Behavioral/Systems/Cognitive

Control of Cannabinoid CB_1 Receptor Function on Glutamate Axon Terminals by Endogenous Adenosine Acting at A_1 Receptors

Alexander F. Hoffman,[1] Nora Laaris,[1] Masahito Kawamura,[2,3] Susan A. Masino,[2] and Carl R. Lupica[1]

[1]Electrophysiology Research Section, National Institute on Drug Abuse Intramural Research Program, National Institutes of Health, Baltimore, Maryland 21224, [2]Department of Psychology and Neuroscience Program, Trinity College, Hartford, Connecticut 06106, and [3]Department of Pharmacology, Jikei University School of Medicine, Tokyo 105-8461, Japan

Marijuana is a widely used drug that impairs memory through interaction between its psychoactive constituent, Δ-9-tetrahydrocannabinol (Δ^9-THC), and CB_1 receptors (CB1Rs) in the hippocampus. CB1Rs are located on Schaffer collateral (Sc) axon terminals in the hippocampus, where they inhibit glutamate release onto CA1 pyramidal neurons. This action is shared by adenosine A_1 receptors (A1Rs), which are also located on Sc terminals. Furthermore, A1Rs are tonically activated by endogenous adenosine (eADO), leading to suppressed glutamate release under basal conditions. Colocalization of A1Rs and CB1Rs, and their coupling to shared components of signal transduction, suggest that these receptors may interact. We examined the roles of A1Rs and eADO in regulating CB1R inhibition of glutamatergic synaptic transmission in the rodent hippocampus. We found that A1R activation by basal or experimentally increased levels of eADO reduced or eliminated CB1R inhibition of glutamate release, and that blockade of A1Rs with caffeine or other antagonists reversed this effect. The CB1R–A1R interaction was observed with the agonists WIN55,212-2 and Δ^9-THC and during endocannabinoid-mediated depolarization-induced suppression of excitation. A1R control of CB1Rs was stronger in the C57BL/6J mouse hippocampus, in which eADO levels were higher than in Sprague Dawley rats, and the eADO modulation of CB1R effects was absent in A1R knock-out mice. Since eADO levels and A1R activation are regulated by homeostatic, metabolic, and pathological factors, these data identify a mechanism in which CB1R function can be controlled by the brain adenosine system. Additionally, our data imply that caffeine may potentiate the effects of marijuana on hippocampal function.

Introduction

Cannabinoid CB_1 receptors (CB1Rs) are members of a large group of G-protein-coupled receptors (GPCRs) extensively expressed in mammalian brain tissue (Matsuda et al., 1990; George et al., 2002). CB1Rs are located largely on axon terminals in the CNS and are activated by diverse agonists, including plant-derived compounds such as Δ-9-tetrahydrocannabinol (Δ^9-THC), synthetic agonists such as WIN55,212-2, and membrane lipid-derived endogenous cannabinoids (eCBs) (Pertwee, 1997; Freund et al., 2003). Activation of CB1Rs on axon terminals by these agonists inhibits neurotransmitter release throughout the CNS (Lévénés et al., 1998; Szabo et al., 1998; Katona et al., 1999; Misner and Sullivan, 1999; Hoffman and Lupica, 2000, 2001; Gerdeman and Lovinger, 2001). Recent studies demonstrate that eCBs are released from hippocampal neurons in an activity-dependent manner to initiate short- and long-term changes in synaptic efficacy following activation of CB1Rs (Wilson and Nicoll, 2001; Alger, 2002; Freund et al., 2003). Furthermore, activation of CB1Rs by acute or long-term exposure to the primary psychoactive constituent of marijuana, Δ^9-THC, disrupts hippocampal function and impairs behaviorally and physiologically defined memory processes in humans and animals (Heyser et al., 1993; Misner and Sullivan, 1999; Ranganathan and D'Souza, 2006; Wise et al., 2009).

Adenosine A_1 receptors (A1Rs) are also members of the GPCR family, activated by synthetic agonists or eADO that is tonically released in brain (Dunwiddie et al., 1981; Dunwiddie and Diao, 1994). Like CB1Rs, A1Rs are located on hippocampal CA3 pyramidal neuron axon terminals, in which they inhibit glutamate release onto CA1 pyramidal neurons (Schubert and Mitzdorf, 1979; Dunwiddie and Hoffer, 1980). Glutamate release from these Schaffer collateral (Sc) axons is inhibited by A1Rs and CB1Rs through inhibition of voltage-dependent Ca^{2+} channels (VDCCs) via $\beta\gamma$ subunits liberated from G_i/G_o G-proteins upon GTP binding (Schubert and Mitzdorf, 1979; Dunwiddie and Hoffer, 1980). Previous studies suggest that P-, Q-, and N-type VDCCs mediate the release of glutamate from Sc terminals and are inhibited by A1Rs and CB1Rs (Wu and Saggau, 1994; Sullivan, 1999; Manita et al., 2004). Also, A1Rs and CB1Rs can couple to similar G-protein α ($G\alpha$) subunits to generate presynaptic inhibition of glutamate release (Straiker et al., 2002). The colo-

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Correspondence should be addressed to Dr. Carl Lupica, National Institute on Drug Abuse Intramural Research Program, Electrophysiology Research Section, National Institutes of Health, 333 Cassell Drive, Baltimore, MD 21224. E-mail: clupica@mail.nih.gov.

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calization of A1Rs and CB1Rs on Sc terminals, and the potential for shared Gα subunits and VDCC mediation of transduction of these signals, suggest points at which A1R–CB1R interactions may occur. In support of this, a study has shown that tonic activation of A1Rs by eADO in brain slices reduced binding of [^{35}S]GTPγS to membranes caused by the cannabinoid agonist WIN55,212-2, and elimination of eADO by the enzyme adenosine deaminase (ADA) increased CB1R-stimulated [^{35}S]GTPγS binding (Moore et al., 2000). This implies a functional interaction between A1Rs and CB1Rs and a role for eADO.

By examining glutamate neurotransmission, we found that A1Rs modulate the effect of cannabinoids in the hippocampus and that this interaction depends upon extracellular levels of eADO. This suggests that adenosine A1Rs can control CB1R modulation of synaptic transmission and that the impact of eCBs on hippocampal function may be regulated by eADO and metabolic and activity-dependent factors that regulate its release.

Materials and Methods

Animals

Animal procedures were according to National Institutes of Health guidelines and based upon the United States Animal Welfare Act. The protocols were approved by the Institutional Animal Care and Use Committee [National Institute on Drug Abuse (NIDA) Intramural Research Program, Baltimore, MD], which is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Wild-type (WT, CB1$^{+/+}$) and CB1R knock-out (KO, CB1$^{-/-}$) littermate C57BL/6J mice (4–12 weeks) were obtained from the NIDA Intramural Research Program transgenic facility colony. These animals were descendants of three heterozygous (CB1$^{+/-}$) breeding pairs, donated by Dr. Andreas Zimmer and the National Institute of Mental Health (Bethesda, MD) (Zimmer et al., 1999). Genotyping of the CB1 mice was performed by Charles River Laboratories. The A1R WT (A1R$^{+/+}$) and KO (A1R$^{-/-}$) mice were also generated on a C57BL/6J background strain (Johansson et al., 2001) and obtained from a congenic breeding colony derived from this original line that is housed at the Trinity College Animal Care Facility (Hartford, CT). Genotyping for A1R mutant mice was performed at Trinity College. Wild-type male Sprague Dawley (SD) rats (4–6 weeks of age) were also used in these studies (Charles River Laboratories).

Brain slice preparation

Hippocampal brain slices were prepared as previously described (Hoffman and Lupica, 2000). Briefly, animals were killed by rapid decapitation, and the brains were removed and immersed in cold (4°C), oxygenated, high-sucrose, low-Ca^{2+} artificial CSF (aCSF) of the following composition (in mM): 87 NaCl, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 glucose, 75 sucrose, 26 $NaHCO_3$. Transverse slices were then cut at 280 μm thickness using a vibrating tissue slicer (VT1000S, Leica Instruments). Hemisectioned brain slices containing the hippocampus were then incubated in normal aCSF consisting of the following (in mM): 126 NaCl, 3.0 KCl, 1.5 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 11.0 glucose, 26 $NaHCO_3$, saturated with 95% O_2 and 5% CO_2, at room temperature for ≥90 min before recordings were initiated. Individual brain slices were placed into a low-volume (~300 μl) recording chamber integrated into the fixed stage of a differential interference contrast microscope (Olympus America) and submerged in continuously flowing (2 ml/min) normal aCSF. This solution was continuously bubbled with 95% O_2 and 5% CO_2 and maintained at 30–32°C using a solution heater (TC-324B, Warner Instruments). For whole-cell experiments using photolysis of α-carboxy-2-nitrobenzyl (CNB)-caged glutamate, a 15 ml volume of oxygenated (95% O_2–5% CO_2) aCSF was recirculated using a peristaltic pump.

Electrophysiology

Field EPSP recordings. Field EPSPs (fEPSPs) were recorded as described previously (Hoffman et al., 2007). Briefly, the tips of whole-cell electrodes filled with 3 M NaCl were placed in the distal stratum radiatum of area CA1 of the hippocampus. Evoked fEPSPs were elicited by stimulating Sc axons with a formvar-insulated, nichrome wire, bipolar electrode at a frequency of 0.033 Hz using single constant-current 0.1 ms pulses. The stimulus intensity was adjusted to produce fEPSPs with peak amplitudes of 0.5–1 mV (30–40% of the maximal response). The signals were acquired with an AC amplifier (A-M Systems model 1800), and were high-pass (10 Hz) and low-pass (10 kHz) filtered. Data were directly acquired to a PC using an analog/digital board (National Instruments PC 6251) and Windows-based software (WinLTP). At least 10 min of stable baseline recording was obtained before the delivery of drugs, and both fEPSP peak amplitudes and slope of 1–1.5 ms of the rising phase of the fEPSP were measured.

Whole-cell recordings. Whole-cell patch-clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices) and electrodes pulled from borosilicate glass (1.5 mm outer diameter, 0.86 mm inner diameter, Sutter Instruments). Data were directly acquired to a personal computer using an A/D board (Instrutech ITC-18) and Windows-based software (WinWCP, courtesy of Dr. John Dempster, University of Strathclyde, Glasgow, UK; http://spider.science.strath.ac.uk/sipbs/software_ses.htm). Electrodes were filled with a solution containing the following (in mM): 100 $CsCH_3SO_3$, 60 CsCl, 0.2 EGTA, 10 HEPES, 2.0 $MgCl_2$, 1.0 Mg^{2+}-ATP, 0.3 Na^+-GTP, and QX-314 (1 mg/ml). This solution was adjusted to pH 7.2–7.4 using CsOH. Series resistance was monitored with a −10 mV voltage step (200 ms), every 30 s. Time versus series resistance was plotted together with the synaptic and photolysis-evoked currents to ensure that changes in these currents were not associated with altered cellular access. Only cells maintaining stable access (<10% change in series resistance over the duration of the recording) were included in analyses. Synaptic EPSCs were evoked using a bipolar stimulator placed on the stratum radiatum of the hippocampus. EPSCs and photolysis-evoked glutamate currents were measured at −60 mV in aCSF containing the $GABA_A$ blocker picrotoxin (PCTx, 100 μM). EPSCs were evoked once per minute and alternated with photolysis-evoked postsynaptic currents throughout the duration of the recordings. Photolysis was performed using a solid-state, pulsed Nd:YAG laser (Minilite I, Continuum). The laser beam output was channeled to a 40× water-immersion microscope objective using a 400-μm-diameter fiber optic light guide. This arrangement yielded a circular illumination area (~25 μm diameter). This spot was focused upon the proximal dendrites of a single CA1 pyramidal neuron, within ~50 μm of the soma. Once whole-cell access was obtained, the objective was focused upon the pyramidal neuron and the laser output was adjusted to yield a postsynaptic glutamate response that was similar in amplitude to a 50% of maximum electrically evoked synaptic response. The settings of the laser and the electrical stimulator were then left undisturbed throughout the remainder of the experiment.

Recording depolarization-induced suppression of excitation. Endocannabinoid effects on excitatory synaptic transmission were assessed by measuring depolarization-induced suppression of excitation (DSE). EPSCs were measured at −70 mV in CA1 pyramidal neurons as described above and were evoked at 0.33 Hz. Following a 45 s baseline period, neurons were depolarized to 0 mV for 3 s and EPSCs monitored for another 45–90 s following the termination of the pulse. EPSC amplitudes were normalized to the mean value obtained during the baseline period. At least two DSE trials were conducted in each cell and averaged to yield a single value per cell.

Drugs

In most experiments, drugs were prepared at 100× final (bath) concentration and were delivered to the flowing aCSF at 20 μl/min, using a calibrated syringe pump (Razel, St. Albans, VT). WIN55,212-2, CGP55845, (*RS*)-dihydroxyphenylglycine (DHPG), and AM251 were purchased from Tocris-Cookson. ADA (200 U/mg) was purchased from Roche. CNB-caged glutamate was purchased from Invitrogen. All other compounds and reagents were purchased from Sigma. Δ^9-THC (200 mg/ml in EtOH) was obtained from the National Institute on Drug Abuse drug supply system (Bethesda, MD) and was diluted to 10 mM (3 mg/ml) in 22.5% randomly methylated β-cyclodextrin (RAMEB) in aCSF and EtOH, and a stock solution was prepared at 1 mM in 10% RAMEB. AM251 and WIN55,212-2 were prepared as 10 mM stock solu-

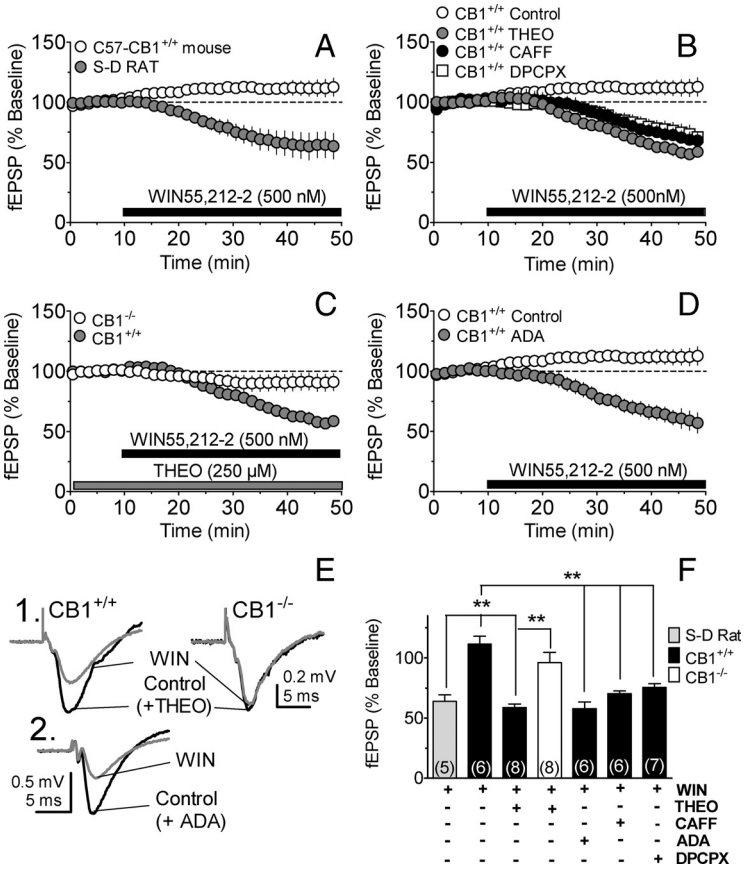

Figure 1. Tonic activation of adenosine receptors by eADO controls CB1R modulation of glutamate release in C57BL/6J mice. ***A***, The cannabinoid agonist WIN55,212-2 (500 nM) inhibited glutamatergic fEPSPs in hippocampal slices from SD rats but not in slices from wild-type (CB1 $^{+/+}$) C57BL/6J mice. In this and all subsequent figures, the duration of drug application is indicated by horizontal bars. ***B***, Antagonism of adenosine receptors by caffeine (CAFF, 50 μM), THEO (250 μM), or DPCPX (0.2 μM) reveals robust inhibition of fEPSPs by WIN55,212-2 in the CB1 $^{+/+}$ mouse hippocampus. Antagonists were applied to the brain slices for 15–20 min before WIN55,212-2 application. ***C***, The inhibition of fEPSPs by WIN55,212-2 during THEO application is mediated by CB1Rs. fEPSP inhibition by WIN55,212-2 was compared in hippocampal slices from CB1 $^{+/+}$ and CB1 $^{-/-}$ littermates following pretreatment with THEO. Responses were inhibited by WIN55,212-2 only in CB1 $^{+/+}$ slices. ***D***, Catabolism of eADO by ADA (2 U/ml) permits inhibition of fEPSPs by WIN55,212-2 in hippocampal slices from CB1 $^{+/+}$ mice. ***E***, Mean fEPSPs ($n = 5$ sweeps in all figures) collected from single hippocampal slices demonstrating the effects of WIN55,212-2 during experiments shown in ***C*** and ***D***. ***E1***, WIN55,212-2 (WIN, 500 nM) inhibits fEPSPs only in CB1 $^{+/+}$ hippocampal slices following THEO pretreatment. ***E2***, Inhibition of fEPSPs by WIN (500 nM) in hippocampal slices from CB1 $^{+/+}$ mice after a 20 min ADA (2 U/ml) pretreatment. ***F***, Summary showing peak effects of WIN (35–40 min of application) from experiments shown in ***A–D***. $^{**}p < 0.01$, RM-ANOVA, and Tukey *post hoc* analysis.

tions in DMSO and then diluted in H_2O containing 0.1% Tween 80 and 0.2% DMSO. Final concentrations of Tween 80 and DMSO in the tissue bath were 0.01% and 0.02%, respectively; these concentrations have no effects on hippocampal synaptic transmission. Vehicle effects of the final bath concentration of 0.1% RAMEB are described in the text.

Data analyses

Data are presented as the mean ± SEM in most cases. However, concentration–response EC_{50} values are given as the mean ± 95% confidence interval (CI). *n* represents the number of slices tested in each case, with no more than three slices obtained from a single animal. Peak amplitudes or slopes of the rising phase of the fEPSP/EPSC during drug application were normalized to the predrug (control) baseline period. All statistical analyses and curve fits were performed using either Prism (v5.02, GraphPad Scientific) or SigmaPlot (v 11, Systat). In all instances in which time courses of drug effects were measured between groups, a two-way repeated-measures ANOVA (RM-ANOVA) was used, with appropriate *post hoc* analyses. The significance level was set at $p = 0.05$ for all tests. Concentration–response curves were fit using the following sigmoidal nonlinear regression function: $y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{(\log EC_{50} - x)})$, where Top and Bottom represent the plateau values of the *y* axis (% inhibition of the fEPSP). Because complete inhibition was observed at the highest concentrations of A1R agonists, the top of the curve was constrained to a value of 100 and the bottom constrained to a value of 0 to optimize curve fits.

Results

Endogenous adenosine and A1Rs control CB1R-mediated inhibition of glutamate release

Previous data from our laboratory demonstrated that the cannabinoid agonist WIN55,212-2 did not affect synaptic glutamate release in hippocampal slices obtained from WT (CB1 $^{+/+}$) C57BL/6J mice, but inhibited these responses in slices from SD rats and CD-1 Swiss-Webster mice (Hoffman et al., 2005). Similarly, in the present study a near-maximal concentration of WIN55,212-2 (500 nM) inhibited fEPSPs measured in rat hippocampal slices, but did not significantly alter these responses in slices that were maintained under identical incubation and recording conditions from C57BL/6J-CB1 $^{+/+}$ mice (Fig. 1*A*) (RM-ANOVA, $F_{(1,32)} = 14.88$, $p < 0.001$ vs rat). However, others have reported strong effects of WIN55,212-2 on synaptic glutamate release in hippocampal slices from WT C57BL/6J mice via activation of CB1Rs (Kawamura et al., 2006; Takahashi and Castillo, 2006).

To address the possible cause of this disparity we noted that several GPCRs are colocalized with CB1Rs on Sc axon terminals, and we hypothesized that one of these GPCRs might limit CB1R effects by an uncharacterized interaction. We also reasoned that such an interaction must occur via either a constitutively active GPCR, or one that is exposed to its endogenous agonist under our recording conditions. Adenosine is a potent inhibitor of synaptic glutamate release in the CA1 region of the hippocampus, and this is mediated by the A1R (Dunwiddie and Masino, 2001). Furthermore, eADO is tonically released in hippocampal brain slices, in which it exerts continuous inhibition of glutamate release via activation of these A1Rs on Sc axon terminals (Dunwiddie et al., 1981). Therefore, to evaluate the possible interaction between CB1Rs and A1Rs we tested the effects of the cannabinoid agonist WIN55,212-2 on fEPSPs in hippocampal slices obtained from C57BL/6J-CB1 $^{+/+}$ mice, that were pretreated with the adenosine receptor antagonists caffeine (50 μM) or theophylline (THEO, 250 μM). In WT C57BL/6J mouse slices treated with these A1R antagonists, WIN55,212-2 (500 nM) now significantly inhibited fEPSPs to a level that was similar to that seen in slices from SD rats (Fig. 1, compare *A*, *B*) (RM-ANOVA, $F_{(64,2)} = 14.48$, $p < 0.001$). Similar results were observed using the selective A1R antagonist DPCPX (200 nM) (Fig. 1*B*,*F*) (RM-ANOVA, $F_{(32,1)} = 10.58$, $p < 0.001$ vs untreated slices). Furthermore, this appeared to be a somewhat selective

effect of A1R antagonism since WIN55,212-2 did not inhibit fEPSPs in mouse slices pretreated with the $GABA_B$ antagonist CGP55845 (2 μM; $n = 4$, 98 ± 3% of control). To determine whether the inhibition produced by WIN55,212-2 during caffeine or theophylline treatment occurred through the activation of CB1Rs the experiment was repeated in hippocampal slices from CB1 $^{-/-}$ mice. The cannabinoid agonist had no effect on fEPSPs in hippocampal slices from these CB1 $^{-/-}$ mice (Fig. 1*C*) (RM-ANOVA, $F_{(32,1)} = 12.05$, $p < 0.001$ vs CB1 $^{+/+}$), indicating that WIN55,212-2 activated CB1Rs to inhibit glutamate release in WT mouse hippocampus following A1R antagonist application.

Caffeine and theophylline are thought to antagonize the tonic effects of continuously released eADO on A1Rs in the hippocampus (Dunwiddie et al., 1981; Dunwiddie and Diao, 1994). However, they may also inhibit phosphodiesterases and stimulate intracellular calcium release at higher concentrations than that used here (Dunwiddie et al., 1981; Snyder, 1981). Therefore, to evaluate the possibility that the lack of CB1R-mediated inhibition of fEPSPs resulted from the effects of eADO acting at adenosine receptors we applied the enzyme ADA to hippocampal slices from C57BL/6J-CB1 $^{+/+}$ mice and evaluated the effects of WIN55,212-2. ADA converts extracellular adenosine to inosine, a metabolite that is inactive at A1Rs (Dunwiddie and Hoffer, 1980; Dunwiddie et al., 1981). Similar to a previous report (Dunwiddie and Hoffer, 1980), and as a result of its ability to reverse the tonic inhibition of glutamate release by eADO, application of ADA (2 U/ml) increased fEPSP amplitudes by 49 ± 11% ($n = 7$) in WT mouse hippocampal slices. Furthermore, during ADA treatment, the cannabinoid agonist WIN55,212-2 (500 nM) inhibited fEPSPs in C57BL/6J-CB1 $^{+/+}$ mouse hippocampal slices to the same extent as that observed in the presence of the A1R antagonists (RM-ANOVA, $F_{(32,1)} = 22.04$, $p < 0.001$ vs control) (Fig. 1*B*,*D*). Together these data suggest that basal levels of eADO exerted a tonic blockade of CB1R signaling in the WT mouse hippocampus that was eliminated following antagonism of adenosine receptors or metabolism of eADO.

There are 3 distinct subtypes of adenosine receptors (A1, $A2_A$, and A3) found in the mammalian hippocampus (Burnstock, 2007). However, it is the A1R that is primarily involved in the presynaptic inhibition of glutamate release (Dunwiddie and Masino, 2001). Therefore, to confirm that the control of the CB1R-mediated inhibition of fEPSPs by eADO resulted from the activation of A1Rs, the effects of WIN55,212-2 were tested in hippocampal slices obtained from WT (A1 $^{+/+}$) and A1R KO (A1 $^{-/-}$) littermate mice that were also developed on the C57BL/6J background strain (Johansson et al., 2001). As seen with the CB1 $^{+/+}$ mice above and in our prior publication (Hoffman et al., 2005), WIN55,212-2 (500 nM) did not inhibit fEPSPs recorded in area CA1 of slices from A1 $^{+/+}$ mice (Fig. 2). However, pretreatment with theophylline (250 μM) permitted robust inhibition of fEPSPs by WIN55,212-2 in hippocampal slices from the C57BL/6J-A1 $^{+/+}$ mice (Fig. 2) (RM-ANOVA, $F_{(23,1)} = 4.87$, $p <$ 0.001 vs untreated slices). More importantly, in the absence of theophylline WIN55,212-2 significantly inhibited fEPSPs in slices obtained from A1 $^{-/-}$ mice (Fig. 2) (RM-ANOVA $F_{(23,1)} =$ 6.10, $p < 0.001$ vs untreated A1 $^{+/+}$ controls). The observations made with these transgenic A1R KO mice were also supported by data in WT mice using the selective A1R antagonist DPCPX. Similar to the effects with the nonselective antagonists, prior treatment with DPCPX was permissive to CB1R-mediated inhibition of fEPSPs (Fig. 1*B*). Together, these data and the observation that the effects of theophylline and caffeine on glutamate release are absent in A1 $^{-/-}$ mice (Johansson et al., 2001) indicate that A1Rs were responsible for the negative modulation of CB1R effects on glutamate release.

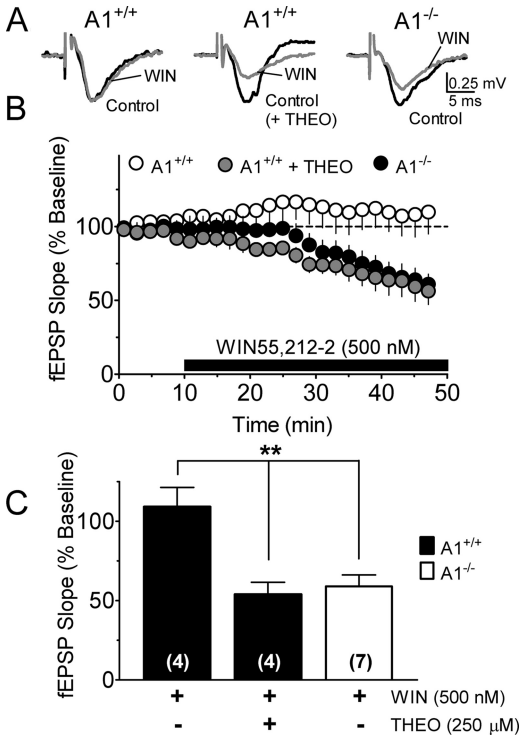

Figure 2. Adenosine A_1 receptors control cannabinoid inhibition of glutamatergic synaptic transmission. ***A***, Mean fEPSPs collected during baseline recordings (Control, black traces) and during application of WIN55,212-2 (WIN, 500 nM, gray trace) in hippocampal brain slices from A1 $^{+/+}$ and A1 $^{-/-}$ C57BL/6J mice. The center control trace shows the effect of WIN after a 15 min treatment with THEO (250 μM). ***B***, Mean time course of the effects of WIN55,212-2 on the initial slope of fEPSPs in hippocampal slices obtained from A1 $^{+/+}$ and A1 $^{-/-}$ mice. Note that WIN55,212-2 inhibited fEPSPs in slices from A1 $^{-/-}$ mice and in slices from A1 $^{+/+}$ mice only after theophylline (250 μM) pretreatment. ***C***, Summary of the effects of the treatment conditions in hippocampal slices from A1 $^{+/+}$ and A1 $^{-/-}$ mice. $^{**}p < 0.01$ RM-ANOVA, Tukey *post hoc* analysis. The mean effects were determined 35–40 min after WIN55,212-2 application and the number of slices in each condition is shown in parentheses.

Although the colocalization of CB1Rs and A1Rs on Sc axon terminals suggested that this is the site of interaction, A1Rs are also located postsynaptically, and previous studies have shown that activation of other postsynaptic GPCRs, such as type I metabotropic glutamate receptors (mGluR) or muscarinic acetylcholine receptors can increase eCB production in the hippocampus (Alger, 2002). Therefore, we hypothesized that postsynaptic A1Rs, activated by high levels of eADO, might stimulate the production of eCBs via a metabotropic action, and thereby occlude the effects of WIN55,212-2 at Sc axon terminals. If this occurred then antagonism of CB1Rs by AM251 should increase fEPSP amplitudes in hippocampal slices from WT C57BL/6J mice. However, similar to our previous data in SD rat hippocampal slices (Hoffman et al., 2007), AM251 (1 μM) had no effect alone on fEPSPs (99 ± 8% of control, $p > 0.05$, paired two tailed t test, $n = 4$). Furthermore, in another group of slices we found that following theophylline (250 μM) pretreatment, AM251 (1 μM) had no effect on fEPSPs (96 ± 10% of control; $p >$ 0.05, paired two tailed t test $n = 4$). Therefore, theophylline did not increase eCB production, nor did it inhibit the uptake of constitutively released eCBs. Collectively, these data demonstrate that control of the CB1R-mediated inhibition of synaptic glutamate release occurred via presynaptic adenosine A1Rs activated by tonic levels of eADO in the C57BL/6J mouse hippocampus.

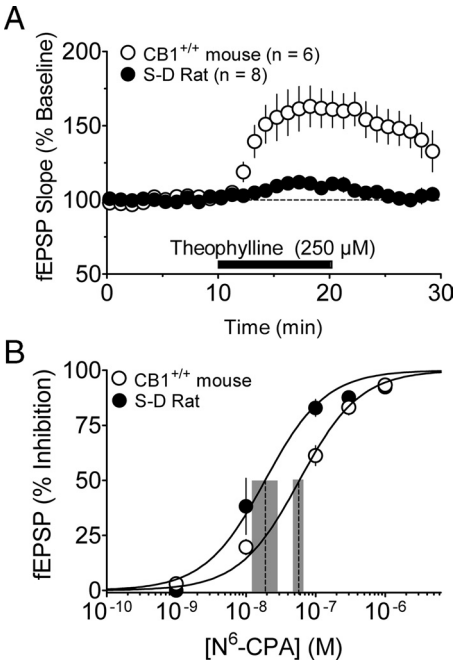

Figure 3. Hippocampal slices from C57BL/6J CB1 $^{+/+}$ mice demonstrate tonic levels of eADO higher than those from SD rats. ***A***, Mean time course of the effects of the adenosine receptor antagonist theophylline on fEPSPs in SD rat or CB1 $^{+/+}$ mouse hippocampal slices. Theophylline caused a significantly larger increase in fEPSPs in mouse brain slices under identical recording conditions ($p < 0.001$, RM-ANOVA). ***B***, Mean concentration–response curves for the effects of the selective A1R agonist N^6-CPA on fEPSPs in hippocampal slices from CB1 $^{+/+}$ mice ($n = 3$–6 slices per concentration) and SD rats ($n = 4$ slices per concentration). N^6-CPA more potently inhibited fEPSPs in slices from SD rats compared with mice. The EC_{50} values (dashed vertical lines) were 189 nM (95% CI = 118–301 nM, vertical shaded bar) in rat hippocampal slices and 587 nM (95% CI = 467–737 nM) in mouse slices. The EC_{50} value CIs obtained in rat and mouse slices did not overlap, indicating a significant difference.

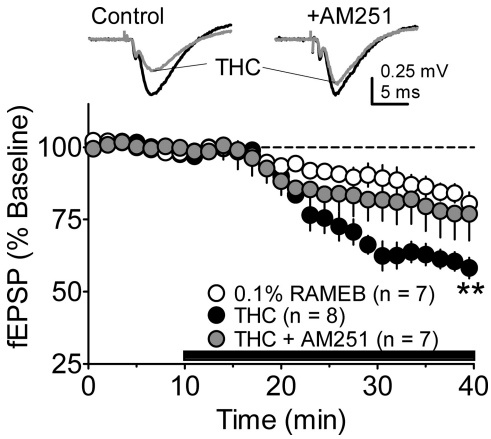

Figure 4. The plant-derived cannabinoid receptor agonist Δ^9-THC inhibits glutamate release through activation of CB1Rs in SD rat hippocampal brain slices. Top, Traces showing the effect of Δ^9-THC on mean fEPSPs in rat hippocampal slices in control aCSF (gray line, left) and following treatment with the CB1R antagonist AM251 (1 μM, gray line at right) in a different rat hippocampal slice. Bottom, Mean time course comparing the effects of Δ^9-THC (10 μM) to those of RAMEB alone and to Δ^9-THC (10 μM) after a 20 min treatment with AM251 (1 μM). Note that whereas RAMEB alone significantly inhibited glutamate release, the effect of Δ^9-THC in the same concentration of vehicle was significantly larger (**$p < 0.01$, RM-ANOVA). Furthermore, the effect of Δ^9-THC on fEPSPs was not significantly different from RAMEB alone, when slices were pretreated with AM251 ($p > 0.05$, RM-ANOVA).

Higher eADO levels in WT C57BL/6J mice explains the lack of CB1R signaling

The data described above show that eADO can influence signaling through CB1Rs on Sc axon terminals in the mouse hippocampus, and provide an explanation for our previous negative results with WIN55,212-2 in this species. However, it remained to be determined why robust CB1R-mediated effects on glutamate release were observed in hippocampal slices from SD rats in the absence of A1R blockade. We hypothesized that the absence of the modulating effects of A1Rs on CB1Rs in rat hippocampus might result from differences in tonic extracellular levels of eADO in brain slices from these species, under the identical incubation and recording conditions.

Previous work has shown that the magnitude of the effect of theophylline on synaptic glutamate release is directly related to the extracellular levels of eADO (Brundege and Dunwiddie, 1996), and that the effects of theophylline on fEPSPs are absent in A1R $^{-/-}$ mice (Johansson et al., 2001). Therefore, we compared the effects of theophylline on fEPSPs in CB1 $^{+/+}$ mouse and SD rat hippocampal slices to determine whether eADO levels differed between these species. Theophylline (250 μM) caused a small increase in fEPSP amplitudes rat hippocampal slices (Fig. 3*A*) that was consistent with previous studies (Dunwiddie and Diao, 1994). However, theophylline caused a much larger increase in fEPSPs in brain slices from mice than that observed in rat slices (Fig. 3*A*) (RM-ANOVA, $F_{(24,1)} = 13.85$, $p < 0.001$).

Although the different effects of theophylline on fEPSPs in mouse versus rat suggested that eADO levels were indeed higher in the mouse hippocampus, it was also possible that there were differences in A1R function or number. Therefore, we examined the effects of the selective A1R agonist N^6-cyclopentyladenosine (N^6-CPA) on fEPSPs in hippocampal slices from SD rats and CB1 $^{+/+}$ mice. N^6-CPA concentration-dependently inhibited fEPSPs in slices from both species, and exhibited a similar maximal effect (Fig. 3*B*). This suggested that, consistent with a previous report (Fastbom et al., 1987), there was not a large disparity in the number of A1Rs mediating the inhibition of fEPSPs between mouse and rat. However, although the maximal responses to N^6-CPA did not differ, the 50% effective concentration of this drug for the inhibition of fEPSPs (EC_{50}) was significantly smaller in brain slices from SD rats (Fig. 3*B*) (rat $EC_{50} = 189$ nM, mouse $EC_{50} = 587$ nM; $p < 0.001$). This is likely explained by the known ability of eADO to compete with N^6-CPA for A1R binding sites (Bruns et al., 1980). Therefore, our data are consistent with the idea that the lower apparent affinity of N^6-CPA for the A1R in the mouse hippocampus is due to higher extracellular levels of eADO in these slices.

Δ^9-THC effects on synaptic glutamate release are controlled by adenosine A1Rs

The cannabinoid agonist WIN55,212-2 has been used extensively in investigations of CB1R function. However, it may also affect glutamatergic synaptic transmission independently of CB1R activation (Hajos et al., 2001). For this reason, and because Δ^9-THC is the cannabinoid agonist that is more relevant to human drug abuse, we sought to determine whether this phytocannabinoid could also alter glutamatergic fEPSPs in the hippocampus, and whether the effects of this drug were similarly affected by eADO. Because of its high degree of insolubility in aqueous solutions, Δ^9-THC was suspended in a vehicle, RAMEB, that greatly increases its water solubility (Hazekamp and Verpoorte, 2006), and permits its *in vitro* effects to be observed (Laaris et al., submitted). Alone, RAMEB (0.1%) inhibited fEPSPs in SD rat hippocampal slices by 16 ± 3.1% at 30 min after application (Fig. 4). In contrast, Δ^9-THC (10 μM), suspended in this RAMEB vehicle,

inhibited fEPSPs to a significantly larger degree at this same time point (Fig. 4) (RM-ANOVA, $F_{(52,2)} = 3.21, p < 0.001$ vs RAMEB, Tukey *post hoc*). Consistent with the activation of CB1Rs, the effect of Δ^9-THC on fEPSPs was prevented by pretreatment (25 min) of the rat hippocampal slices with the antagonist AM251 (1 μM) (Fig. 4) (RM-ANOVA, $F_{(52,2)} = 3.21$, $p < 0.001$ vs THC, Tukey *post hoc*). These data indicated that Δ^9-THC is an agonist at CB1Rs located on Sc axon terminals in the rat hippocampal slice, and can inhibit synaptic glutamate release.

To determine whether Δ^9-THC similarly inhibited glutamate release in the WT C57BL/6J mouse hippocampus, and to determine whether, like WIN55,212-2, its effects were occluded by A1R activation, we measured Δ^9-THC effects on whole-cell glutamatergic EPSCs evoked by Sc stimulation in CA1 pyramidal neurons. Also, to confirm that the effects of Δ^9-THC were presynaptic its effects on postsynaptic ionotropic glutamate receptor currents were measured using laser photolysis of CNB-caged glutamate in the same neurons. Thus, the synaptic EPSCs were alternated every 60 s with single UV-flash-activated glutamatergic postsynaptic currents throughout each experiment. This approach thereby permitted a comparison of potential presynaptic and postsynaptic effects of Δ^9-THC simultaneously in the same CA1 pyramidal neurons.

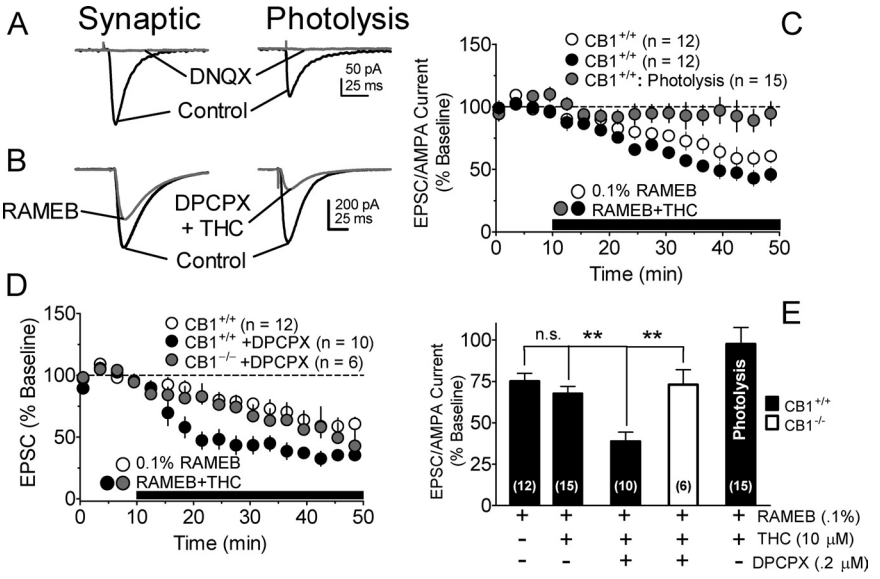

Figure 5. Adenosine A1Rs control CB1R inhibition of hippocampal EPSCs in the C57BL/6J mouse hippocampus. ***A***, Mean evoked EPSCs and currents elicited through photolysis of CNB-caged glutamate in the same CA1 neuron from a $CB1^{+/+}$ mouse. Both synaptic and photolysis-evoked glutamate responses were blocked by the AMPAR antagonist DNQX (10 μM). ***B***, Mean EPSCs recorded from a pyramidal neuron in $CB1^{+/+}$ mouse hippocampus showing the effect of the RAMEB vehicle (left) and Δ^9-THC (10 μM) after treatment with the selective adenosine A_1 receptor antagonist DPCPX (200 nM). ***C***, Mean time course showing the effects of RAMEB on EPSCs and of Δ^9-THC (10 μM) dissolved in this vehicle on EPSCs and photolysis-evoked currents in $CB1^{+/+}$ mouse CA1 pyramidal neurons. The difference between the effects of RAMEB and Δ^9-THC plus RAMEB was not statistically significant ($p > 0.5$, RM-ANOVA), and Δ^9-THC plus RAMEB had no effect on the photolysis-evoked glutamate currents. ***D***, Mean time course comparing the effects of RAMEB to Δ^9-THC in DPCPX-pretreated pyramidal neurons in hippocampal slices from $CB1^{+/+}$ and $CB1^{-/-}$ mice. A1R antagonism with DPCPX significantly increased the inhibition of EPSCs by Δ^9-THC ($p < 0.01$, RM-ANOVA) in the $CB1^{+/+}$ slices, but Δ^9-THC effects were not significantly different from vehicle in $CB1^{-/-}$ slices, despite DPCPX pretreatment. In the DPCPX experiments, the data were normalized after the antagonist effects had stabilized, before Δ^9-THC application. Therefore, the effect of DPCPX on EPSCs is not shown. ***E***, Summary of the effects of Δ^9-THC and RAMEB on EPSCs or photolysis-evoked AMPA currents recorded in mouse hippocampus. The mean effects were obtained 20 min after Δ^9-THC or RAMEB application (** = $p < 0.01$, RM-ANOVA). Picrotoxin was used to block $GABA_A$ receptors throughout these experiments.

The synaptic EPSC and the photolysis-activated glutamate currents, measured in the presence of picrotoxin to block $GABA_A$ currents, were completely blocked by the AMPA/kainate receptor antagonist DNQX (10 μM) (Fig. 5*A*). Furthermore, like fEPSPs in SD rat hippocampal slices, RAMEB (0.1%) alone significantly inhibited Sc-evoked EPSCs in $CB1^{+/+}$ mouse CA1 pyramidal neurons, without altering the photolysis-evoked glutamate currents (RM-ANOVA, $F_{(16,1)} = 3.19$, $p < 0.001$) (Fig. 5*B*,*C*,*E*). Also, whereas the effect of Δ^9-THC (10 μM) on EPSCs was slightly larger than that observed with RAMEB alone (Fig. 5*C*), this was not significantly different (RM-ANOVA, $F_{(16,1)} = 1.20$, $p > 0.05$ vs RAMEB, Tukey *post hoc*), suggesting that, like WIN55,212-2, Δ^9-THC was not an effective agonist in the WT C57BL/6J mouse hippocampus under these control recording conditions. Additionally, the absence of Δ^9-THC effects on the photolysis-evoked glutamate currents indicated that there was likely no postsynaptic effect of this drug or RAMEB on either glutamate receptors, or glutamate uptake (Fig. 5*C*,*E*).

We next determined whether CB1R-dependent effects of Δ^9-THC could be observed in hippocampal slices from $CB1^{+/+}$ mice during blockade of A1Rs. Thus, brain slices were pretreated with the selective A1R antagonist DPCPX (200 nM) for 20 min before Δ^9-THC application. When applied alone, DPCPX caused a significant increase (187.7 ± 35.5% of control, $n = 8$ neurons; $p < 0.001$, paired *t* test) in the amplitudes of EPSCs recorded in WT C57BL/6J mouse hippocampal slices. Following 20 min of DPCPX pretreatment the subsequent EPSCs were normalized to this new baseline. In the presence of DPCPX, Sc-evoked EPSCs were now significantly inhibited by Δ^9-THC (10 μM) (Fig. 5*B*,*D*,*E*), and this was significantly larger than the effect of RAMEB alone (Fig. 5*D*,*E*) (RM-ANOVA, $F_{(16,1)} = 5.28$, $p < 0.001$, Tukey *post hoc*). Furthermore, to confirm that the inhibition of EPSCs by Δ^9-THC during A1R blockade was mediated by CB1Rs, we repeated the previous experiment in hippocampal slices from $CB1^{-/-}$ mice. In these pyramidal neurons, the effect of Δ^9-THC (10 μM) on EPSCs was not significantly different from the inhibition produced by RAMEB alone (RM-ANOVA $F_{(16,1)} = 0.285$, $p > 0.05$) (Fig. 5*D*,*E*).

A1R antagonism increases endocannabinoid function

The above data demonstrated that tonic activation of A1Rs by eADO blocked signaling through CB1Rs located on Sc axon terminals in the C57BL/6J mouse hippocampus, and that this was observable with both synthetic and plant-derived CB1R agonists. To extend these results and to further determine the potential functional significance of this interaction, we examined the effect of A1R antagonism on eCB signaling. The phenomenon known as DSE occurs when the eCB 2-arachidonoyl glycerol (2-AG) is released from CA1 pyramidal neurons during depolarization (Straiker and Mackie, 2005). 2-AG is thought to then act in a retrograde manner to activate CB1Rs located on Shaffer collateral axon terminals to reduce glutamate release (Ohno-Shosaku et al., 2002b; Straiker and Mackie, 2005). We attempted to generate DSE during whole-cell voltage-clamp recordings of electrically evoked EPSCs from CA1 pyramidal neurons in $CB1^{+/+}$ mouse

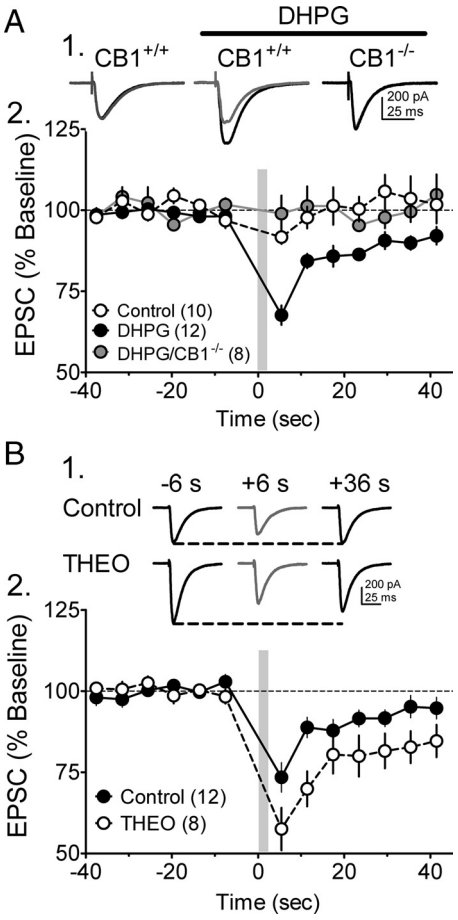

Figure 6. A1R antagonism increases endocannabinoid-dependent DSE in mGluR agonist-treated mouse brain slices. ***A***, DSE was induced using a 3 s voltage step to 0 mV from a −70 mV holding potential (gray vertical bars in ***A2*** and ***B2***). ***A1***, Mean EPSCs collected 6 s before (black line) and 6 s after (gray line) DSE in neurons from $CB1^{+/+}$ and $CB1^{-/-}$ mice, in the absence or presence of the mGluRI agonist DHPG (10 μM). Note that DSE was observed in neurons from $CB1^{+/+}$ mice only during DHPG application and was absent in DHPG-treated $CB1^{-/-}$ neurons. ***A2***, Mean time course showing the effect of DHPG on DSE in $CB1^{+/+}$ and $CB1^{-/-}$ neurons. ***B***, Effects of A1R antagonism by THEO (250 μM) on endocannabinoid-mediated DSE in CA1 pyramidal neurons from $CB1^{+/+}$ mice pretreated with DHPG (10 μM). ***B1***, Mean EPSCs demonstrating DSE in the absence (Control) and presence of THEO in DHPG-pretreated slices. The EPSCs are averages of three traces collected every 3 s before the indicated time point, before or after the voltage step. ***B2***, Mean time course of the effect of A1R antagonism by THEO on DSE. THEO significantly increased the level of endocannabinoid-mediated DSE (RM-ANOVA; $p < 0.01$, Tukey *post hoc* analysis). However, the degree of potentiation of DSE by THEO is likely underestimated due to desensitization of A1Rs by DHPG (de Mendonça and Ribeiro, 1997; Shahraki and Stone, 2003).

brain slices by depolarizing the membrane potential from −70 to 0 mV for 3 s. However, as reported previously, DSE was not observed using this relatively short depolarization under baseline conditions (Ohno-Shosaku et al., 2002b) (Fig. 6*A*), and further, was not seen during theophylline application (data not shown). The activation of group I metabotropic glutamate receptors (mGluRI) has been shown to enhance the production of eCBs (Ohno-Shosaku et al., 2002a; Straiker and Mackie, 2007). Therefore, we attempted to elicit DSE following activation of mGluRIs with the agonist DHPG (10 μM). In the presence of DHPG, a significant level of DSE was now observed in $CB1^{+/+}$ slices (Fig. 6*A*) (RM-ANOVA, $F_{(29,1)} = 2.23$, $p < 0.001$ vs no DHPG treatment, Tukey *post hoc*). However, DSE was not observed in neurons recorded in slices from $CB1^{-/-}$ mice, despite the presence of the mGluRI agonist (Fig. 6*A*) (RM-ANOVA, $F_{(29,1)} = 2.30$, $p < 0.001$ vs $CB1^{+/+}$, Tukey *post hoc*). Thus, eCB-mediated DSE was observed in the $CB1^{+/+}$ mouse hippocampus only when mGluRIs were activated.

To determine whether A1R antagonism could facilitate eCB-mediated DSE, the above experiment was repeated in hippocampal slices pretreated with DHPG (10 μM) alone, or with DHPG and theophylline (250 μM). Consistent with the proposed modulatory effect of A1Rs on CB1Rs, DSE was significantly larger in CA1 pyramidal neurons pretreated with theophylline, than those pretreated with DHPG alone (Fig. 6*B*) (RM-ANOVA, $F_{(12,1)} = 3.29$, $p < 0.001$). Collectively, the present data suggest that A1Rs regulate CB1R function, whether activated by synthetic, plant-derived or endogenously released cannabinoid agonists.

Blockade of $GABA_A$ receptors elevates eADO and reduces CB1R signaling

Extracellular eADO levels are regulated by a variety of physiological stimuli in the mammalian CNS. For example, brain adenosine concentrations increase under conditions of high metabolic demand, including seizures, hypoxia, high-frequency stimulation, ischemia, or during transient increases in brain temperature (Rubio et al., 1975; Winn et al., 1980; Mitchell et al., 1993; Masino and Dunwiddie, 1999; Berman et al., 2000). The present findings demonstrating that eADO and the activation of presynaptic A1Rs can control CB1R function suggest that CB1R signaling may be tied to the same metabolic processes regulating eADO release. Therefore, to determine the physiological significance of the eADO control of CB1Rs we increased eADO levels by increasing cellular activity through the blockade of $GABA_A$ receptors with PCTx (Dulla et al., 2009). First, to confirm that PCTx increased eADO levels we examined the effects of theophylline (250 μM) on fEPSPs in brain slices from SD rats, before and during PCTx (50 μM) treatment. In the absence of PCTx theophylline caused a modest increase in the slope of the fEPSP in SD rat hippocampal slices (Fig. 7*A*) that was comparable to that described in Figure 3*A*. In contrast, the increase in fEPSP slope caused by theophylline in the same slices was much larger after PCTx was applied (RM-ANOVA, $F_{(19,3)} = 32.96$, $p < 0.001$) (Fig. 7*A*). This suggests that the increase in cellular activity caused by PCTx increased extracellular eADO levels, and this caused greater A1R-mediated inhibition of fEPSPs.

Since the PCTx-induced increase in cellular activity might release presynaptic modulators of glutamate release other than adenosine, we also examined the ability of antagonists for $GABA_B$ and CB1Rs to modify fEPSPs during PCTx application. However, whereas the $GABA_B$ antagonist CGP55845 (2 μM) and the CB1R antagonist AM251 (1 μM) did not alter the fEPSP slope after PCTx application, the selective A1R antagonist DPCPX (200 nM) significantly increased this measure of glutamate release (Fig. 7*B*) (RM-ANOVA, $F_{(44,2)} = 3.59$, $p < 0.001$ vs AM251 and CGP, Tukey *post hoc*). This suggested that PCTx did not elevate extracellular GABA or eCB levels to a degree that was sufficient to activate $GABA_B$ and CB1Rs located on Sc axon terminals, but it did increase the eADO concentration, permitting increased activation of A1Rs.

As described above, Δ^9-THC can inhibit Sc-evoked fEPSPs in rat hippocampal slices via activation of CB1Rs (Fig. 4). Therefore, we hypothesized that the elevation of eADO levels by PCTx, and subsequent activation of A1Rs, would limit the effect of Δ^9-THC on these responses. In control hippocampal slices that were not pretreated with PCTx, Δ^9-THC (10 μM) again robustly inhibited the slope of the fEPSP (Fig. 7*C*). However, during PCTx (50 μM) application the effect of Δ^9-THC on fEPSP slope was signifi-

cantly reduced (RM-ANOVA, $F_{(78,3)} = 2.69$, $p < 0.001$, Tukey *post hoc* vs THC alone), and this attenuated response to Δ^9-THC in the presence of PCTx was completely reversed by pretreatment with the A1R antagonist DPCPX (200 nM) (Fig. 7*C*). In contrast, the $GABA_B$ antagonist CGP55845 did not alter the reduced response to Δ^9-THC caused by PCTx (Fig. 7*C*). Therefore, the inhibitory effects of Δ^9-THC on fEPSPs were blocked by the activation of A1Rs by eADO that was released during increased cellular activity caused by PCTx (Dulla et al., 2009).

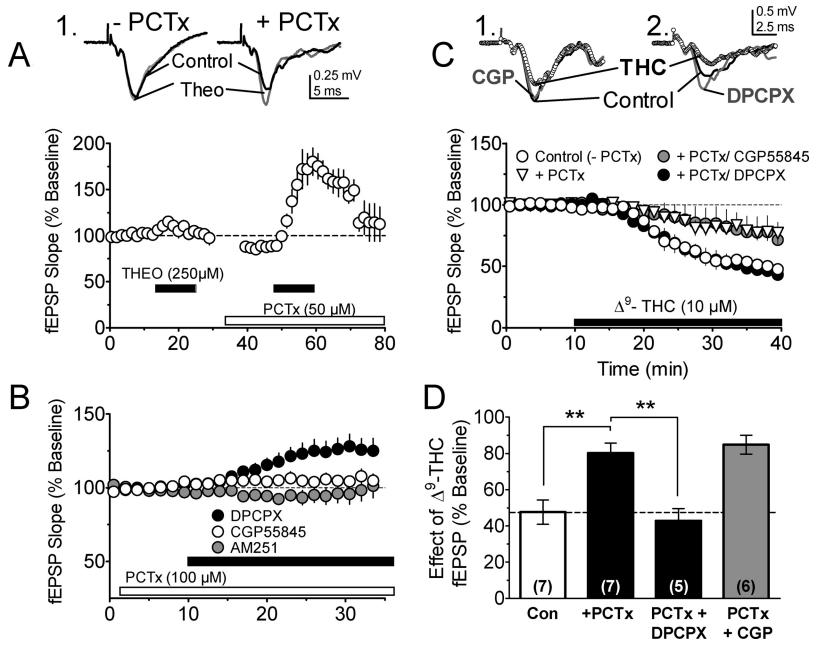

Figure 7. PCTx (50 μM) increases eADO release and the activation of A1Rs and reduces CB1R signaling in rat hippocampus. ***A1***, Mean fEPSPs recorded before (Control) and during application of the adenosine receptor antagonist theophylline (250 μM, gray lines), in the absence (−PCTx) and presence (+PCTx) of PCTx. ***A***, Mean time course ($n = 4$) of the effect of theophylline before and during PCTx treatment. The effect of theophylline is significantly larger in during PCTx treatment ($p < 0.1$, RM-ANOVA). ***B***, The selective A1R antagonist DPCPX (200 nM), but not the $GABA_B$ antagonist CGP55845 (2 μM) or the CB1R antagonist AM251 (1 μM), significantly increased fEPSPs during PCTx treatment ($p < 0.1$, RM-ANOVA). ***C***, Antagonism of A1Rs restores CB1R inhibition of fEPSPs caused by Δ^9-THC in PCTx-treated SD rat hippocampal slices. ***C1***, fEPSPs obtained in rat hippocampus during PCTx application. The $GABA_B$ antagonist CGP55845 (2 μM) had no effect on the fEPSP, and subsequent application of Δ^9-THC (10 μM, dotted line) produced only a small decrease in the response. ***C2***, During PCTx application, DPCPX (200 nM) increased the fEPSP amplitude and permitted a larger inhibition of the fEPSP by Δ^9-THC (10 μM, dotted line). ***C***, Mean time course of the effects of Δ^9-THC under each condition demonstrating decreased inhibition of fEPSPs by Δ^9-THC in PCTx and the restoration of the Δ^9-THC inhibition by DPCPX (200 nM; $n = 5$). Also shown is that $GABA_B$ antagonism by CGP55845 (2 μM, $n = 6$) did not reverse the attenuation of the Δ^9-THC effect caused by PCTx ($p < 0.01$, RM-ANOVA). ***D***, Summary of the data shown in ***C***. Mean effects of 10 μM Δ^9-THC were determined by averaging data acquired 35–40 min after its application in slices pretreated with the indicated drugs. $^{**}p < 0.01$, ANOVA. The number of brain slices under each condition is noted in parentheses.

Discussion

CB1Rs and A1Rs are located on CA3 to CA1 pyramidal neuron Sc axon terminals, where they exert inhibitory control of glutamate release. However, there have been few reports of functional interactions between these GPCRs. A previous study showed that metabolism of eADO by ADA increased cannabinoid agonist-stimulated binding of [^{35}S]-GTPγS to activated G-proteins in the rat hippocampus, demonstrating a potential molecular basis for negative modulation of CB1Rs by eADO (Moore et al., 2000). The present results demonstrate a novel functional interaction between these GPCRs by which tonically activated A1Rs can control responses to cannabinoid receptor agonists mediated by CB1Rs.

In mouse hippocampus, the control of CB1R-dependent inhibition of glutamate neurotransmission by A1Rs was observed using synthetic (WIN55,212-2) and plant-derived (Δ^9-THC) cannabinoid agonists, as well as during eCB-mediated DSE. Furthermore, the attenuation of CB1R inhibition by eADO was absent in brain slices from A1 $^{-/-}$ C57BL/6J mice, providing strong evidence for the involvement of these receptors in this interaction. Endocannabinoid-dependent DSE was observed when mGluRIs were activated with the agonist DHPG, and this was significantly increased during A1R antagonism. This provides evidence that A1Rs may also control eCB function in the hippocampus. However, the enhancement of DSE by theophylline may have been underestimated because mGluRIs are also known to desensitize A1Rs at this glutamate synapse (de Mendonça and Ribeiro, 1997; Shahraki and Stone, 2003).

In the C57BL/6J mouse hippocampus the A1R–CB1R interaction resulted from tonic activation of A1Rs by eADO causing strong basal inhibition of glutamate release at CA3–CA1 synapses (Dunwiddie et al., 1981; Dunwiddie and Diao, 1994). However, in the SD rat hippocampus, attenuation of CB1R function was observed only when eADO levels and A1R activation were increased via disinhibition by the $GABA_A$ Cl^- channel blocker PCTx (Fig. 6) (Dulla et al., 2009). This species difference likely resulted from higher basal eADO in the C57BL/6J mouse hippocampus, despite the identical preparation and handling of the brain slices. The evidence for higher levels of eADO in WT C57BL/6J mouse hippocampus included larger increases in fEPSPs caused by A1R antagonists, which is consistent with studies demonstrating that the increase in EPSP amplitude caused by theophylline was proportional to the extracellular concentration of eADO acting at A1Rs in the hippocampus (Dunwiddie and Diao, 1994; Brundege and Dunwiddie, 1996). We also observed a significantly higher EC_{50} for the A1R agonist N^6-CPA in inhibiting fEPSPs in the mouse hippocampus, compared with that observed in rat (Fig. 3). Receptor binding experiments have demonstrated that eADO can decrease the apparent affinity of A1R agonists by competing for binding sites, unless eADO is removed by ADA (Bruns et al., 1980). Therefore, it is likely that the higher eADO concentrations in the mouse slices caused a reduction in the apparent affinity of N^6-CPA for A1Rs in our study. However, despite this difference in apparent affinity, the maximum inhibition of fEPSPs by N^6-CPA was similar between mouse and rat hippocampus, arguing against different numbers of functional A1Rs. This is further supported by studies reporting similar numbers of A1Rs in the hippocampi of several species, including mouse and rat (Fastbom et al., 1987). Therefore, in light of the present data and these previous binding studies, we suggest that the differences in sensitivity to A1R antagonists between rat and mouse hippocampus resulted from differences in extracellular eADO concentrations, and not A1R number or inherent affinity.

We previously reported that the cannabinoid agonist WIN55,212-2, was ineffective at inhibiting fEPSPs in C57BL/6J-

$CB1^{+/+}$ mice, but inhibited GABAergic IPSCs in these animals (Hoffman et al., 2005). Furthermore, robust inhibition of fEPSPs by WIN55,212-2 was observed in slices from SD rats and CD-1 mice maintained under identical incubation and recording conditions (Hoffman et al., 2005). Subsequent work in other laboratories demonstrated that WIN55,212-2 inhibited synaptic glutamate release in the C57BL/6J-$CB1^{+/+}$ mouse hippocampus, but not in hippocampi from $CB1^{-/-}$ littermates (Kawamura et al., 2006; Takahashi and Castillo, 2006). Also, using a highly sensitive CB1R antibody, CB1Rs were found at low levels on Sc axon terminals near molecular components necessary for eCB production (Katona et al., 2006; Kawamura et al., 2006). These data therefore provided evidence for CB1Rs on Sc terminals, and implied that an additional factor was responsible for the absence of WIN55,212-2 effects on glutamate release in the C57BL/6J mouse hippocampus in our laboratory. The present results suggest that this disparity can be explained by differences in basal levels of eADO and A1R-mediated tonic inhibition between these laboratories, likely resulting from different procedures used to prepare and maintain hippocampal slices. Although the precise reason for this difference in eADO levels in C57BL/6J mouse brain slices among laboratories is unclear, it is well known that eADO concentrations are controlled by a variety of metabolic, and activity-dependent neuronal factors (Dunwiddie and Masino, 2001; Masino et al., 2002). Thus, eADO concentrations can increase dramatically during periods of ischemia, and hypoxia, and during decreases or increases in brain slice temperature (Rubio et al., 1975; Winn et al., 1980; Masino and Dunwiddie, 1999). Although any of these variables may explain differences in eADO concentrations, constitutive differences must also exist between species, since eADO levels, as defined by sensitivity to A1R antagonists, differed between C57BL/6J mouse and SD rat hippocampal slices in our study.

There are several points in the control of eADO metabolism at which differences might be found among species. For example, facilitated transport mechanisms that maintain eADO levels in the extracellular space differ both within (Short et al., 2006) and across species (Johnston and Geiger, 1990). Furthermore, species differences exist in the distribution of 5′-nucleotidase, the enzyme that catalyzes the final step of nucleotide (ATP, ADP, and AMP) conversion to adenosine (Lee et al., 1986; Fastbom et al., 1987; Dunwiddie et al., 1997), and in the subcellular localization of ADA (Yamamoto et al., 1987). Thus, the net balance between eADO formation, uptake and metabolism likely differs across species. In the present study this may be reflected by higher eADO levels in the C57BL/6J mouse hippocampus compared with other species and strains of rodent examined in our laboratory.

Although the mechanism for the interaction between A1Rs and CB1Rs was not identified in the present study, previous investigations have demonstrated that these receptors can use overlapping sets of G-protein Gα subunits (Straiker et al., 2002), and tonic A1R activation by eADO can inhibit CB1R-mediated activation of G-proteins (Moore et al., 2000). This may provide prima facie evidence that tonically activated A1Rs can appropriate G-proteins necessary for CB1R signal transduction at Sc axon terminals. Alternatively, A1Rs and CB1Rs also inhibit glutamate release at Sc axon terminals through inhibition of overlapping classes of VDCCs (Wu and Saggau, 1994; Sullivan, 1999; Manita et al., 2004), and A1Rs tonically inhibit a significant proportion of these channels (Manita et al., 2004), perhaps removing them from CB1R influence. It also remains to be determined whether the A1R–CB1R interaction that we have identified here is reciprocal. However, unlike tonic eADO tone, there appears to be no basal eCB release at Sc synapses (this study and Hoffman et al., 2007). Therefore, we would predict that CB1R-mediated inhibition of A1R effects might only be observed during cellular processes causing relatively substantial increase in eCB release. Although the molecular mechanism for this A1R–CB1R interaction awaits additional research, it seems to be selective for these GPCRs since antagonism of $GABA_B$ receptors that also inhibit Sc glutamate release, did not alter CB1R-mediated inhibition of fEPSPs in the mouse hippocampus, nor did it reverse the attenuation of the effect of Δ^9-THC by PCTx in rat hippocampus (Fig. 6).

Despite the fact that CB1Rs are more densely expressed on axon terminals of cholecystokinin-containing GABAergic interneurons in area CA1 of the hippocampus (Katona et al., 1999; Tsou et al., 1999; Hoffman and Lupica, 2000), the interaction between CB1Rs and A1Rs that we describe would likely occur only at glutamatergic axon terminals. This is because GABAergic axon terminals lack A1Rs, and adenosine does not directly modulate GABA-mediated IPSPs in CA1 pyramidal neurons (Kamiya, 1991; Yoon and Rothman, 1991). Therefore, eADO should selectively restrict CB1R function at glutamatergic and not GABAergic synapses in the hippocampus. Consistent with this idea, robust inhibition of IPSCs by WIN55,212-2 is observed in the C57BL/6 mouse hippocampus in the absence of A1R antagonists (Hoffman et al., 2005), and compared with glutamate release, IPSCs are more sensitive to WIN55,212-2 (Ohno-Shosaku et al., 2002b; Hoffman et al., 2007). Therefore, this difference in sensitivity to WIN55,212-2 may result from the interaction between CB1Rs and A1Rs on glutamate but not GABA axon terminals.

The different sensitivities to cannabinoids at excitatory and inhibitory axon terminals in the hippocampus, and differences in eADO levels among rodent species may also help explain inconsistencies observed in the behavioral effects of cannabinoids. For example, cannabinoid agonists can be anxiolytic or anxiogenic in mice and rats, respectively, and this has been related to different sensitivities of GABAergic and glutamatergic synapses to cannabinoids (Haller et al., 2007). Studies cited above suggest that species differences in brain adenosine systems exist, and our data show that eADO also alters the sensitivity of glutamatergic pathways to cannabinoids. Therefore, the interaction between A1Rs and CB1Rs may help explain some of the discrepant actions of cannabinoids on anxiety and other complex behaviors.

The present findings suggest that the degree of cannabinoid signaling and control of glutamatergic synaptic transmission will be limited by tonic levels of eADO and therefore indirectly subject to the same homeostatic controls regulating extracellular levels of this purinergic neuromodulator. Because of this, we predict that CB1R inhibition of glutamate neurotransmission will be more strongly limited when eADO is elevated, such as during epileptiform activity, during increased or reduced brain temperature, and during ischemia, hypoxia, and perhaps sleep (Rubio et al., 1975; Winn et al., 1980; Mitchell et al., 1993; Masino and Dunwiddie, 1999; Berman et al., 2000). Furthermore, our data demonstrating that the effects of Δ^9-THC in the hippocampus are attenuated when eADO levels are elevated and enhanced when A1Rs are antagonized suggest the intriguing possibility that the effects of marijuana on hippocampal-dependent memory and cognition in humans might be increased during the simultaneous consumption of marijuana and caffeine.

References

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