. **(a)** Effects of WIN55,212-2 (WIN, 1 and 10  $\mu\text{M}$ ) and of its  $\text{CB}_1$  receptor-inactive enantiomer, WIN55,212-3 (WIN, 3, 10  $\mu\text{M}$ ), in 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  $\mu\text{M}$  WIN (\*\* $p < 0.01$ , compared to the effect of WIN in absence of CPA, in the same experiments), while the effect of WIN-3 (10  $\mu\text{M}$ ) was unaffected by CPA ( $p > 0.05$ ). **(b)** WIN (1 and 10  $\mu\text{M}$ ) 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  $\mu\text{M}$ ) was tested in the absence or in the presence of the selective  $\text{GABA}_B$  receptor agonist, baclofen (10  $\mu\text{M}$ ), 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  $\mu\text{M}$ ) ( $p > 0.05$ ). Bars represent the mean  $\pm$  s.e.m. 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 to 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 Methods for further details).

**Figure 3.**  $A_1$  receptors modulate the  $CB_1$ -mediated inhibition of [ $^3H$ ]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  $\mu$ M). **(b)** The inhibition induced by WIN (1  $\mu$ M) 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 presence of WIN (1  $\mu$ M). Bars represent the mean  $\pm$  s.e.m. 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 to corresponding controls within the same batch of synaptosomes.

**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 THC-induced [ $^{35}S$ ]GTP $\gamma$ S binding. Rat hippocampal membranes (10  $\mu$ g protein) were incubated for 30 min at 37° C with 30  $\mu$ M GDP, 0.1 nM [ $^{35}S$ ]GTP $\gamma$ S and varying concentrations of **(a)** WIN55,212-2 (0.1 nM – 10  $\mu$ M) or **(b)** THC (0.1 nM – 1  $\mu$ M), alone ( $\circ$ ) or in combination with 100 nM CPA ( $\blacksquare$ ), or 100  $\mu$ M baclofen ( $\blacklozenge$ ).  $E_{max}$  and log  $EC_{50}$  values are shown in Table 1. Data represent mean percentage of basal stimulation  $\pm$  s.e.m. of  $n=7$  ( $\circ$ ) and  $n=4$  ( $\blacksquare$ ,  $\blacklozenge$ ), performed in duplicate. Non-visible error bars are within symbols.



**Figure 5.** Influence of chronic caffeine administration upon the 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, while **(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 one 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  $\pm$  s.e.m. of n=7-8. \* $p$ <0.05, 2-way analysis of variance, followed by Tukey *post hoc* test (see text for more details).

**Figure 6.** Influence of chronic caffeine administration, and involvement of the adenosine A<sub>1</sub> receptors, upon the acute effects of 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, while **(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 to 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  $\pm$  s.e.m. of  $n=7$ . \* $p<0.05$ , \*\* $p<0.01$ , 2-way analysis of variance, followed by Tukey *post hoc* tests (see text for more details).

**Figure 7.** Saturation analysis of specific [ $^3$ H]DPCPX binding (0.1 – 10 nM) to cortico-hippocampal membranes (40  $\mu$ g protein) from chronic caffeine (3 mg/kg/day, for 22 days,  $\blacklozenge$ ) and vehicle (O) treated mice. Inset:  $B_{max}$  obtained from non-linear regression analysis. Non-specific binding was determined at all [ $^3$ H]DPCPX concentrations by addition of 2  $\mu$ M XAC. All points represent mean  $\pm$  s.e.m. of  $n=6$ , and each saturation experiment was performed in duplicate. \* $p<0.05$ , versus control, calculated using the extra sum-of-squares F test.

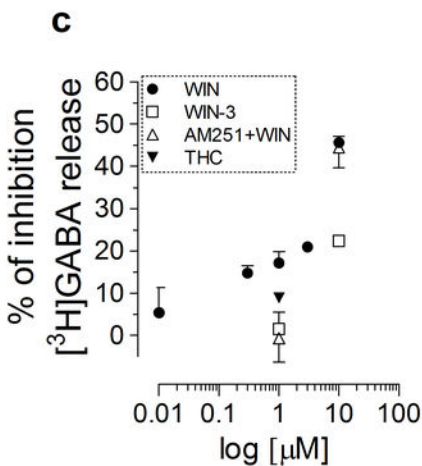
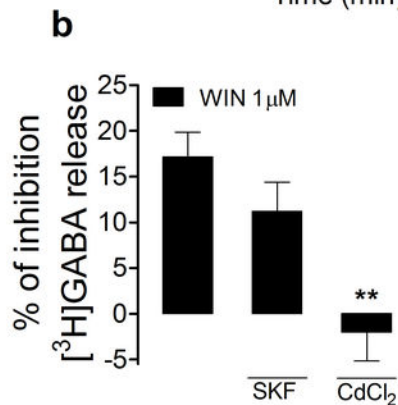
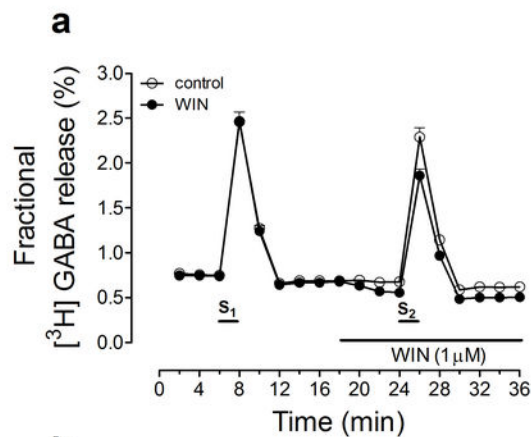
**Figure 8.** Influence of the adenosine  $A_1$  receptor agonist, CPA (100 nM), and of chronic caffeine administration on the  $CB_1$ -mediated inhibition of  $K^+$ -evoked [ $^3$ H]GABA release from mouse cortico-hippocampal synaptosomes. WIN55,212-2 (WIN, 1  $\mu$ M) was tested in the absence and 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  $\pm$  s.e.m. of 4 experiments, performed in duplicate. \*\* $p < 0.01$ , compared to effect of WIN alone; \* $p < 0.05$ , compared to effect of WIN in vehicle-treated mice (paired Student's t-test).

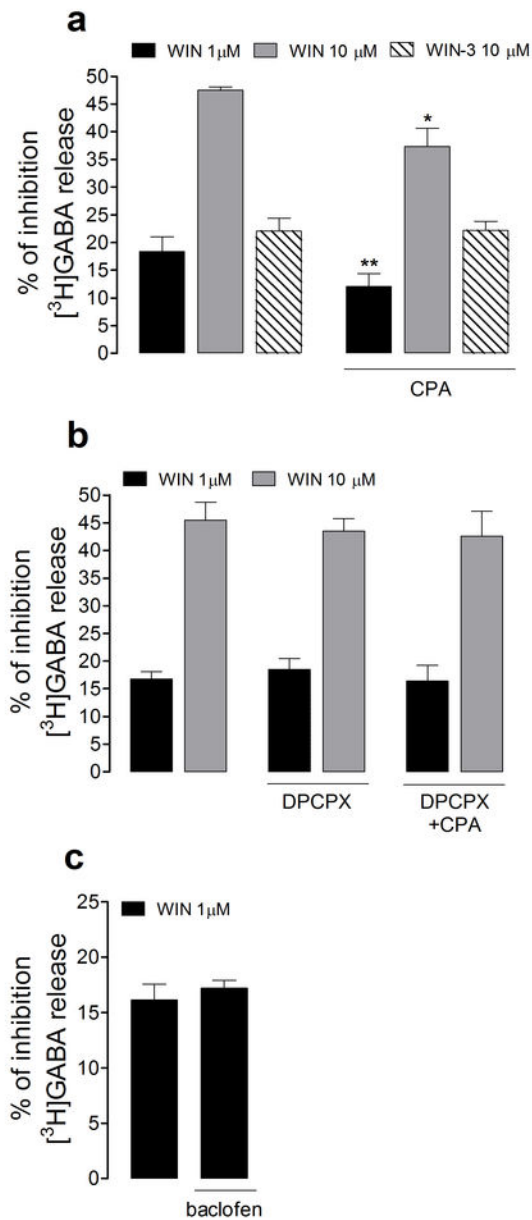
**Figure 9.** Influence of chronic caffeine administration on CB<sub>1</sub>-induced stimulation of G-proteins, as assayed by WIN55,212-2 or THC-induced [<sup>35</sup>S]GTP $\gamma$ S binding. Cortico-hippocampal membranes (10  $\mu$ g protein) from chronic caffeine ( $\blacklozenge, \blacktriangledown$ ) and vehicle (O,  $\triangle$ ) treated mice were incubated for 30 min at 37° C with 30  $\mu$ M GDP, 0.1 nM [<sup>35</sup>S]GTP $\gamma$ S and varying concentrations of WIN (0.1 nM-10  $\mu$ M) in the absence (**a**) or in the presence of 100 nM CPA (**b**), or varying concentrations of THC (0.1 nM – 1  $\mu$ M) (**c**). E<sub>max</sub> and log EC<sub>50</sub> values are shown in Table 2. Data represent mean percentage of basal stimulation  $\pm$  s.e.m. of, n=5 (**a,b**) and n=4 (**c**), performed in duplicate. Non-visible error bars are within symbols.

**Figure 10.** Saturation analysis of specific [<sup>3</sup>H]SR141716A binding (0.1 – 4 nM) to (**a**) cortical and (**b**) hippocampal membranes (50  $\mu$ g protein) from chronic caffeine (3 mg/kg/day, for 22 days), and vehicle treated mice. Insets: B<sub>max</sub> values, obtained from non-linear regression analysis. Non-specific binding was determined at all [<sup>3</sup>H]SR141716A concentrations by addition of 1  $\mu$ M AM251. All points represent mean  $\pm$  s.e.m. of 5-10 experiments, each performed in duplicate. \* $p < 0.05$ , versus control, calculated using the extra sum-of-squares F test.

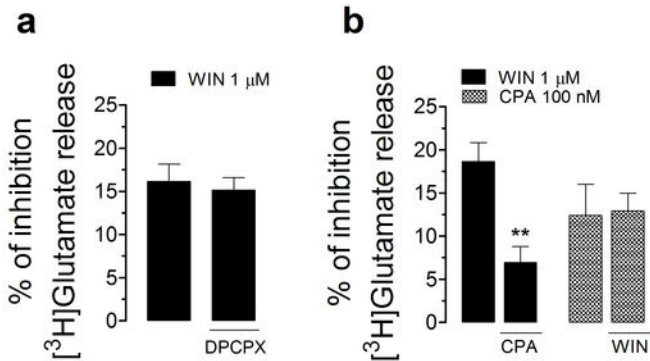
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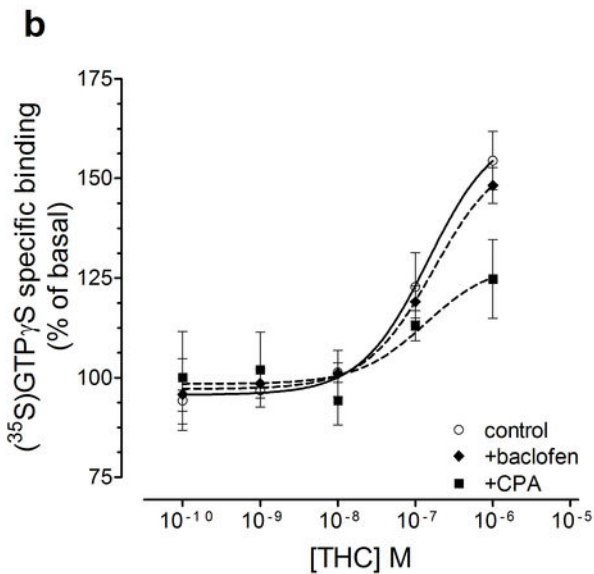
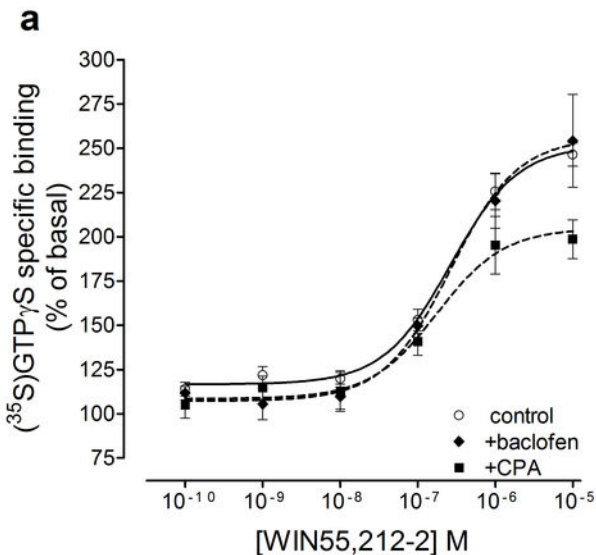
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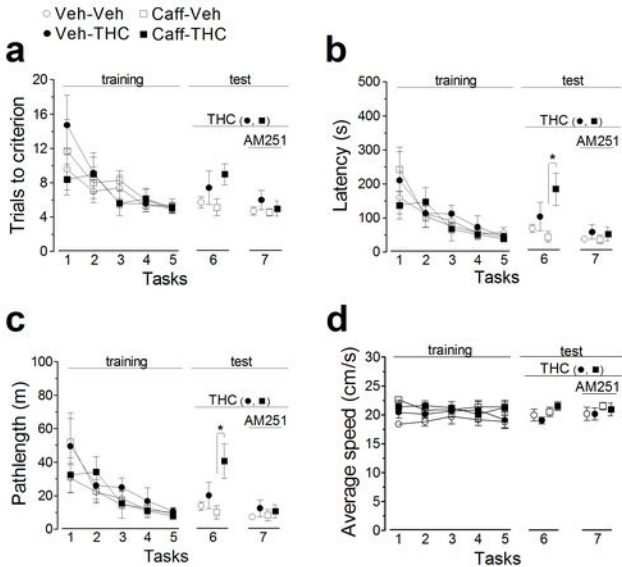
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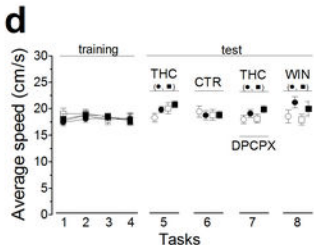
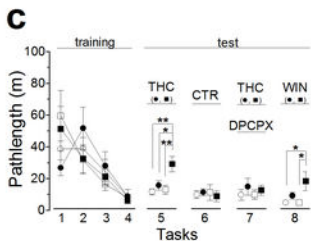
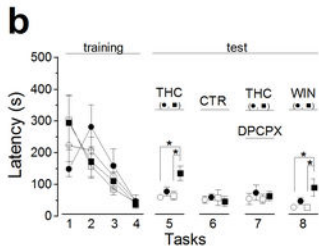
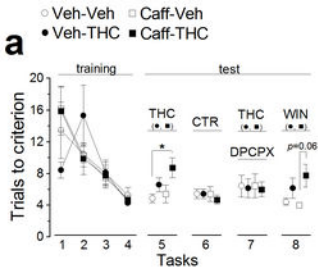


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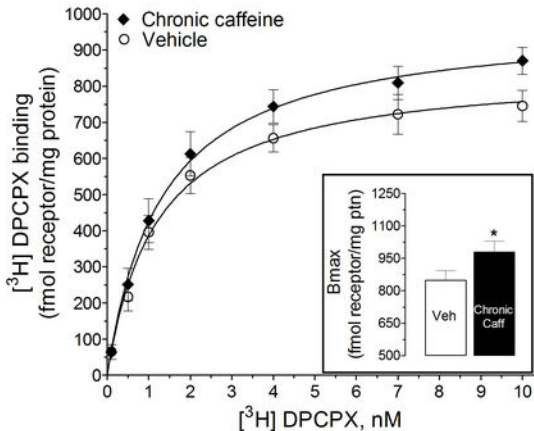




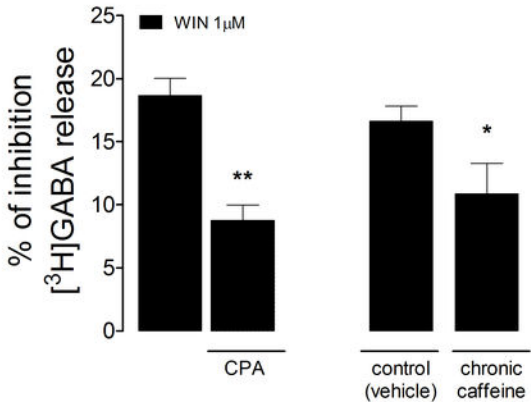
# Figure 6.



# Figure 7.

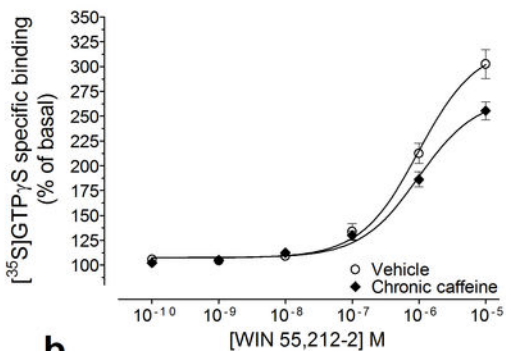


# Figure 8.

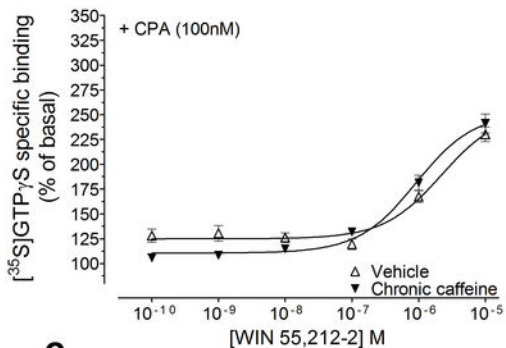


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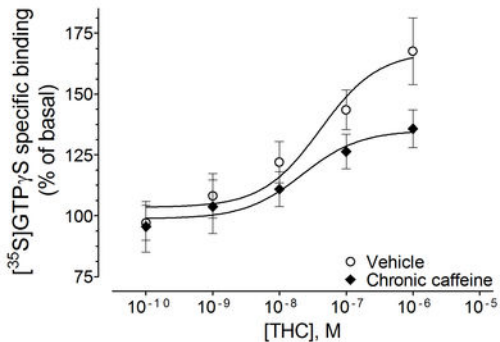
**a**



**b**



**c**



# Figure 10.

