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Δ^9-tetrahydrocannabinol is a full agonist at CB1 receptors on GABA neuron axon terminals in the hippocampus

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Summary

Marijuana impairs learning and memory through actions of its psychoactive constituent, delta-9-tetrahydrocannabinol (Δ^9-THC), in the hippocampus, through activation of cannabinoid CB1 receptors (CB1R). CB1Rs are found on glutamate and GABA neuron axon terminals in the hippocampus where they control neurotransmitter release. Previous studies suggest that Δ^9-THC is a partial agonist of CB1Rs on glutamate axon terminals in the hippocampus, whereas its effects on GABA terminals have not been described. Using whole-cell electrophysiology in brain slices from C57BL6/J mice, we examined Δ^9-THC effects on synaptic GABA IPSCs, and postsynaptic GABA currents elicited by laser-induced photo-uncaging (photolysis) of α-carboxy-2-nitrobenzyl (CNB) caged GABA. Despite robust inhibition of synaptic IPSCs in wildtype mice by the full synthetic agonist WIN55,212-2, using a Tween-80 and DMSO vehicle, Δ^9-THC had no effects on IPSCs in this, or in a low concentration of another vehicle, randomly-methylated β-cyclodextrin (RAMEB, 0.023%). However, IPSCs were inhibited by Δ^9-THC in 0.1% RAMEB, but not in neurons from CB1R knockout mice. Whereas Δ^9-THC did not affect photolysis-evoked GABA currents, these responses were prolonged by a GABA uptake inhibitor. Concentration-response curves revealed that the maximal effects of Δ^9-THC and WIN55,212-2 were similar, indicating that Δ^9-THC is a full agonists at CB1Rs on GABA axon terminals. These results suggest that Δ^9-THC inhibits GABA release but does not directly alter $GABA_A$ receptors or GABA uptake in the hippocampus. Furthermore, full agonist effects of Δ^9-THC on IPSCs likely result from a much higher expression of CB1Rs on GABA versus glutamate axon terminals in the hippocampus.

Keywords

marijuana; brain slice; learning; memory; synaptic plasticity; cannabinoid

Introduction

Marijuana (*Cannabis sativa*) is a psychoactive plant that is widely used throughout the world. The marijuana plant contains approximately 70 cannabinoid compounds (Burns & Ineck, 2006). However, Δ^9-THC is thought to be the main psychoactive cannabinoid responsible for mood alterations, feelings of euphoria, and cognitive impairments that are hallmarks of its effects in humans (Gaoni & Mechoulam, 1964). Δ^9-THC exerts its effects on cellular processes

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by activating cannabinoid CB1 and CB2 receptors that are members of the G-protein coupled receptor family (Pertwee, 1997). However, compared to CB2Rs, CB1Rs are highly expressed in brain tissue, and mediate most of the central actions of marijuana in humans (Huestis *et al.*, 2001). The discovery of the cannabinoid receptors (Matsuda *et al.*, 1990; Munro *et al.*, 1993), and identification of endogenous ligands (Devane *et al.*, 1992; Mechoulam *et al.*, 1995; Stella *et al.*, 1997) demonstrated the existence of a brain endocannabinoid system.

An important cognitive effect of marijuana in humans is the impairment of memory through the disruption of information encoding and recall of newly acquired information (Abel, 1971a; Abel, 1971b; Ranganathan & D'Souza, 2006; Wilson *et al.*, 1994). In addition, working memory is necessary for normal performance in many cognitive tasks in humans and animals, and it relies upon intact hippocampal function (Hampson & Deadwyler, 1998; Ranganathan & D'Souza, 2006). In animals, spatial working memory is profoundly impaired by Δ^9-THC given systemically, injected directly into the hippocampus (Lichtman *et al.*, 1995), or by exposure to marijuana smoke (Niyuhire *et al.*, 2007), and this appears to be mediated exclusively by CB1Rs in the hippocampus (Varvel & Lichtman, 2002; Wise *et al.*, 2009). In addition to disruption of spatial working memory, non-spatial operant learning and memory is also disrupted by Δ^9-THC (Hampson & Deadwyler, 1998; Heyser *et al.*, 1993). In support of the central role of the hippocampus in mediating the cognitive effects of marijuana, this structure contains a dense population of CB1Rs that mediate the effects of exogenous and endogenous cannabinoids (Herkenham *et al.*, 1990; Katona *et al.*, 1999), and a recent study has provided strong evidence for the involvement of CB1Rs on GABA axon terminals in the effects of Δ^9-THC on spatial memory in mice (Puighermanal *et al.*, 2009).

At the cellular level, cannabinoids presynaptically inhibit the release of both GABA and glutamate in the hippocampus and throughout the brain (Gerdeman & Lovinger, 2001; Hoffman *et al.*, 2003; Hoffman & Lupica, 2000; Hoffman & Lupica, 2001; Levenes *et al.*, 1998; Robbe *et al.*, 2001; Wilson & Nicoll, 2002). Furthermore, it is likely that this is the primary means through which cannabinoids alter hippocampal neuronal network activity (Hajos *et al.*, 2000; Robbe *et al.*, 2006). Another mechanism that may be involved in the disruption of memory by cannabinoids is the inhibition of forms of synaptic plasticity, such as long-term potentiation and depression (LTP, LTD), that are proposed cellular correlates of learning and memory (Bliss & Collingridge, 1993; Lynch, 2004). Acutely, synthetic cannabinoids, endocannabinoids, and Δ^9-THC block LTP in the CA1 region of the hippocampus in vitro (Misner & Sullivan, 1999; Nowicky *et al.*, 1987; Stella *et al.*, 1997), and long-term exposure to Δ^9-THC in vivo can block LTP in vitro during withdrawal, despite the absence of detectable tissue levels of Δ^9-THC at the time of LTP induction (Hoffman *et al.*, 2007; Fan *et al.*, 2010).

Although ample evidence implicates the hippocampus as a site for the actions of Δ^9-THC in the disruption of memory, surprisingly little is known of its physiological actions on specific intact neural pathways in the CNS. Although a some studies have described the effects of Δ^9-THC in adult hippocampal brain slices (Foy *et al.*, 1982; Nowicky *et al.*, 1987), these were conducted prior to identification of CB1Rs, and the development of antagonists and CB1R knockout animals. More recent functional studies with Δ^9-THC in vitro have utilized immature hippocampal neurons maintained in culture to demonstrate that this phytocannabinoid can act as an agonist, partial agonist or antagonist at CB1Rs coupled to the inhibition of glutamate release (Kelley & Thayer, 2004; Roloff & Thayer, 2009; Shen & Thayer, 1999; Straiker & Mackie, 2005). However, these studies have not examined the effects of Δ^9-THC in mature hippocampal circuits, nor have they directly examined its effects on GABAergic neurotransmission, despite the much higher density of CB1Rs on these axon terminals compared to glutamate terminals (Kawamura *et al.*, 2006; Marsicano & Lutz, 1999). In addition to the paucity of information of Δ^9-THC effects on GABA release in the hippocampus, it has

also been proposed to inhibit the uptake of GABA and other neurotransmitters (Banerjee *et al.*, 1975; Coull *et al.*, 1997; Maneuf *et al.*, 1996). Furthermore, our limited understanding of the effects of Δ^9-THC in specific CNS circuits might be due to its high lipophilicity and poor solubility in aqueous media (Banerjee *et al.*, 1975; Jarho *et al.*, 1998).

In an attempt to identify the specific sites at which Δ^9-THC acts to alter hippocampal function, we have examined its effects on GABA release in mature hippocampal slices obtained from $CB1^{+/+}$ and $CB1^{-/-}$ mice, and compared these actions to those of a full synthetic agonist. Additionally, since Δ^9-THC proved to be highly insoluble for *in vitro* use, we describe procedures permitting is solubilization.

Methods

Animals

Animal protocols were approved by the Animal Care and Use Committee of the NIDA Intramural Research Program, and were conducted in strict accordance with NIH guidelines to minimize the number of animals used in these studies. Wildtype (WT, $CB1^{+/+}$) and CB1 receptor knockout (KO, $CB1^{-/-}$) littermate C57BL6\J mice (4-10 weeks) were obtained from the NIDA Intramural Research Program transgenic facility colony. These animals were descendants of 3 heterozygous ($CB1^{+/-}$) breeding pairs, donated by Dr. Andreas Zimmer and the National Institute of Mental Health (Bethesda, MD, USA). Genotyping was performed by Charles River Laboratories (Raleigh, NC, USA). Several of the observations made in mice were confirmed using a smaller number of wildtype male Sprague-Dawley rats (4-6 weeks of age), obtained from Charles River Laboratories (Raleigh, NC, USA).

Brain slice preparation

Hippocampal brain slices were prepared as previously described (Hoffman & Lupica, 2000). Briefly, animals were killed by cervical dislocation followed by decapitation. The brains were rapidly removed and immersed in cold (4°C), oxygenated high-sucrose, low-Ca^{2+}-containing artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl, 87; KCl, 2.5; $MgCl_2$, 7; $CaCl_2$, 0.5; NaH_2PO_4, 1.25; glucose, 25; sucrose 75; $NaHCO_3$, 25. Coronal slices were then cut at 280 μm thickness using a vibrating tissue slicer (VT1000S, Leica Instruments, Germany). Hemi-sectioned brain slices containing the hippocampus were then incubated in a solution composed of 50% high-sucrose and 50% normal aCSF of the following composition (mM): NaCl, 126; KCl, 3.0; $MgCl_2$, 1.5; $CaCl_2$, 2.4; NaH_2PO_4, 1.2; glucose, 11.0; $NaHCO_3$, 26, saturated with 95% O_2 and 5% CO_2, at room temperature for ≥ 90 minutes before recordings. Individual brain slices were placed into a low-volume (~300 μL) recording chamber integrated into the fixed stage of a differential interference (DIC) contrast microscope (Olympus America, Center Valley, PA, USA), and submerged in normal aCSF of a fixed volume (~15 mL) that was recirculated at 2 mL/min using a peristaltic pump. 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, Hamden, CT).

Electrophysiology

Whole-cell electrophysiological recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Foster CA) and electrodes pulled from borosilicate glass (1.5 mm O.D., 0.86 mm I.D., Sutter Instruments, Burlingame, CA). Electrodes were filled with a solution containing (mM): D-gluconic acid, 125.0; HEPES, 10.0; EGTA, 1.0; $CaCl_2$, 0.1; KCl, 10.0; Mg^{2+}-ATP, 1.0; Na^+-GTP, 0.2, or $CsCH_3SO_3$, 100; CsCl, 60; EGTA 0.2; HEPES, 10; $MgCl_2$, 2.0; Mg^{2+}-ATP, 1.0; Na^+-GTP, 0.3. All intracellular solutions also contained the quaternary lidocaine derivative, QX-314 (Sigma, St. Louis, MO; 1 mg/ml), to block action potentials only in the recorded cells. The internal solutions were adjusted to pH 7.2-7.4 using

CsOH. Series resistance was monitored with a -10 mV voltage step (200 ms), initiated every 30 sec. Series resistance measurements and the synaptic and photolysis-evoked current amplitudes were all plotted versus time on the same graph to determine whether the observed changes in these currents were associated with altered cellular access resistance. Only cells maintaining stable series resistance (< 10% change over the duration of the recording) were included in analyses. Data were directly acquired to a personal computer using an A/D board (Instrutech ITC-18, Bellmore, NY) 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).

GABAergic currents were measured in hippocampal CA1 pyramidal neurons identified under visual control using differential-interference contrast (DIC) videomicroscopy and infrared illumination. To measure evoked IPSCs, CA1 pyramidal neurons were voltage clamped at −20 mV when using the gluconic acid intracellular solution (outward currents), and at -70 mV when the $CSCH_3SO_3$ intracellular solution (inward currents) was used. IPSCs were evoked using a custom-built bipolar formvar-insulated nickel-chromium stimulating electrode placed near stratum pyramidale. Synaptic IPSCs and photolysis-evoked $GABA_A$-mediated Cl^- currents were pharmacologically isolated using the glutamate receptor antagonists D-(-)-2-amino-5-phosphonopentanoic acid (APV, 40 μM) to block NMDA receptors, and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM) to block AMPA/kainate receptors. Synaptic IPSCs were evoked once per minute and alternated with photolysis-evoked postsynaptic GABA currents throughout the duration of the recordings. Photolysis was performed using a solid state, pulsed Nd:YAG laser (Minilite I, Continuum, Santa Clara, CA, USA). The laser output beam 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, approximately 25 μm in diameter. This spot was focused upon the pyramidal neuron soma to uncage α-carboxy-2-nitrobenzyl (CNB)-caged GABA (Invitrogen, Carlsbad, CA, USA). Once whole-cell access was obtained, the objective was focused upon the pyramidal neuron and the laser output was adjusted to yield a postsynaptic 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.

Drugs

WIN55,212-2 and AM251 were purchased from Tocris-Cookson (Ballwin, MO, USA). DNQX, APV, picrotoxin and randomly-methylated β-cyclodextrin (RAMEB) were purchased from Sigma (St. Louis, MO). CNB-caged GABA was purchased from Invitrogen (Carlsbad, CA, USA). Δ^9-THC (200 mg/ml in EtOH) was obtained from the National Institute on Drug Abuse drug supply system (Bethesda, MD). The Δ^9-THC resin was suspended in an equivalent volume of DMSO. The Δ^9-THC solution was then diluted to 10 mM (3 mg/ml) in 22.5% RAMEB in 50% EtOH, and a stock solution prepared at 1 mM in 10% or 2.3% RAMEB. The final bath concentration of RAMEB was 0.1% in all experiments, unless indicated. For experiments described in Figure 1B, Δ^9-THC was also prepared in Tween-80 (10%), DMSO (20%), and saline (70%). WIN55,212-2 was initially prepared as 10 mM stock solution in DMSO, and then diluted in Tween-80 and normal aCSF. Final concentrations of Tween-80 and DMSO in the tissue bath were 0.01% and 0.02%, respectively, and have been found have no effects on the hippocampal synaptic transmission at these concentrations.

Data analysis

All data are presented either as the mean ± SEM, or the mean ± C.I. (95% confidence interval). The *n* represents the number of neurons tested, with no more than 2 neurons obtained from a single animal. Peak amplitudes of IPSCs during drug application were normalized to the pre-drug (control) baseline period. All statistical analyses and curve fits were performed using

Prism (v5.02, GraphPad Scientific, San Diego, CA). In all instances where time courses of drug effects were measured, a repeated measures analysis of variance (RM-ANOVA) was used, with appropriate post hoc analyses. The Δ^9-THC concentration-response curve was fit using the sigmoidal non-linear regression function:

$$Y=\text{Bottom}+(\text{Top}-\text{Bottom})/(1+10\hat{}((\text{LogEC50}-X)))$$

where Top and Bottom represent the plateau values of the Y-axis (% inhibition of the IPSC). IPSC decay time constants were fit using a single phase exponential decay function:

$$Y=(Y0-\text{Plateau})*\exp(-K*X)+\text{Plateau}$$

Where Y0 is the Y value when X (time) = zero, K is the rate constant, and tau (time constant of decay) = 1/K.

Results

Vehicle-dependent effects of Δ^9-THC on GABA currents

Synaptic currents (IPSCs) and those activated by laser photolysis of caged GABA (CNB-GABA) were recorded in the same CA1 pyramidal neurons during application of the ionotropic glutamate receptor antagonists DNQX (10 μM), and APV (40 μM), in hippocampal slices from WT C57BL6/J mice. In general, the CNB-GABA photolysis currents exhibited kinetic properties that were similar to those of the synaptic IPSCs (Fig. 1A). In addition, both the synaptic IPSCs and the photolysis-evoked GABA currents were completely abolished by the $GABA_A$ blocker picrotoxin (100 μM, Fig. 1A). The cannabinoid receptor agonist WIN55,212-2 (5 μM), solubilized in a Tween-80/DMSO vehicle (Hoffman & Lupica, 2000), caused robust inhibition of the synaptic IPSC in wildtype mice, whereas the photolysis-evoked $GABA_A$ current was only slightly affected (Fig. 1B,1C). We have shown previously that this effect of WIN55,212-2 on synaptic IPSCs in hippocampal CA1 pyramidal neurons is mediated by presynaptic CB1Rs and is absent in $CB1^{-/-}$ mice (Hoffman *et al.*, 2005). In contrast to the effects of WIN55,212-2 on IPSCs, Δ^9-THC (10 μM) had no effect on these responses or on photolysis-evoked GABA currents when it was solubilized in the same Tween-80/DMSO vehicle (Fig. 1D).

Since Δ^9-THC is highly insoluble in aqueous solutions, we hypothesized that unlike WIN55,212-2, the Tween-80/DMSO vehicle may not permit adequate suspension of the phytocannabinoid. Recent chemical studies have shown that the solubility of Δ^9-THC can be greatly increased by suspension in randomly-methylated-β-cyclodextrin (RAMEB) (Hazekamp & Verpoorte, 2006). Therefore, the effects of Δ^9-THC dissolved in this vehicle were determined on IPSCs and photolysis-GABAergic currents. Δ^9-THC (10 μM) dispersed in 0.023% RAMEB did not significantly affect IPSCs or photolysis-evoked GABA currents recorded in CA1 pyramidal neurons (Fig. 2A). However, when the same concentration of Δ^9-THC was suspended in a higher concentration of RAMEB (0.1%) synaptic IPSCs were significantly inhibited (Fig. 2B). Also, this concentration of RAMEB alone had no effect on these currents (103 ± 16.9% of control at 50 min of application, n = 6 neurons). The inhibition of GABAergic IPSCs by Δ^9-THC differed from that of WIN55,212-2 in that it was much slower to develop (compare Fig. 1C to Fig. 2B). Thus, a mean single exponential time constant for inhibition of IPSCs for Δ^9-THC was 25.5 min, whereas that for by WIN55,212-2 (5 μM) was 9.8 min. This delay in the pharmacological effect of Δ^9-THC likely resulted from the slower

partition of the Δ^9-THC-RAMEB complex in the brain slice, as compared to that for WIN55,212-2 in Tween-80/DMSO. Since the 0.1% RAMEB effectively permitted the inhibition of evoked synaptic IPSCs by Δ^9-THC, but did not affect these responses by itself, this concentration of the vehicle was used for the remainder of the Δ^9-THC experiments.

Full agonist properties of Δ^9-THC at inhibitory axon terminals in the hippocampus

The inhibition of synaptic GABAergic IPSCs by Δ^9-THC was dependent upon the concentration of this agonist (Fig. 2C). Thus, 50 μM Δ^9-THC produced a mean maximal inhibition of the IPSCs of 42.9 ± 1.8%, and exhibited an EC_{50} value of 1.22 μM (95% C.I. = 0.86 μM to 1.73 μM). For comparison with the effects of Δ^9-THC, a concentration-response relationship was also determined with the full CB1R agonist, WIN55,212-2. The maximal inhibition of IPSCs by WIN55,212-2 (10 μM, Fig 2C) suspended in DMSO/Tween 80, was, 45.5 ± 1.9%, which is comparable to the maximal effect of this drug in rat brain slices in our laboratory (46.6 ± 5.3%, Hoffman & Lupica, 2000). Since this value did differ significantly from the maximal inhibition of IPSCs produced by 50 μM Δ^9-THC described above ($p > 0.05$, t-test), we conclude that WIN55,212-2 and Δ^9-THC acted as full agonists in the inhibition of GABA release in the hippocampus.

Δ^9-THC inhibits GABA neurotransmission via CB1R activation

To determine whether the inhibition of GABAergic IPSCs by Δ^9-THC occurred through activation of CB1Rs, we examined its effects in hippocampal brain slices obtained from $CB1^{-/-}$ mice that were littermates of the WT mice described in the experiments above. No effects of Δ^9-THC on evoked IPSC amplitudes were observed in neurons from $CB1^{-/-}$ mice (Fig. 3B), and, as described above in WT hippocampal slices, Δ^9-THC had no effect upon photolysis-evoked $GABA_A$ current amplitudes in CA1 pyramidal neuron recordings obtained from the $CB1^{-/-}$ mice (Fig. 3B).

The effect of Δ^9-THC on GABA neurotransmission is presynaptic

Although our observation that Δ^9-THC inhibited synaptic IPSC amplitudes without altering photolysis-evoked GABA currents provided good evidence for a presynaptic action of this drug, others have suggested that Δ^9-THC affects GABAergic neurotransmission by inhibiting GABA uptake and prolonging the duration of action of this neurotransmitter at the synapse (Banerjee *et al.*, 1975; Coull *et al.*, 1997; Maneuf *et al.*, 1996). To address this we examined the effects of Δ^9-THC, at a concentration that was maximal (10 μM) for inhibition of IPSCs via CB1R activation, on the decay kinetics of synaptic and photolysis-evoked GABA currents. In addition, the effects of Δ^9-THC were compared with the GABA uptake inhibitor, nipecotic acid (10 μM). The decay phase of averaged IPSCs and photolysis-evoked currents were fitted with single exponential curves using an iterative best fit algorithm, and decay time constants (tau) were determined from this fit. The change in tau was then determined in each neuron by calculating a ratio of tau in normal aCSF to that measured 30 min after Δ^9-THC or nipecotic acid was applied to the hippocampal brain slices (Fig. 4C). Whereas nipecotic acid significantly prolonged the duration of both synaptic and photolysis-evoked $GABA_A$ receptor-mediated currents (Fig. 4C), Δ^9-THC (10 μM) had no effect on tau values of either the synaptic or photolysis-evoked currents (Fig. 4). This suggests that Δ^9-THC did not directly alter GABA uptake, nor GABAa receptor/Cl^- channel function on CA1 pyramidal neurons.

Discussion

The central and peripheral effects of marijuana and its primary psychoactive constituent Δ^9-THC have been the subject of intense study for many years. Furthermore, marijuana is a widely used illicit drug whose potency has increased in recent years due to selective hybridization to increased Δ^9-THC levels (McLaren *et al.*, 2008). Despite its widespread use and increased

potency, few modern studies have examined the pharmacological and physiological actions of Δ^9-THC on intact mature neuronal pathways. Additionally, although most studies show that cannabinoid agonists act at presynaptic CB1Rs to inhibit neurotransmitter release in the CNS, several groups report other actions of Δ^9-THC, including inhibition of neurotransmitter uptake (Banerjee *et al.*, 1975; Coull *et al.*, 1997; Maneuf *et al.*, 1996). The relative lack of knowledge regarding the effects of Δ^9-THC and interest in the specific sites at which this widely used drug acts to disrupt memory processes (Hoffman *et al.*, 2003; Hoffman *et al.*, 2007), prompted us to examine these issues more directly using transgenic mice and optical uncaging methods to permit unequivocal distinction between pre- and postsynaptic effects of Δ^9-THC in the hippocampus. Additionally, a recent study has demonstrated that selective deletion of the CB1R gene on GABA, but not glutamate neurons in the hippocampus can disrupt spatial learning in mice (Puighermanal *et al.*, 2009), suggesting that this may be the primary site of Δ^9-THC action in this brain structure.

Our initial experiments in hippocampal brain slices demonstrated that whereas the synthetic cannabinoid agonist WIN55,212-2 inhibited GABAergic IPSCs, no effect of Δ^9-THC was observed when it was suspended in the same DMSO/Tween-80 vehicle (Fig. 1D). This vehicle is commonly used in this (Hoffman & Lupica, 2000), and other laboratories to suspend hydrophobic cannabinoid agonists that exhibit poor solubility in aqueous solutions (Banerjee *et al.*, 1975). Cyclodextrins are naturally occurring water-soluble oligosaccharides that form inclusion complexes with hydrophobic molecules, greatly increasing their solubility (Fahr & Liu, 2007), and the cyclodextrin RAMEB greatly increases the solubility of Δ^9-THC in water (Hazekamp & Verpoorte, 2006). Although these compounds increase lipophilic drug solubility, they can also alter baseline synaptic transmission in the hippocampus, secondary to extraction of cholesterol from cellular membranes (Frank *et al.*, 2008). Interestingly, RAMEB-mediated cholesterol depletion selectively reduces glutamatergic and not GABAergic synaptic transmission (Frank *et al.*, 2008;Hoffman *et al.*, 2010). We also found that baseline GABergic synaptic transmission was unaltered by RAMEB, and that Δ^9-THC significantly inhibited synaptic $GABA_A$ receptor-mediated IPSCs when it was suspended in this vehicle. The fact that RAMEB was necessary to solubilize Δ^9-THC suggests that, even in comparison with other hydrophobic cannabinoid agonists like WIN55,212-2, the chemical properties of this molecule render it highly insoluble in standard vehicles used for studies in brain slices. This insolubility, and the relatively slow onset of Δ^9-THC effects compared to compounds such as WIN55,212-2 should be taken into consideration when examining the pharmacological effect of this drug *in vitro*.

In contrast to its effects on IPSCs in pyramidal neurons from $CB1^{+/+}$ mice, Δ^9-THC did not affect these synaptic currents in brain slices obtained from $CB1^{-/-}$ mice. This, together with the lack of Δ^9-THC effects on postsynaptic currents activated by the photolysis of caged GABA in the same neurons, strongly suggests that Δ^9-THC acted at CB1Rs located on inhibitory axon terminals to affect GABA neurotransmission. This is consistent with the effects of synthetic cannabinoids on IPSCs in pyramidal neurons (Hoffman & Lupica, 2000; Katona *et al.*, 1999), and with the dense expression of CB1Rs on GABAergic axon terminals in the CA1 region of the hippocampus (Marsicano & Lutz, 1999; Tsou *et al.*, 1998; Tsou *et al.*, 1999).

Previous studies of Δ^9-THC's effects in hippocampal brain slices have demonstrated relatively complex actions on pyramidal neuron population responses. Thus, at a very low concentration (10 pM) Δ^9-THC increased population spike amplitude, whereas it inhibited these responses at concentrations as high as 10 nM (Foy *et al.*, 1982; Nowicky *et al.*, 1987). However, in the latter study, population EPSPs (field EPSPs) were uniformly inhibited by Δ^9-THC across this same range of concentrations (Nowicky *et al.*, 1987). In the present study, we found that significant effects of Δ^9-THC on GABAergic IPSCs were not observed at concentrations below ~100 nM (Fig 2C). At present the reason for this discrepancy is unknown. However, one

subsequent study reported an EC_{50} value of 20 nM for Δ^9-THC's inhibition of glutamate synaptic responses in cultured hippocampal neurons (Shen & Thayer, 1999), whereas another reported no inhibition of these responses in cultured hippocampal cells (Straiker & Mackie, 2005). This high degree of variability in the pharmacological effects of Δ^9-THC may relate to its insolubility in aqueous buffers used during in vitro physiological experiments, and the relatively high EC_{50} in the present study (~1.2 μM) may reflect the comparatively poor degree of agonist penetration in the hippocampal brain slice, compared to more accessible receptor sites in cell culture systems.

Based on its relatively limited effects on glutamate release from axon terminals in hippocampal cultures (Shen & Thayer, 1999, and see Sim *et al.*, 1996), Δ^9-THC has been proposed to act as a partial agonist in the hippocampus. In addition, it has also been shown that that Δ^9-THC was without agonist activity at hippocampal glutamate terminals, but could antagonize the effects of full CB1R agonists at this site (Straiker & Mackie, 2005). This observation prompted the hypothesis that some of the psychotropic effects of Δ^9-THC may be mediated, not by the activation of CB1Rs, but rather by antagonism of endogenously released cannabinoids (Straiker & Mackie, 2005). Classical receptor theory predicts that low efficacy ligands like Δ^9-THC will behave as pure antagonists or partial agonists in systems where the stimulation of a large proportion of receptors is required to see a physiological response, or if there is a limited spare receptor reserve (Hoyer & Boddeke, 1993; Morissette *et al.*, 2007). In contrast, the same ligand can exhibit full efficacy when there is a large receptor reserve, and a smaller fraction of receptor occupancy is required to see a complete physiological response. Since CB1Rs are much more densely expressed on GABAergic versus glutamatergic axon terminals in the hippocampus (Kawamura *et al.*, 2006), one would predict that Δ^9-THC should exhibit higher efficacy in inhibiting GABA release than that observed for the inhibition of glutamate release. Commensurate with this hypothesis, Δ^9-THC's maximal inhibition of GABAergic IPSCs was similar to the full agonist WIN55,212-2 in the present study (and see Hoffman & Lupica, 2000), and the maximal effects of Δ^9-THC on EPSCs was smaller than WIN55,212-2 using the same brain slices under identical recording conditions, when the independent effects of RAMEB on EPSCs were considered (Hoffman *et al.*, 2010).

This differential pharmacological profile of Δ^9-THC at glutamate and GABAergic axon terminals has important implications for interpreting its effects on hippocampal circuitry, and for a more complete understanding of its ability to disrupt hippocampal function. Since Δ^9-THC would preferentially activate CB1Rs on inhibitory axon terminals to inhibit GABA release, and have smaller effects on glutamate release, it is likely that the deleterious effects of marijuana on learning and memory are primarily mediated through GABAergic mechanisms. This may occur as a result of the disruption of hippocampal network activity that is driven by synchronized GABA neuron activity, which is known to be disrupted by cannabinoids (Hajos *et al.*, 2000; Robbe *et al.*, 2006). Further support for hippocampal GABAergic networks in mediating the effects of Δ^9-THC is also found in a recent study showing that disruption of hippocampal-dependent behavior by Δ^9-THC was absent in transgenic animals containing a targeted deletion of the CB1R gene in GABAergic, but not glutamatergic neurons (Puighermanal *et al.*, 2009). This suggests that alterations in hippocampal GABAergic transmission are primarily responsible for the memory disrupting effects of Δ^9-THC, and the present results demonstrate that this is likely through full agonist actions of this drug at these abundant CB1Rs.

Several studies have demonstrated that Δ^9-THC can inhibit the clearance of neurotransmitters, including GABA, from the extracellular space in the CNS (Banerjee *et al.*, 1975; Coull *et al.*, 1997; Maneuf *et al.*, 1996). However, we found that despite clear effects of the GABA uptake inhibitor nipecotic acid on the rates of decay of synaptic and photolysis-evoked $GABA_A$ currents, Δ^9-THC had no effect on these measures at concentrations that strongly inhibited

IPSCs via CB1Rs. This suggests that whereas the synaptic release of GABA is inhibited by Δ^9-THC acting at CB1Rs, it is unlikely that the influence of GABA will be prolonged at this synapse by the inhibition of GABA uptake at these synapses.

Conclusion

Marijuana can profoundly impair human memory through interference with the acquisition of new information and its recall (Abel, 1971a; Abel, 1971b; Ranganathan & D'Souza, 2006; Wilson *et al.*, 1994), and working memory is profoundly impaired in animals exposed to marijuana smoke, or by hippocampal injection of Δ^9-THC (Lichtman *et al.*, 1995; Niyuhire *et al.*, 2007). More recently it has been shown that intrahippocampal injection of a CB1R antagonist can block the memory disruptive effects of systemic Δ^9-THC, strongly supporting the involvement of CB1Rs and the hippocampus in this effect of the drug (Wise *et al.*, 2009). The present study demonstrates that Δ^9-THC inhibits GABA release via presynaptic CB1Rs in the absence of postsynaptic effects and that, unlike its actions at glutamate axon terminals in the hippocampus, it appears to act as a full agonist at GABA terminals. Thus, the modulation of the inhibitory hippocampal circuitry should be considered as a primary site for actions of Δ^9-THC in this brain structure.

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Abbreviations

aCSF	artificial cerebrospinal fluid
APV	D-(-)-2-amino-5-phosphonopentanoic acid
$CB1^{+/+}$	wildtype C57BL6/J mice
$CB1^{-/-}$	C57BL6/J mice lacking the gene for the CB1 receptor
CB1R	cannabinoid type 1 receptor
CNB	of α-carboxy-2-nitrobenzyl
Δ^9-THC	delta-9-tetrahydrocannabinol
DIC	differential interference contrast microscopy
DNQX	6,7-dinitroquinoxaline-2,3-dione
Nd	YAG, neodymium-doped yttrium aluminum garnet
LTD	long-term depression
LTP	long-term potentiation
RAMEB	randomly methylated β-cyclodextrin
WIN55212-2	(*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

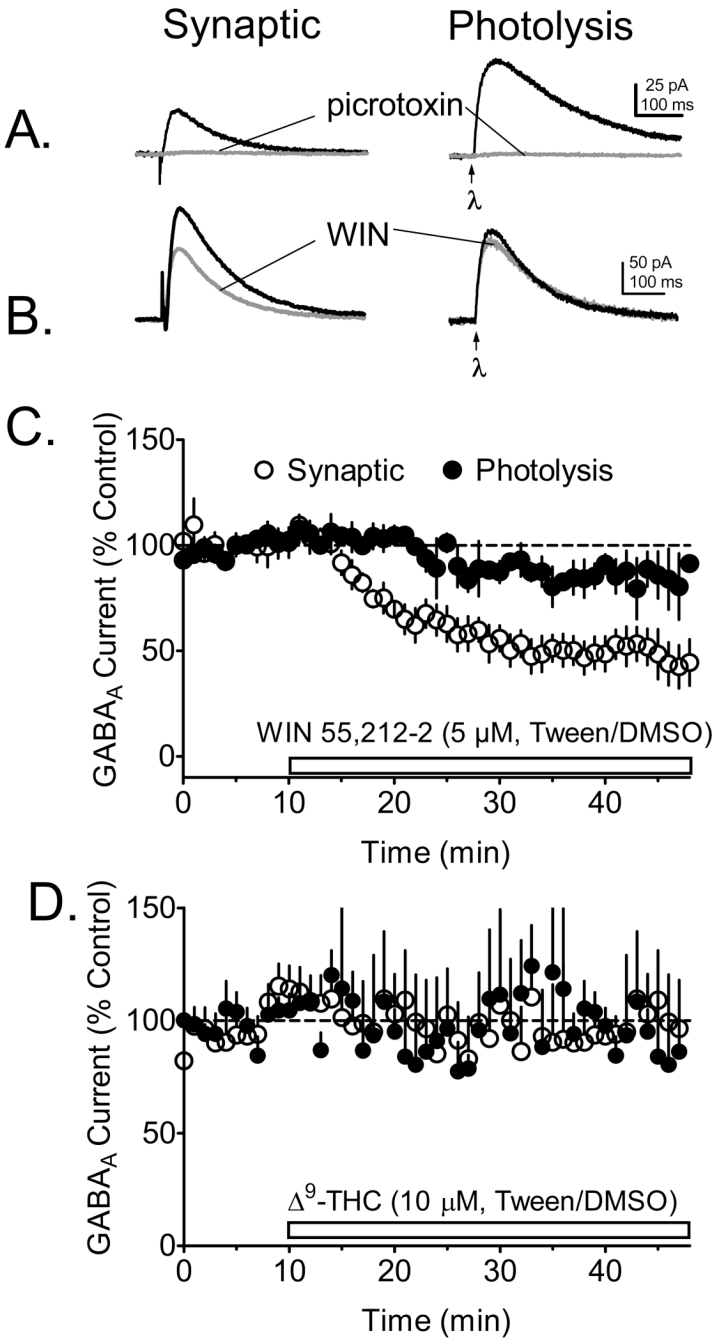

Figure 1.

Effects of cannabinoid agonists on $GABA_A$ receptor-mediated currents evoked synaptically, or through *uv* laser photolysis of CNB-caged GABA (50 μM) in WT mouse hippocampal CA1 pyramidal neurons. All recordings were performed during blockade of AMPA/kainate receptors with DNQX (10 μM) and NMDA receptors with APV (40 μM). A. Representative signal averages of outward $GABA_A$ receptor currents activated through either electrical stimulation (synaptic) or local photolysis of caged GABA by *uv* light (λ, applied at arrow) focused through the microscope objective. Note that both the synaptic and the photolysis GABA responses were completely blocked by the $GABA_A$ channel blocker picrotoxin (100 μM). B. Effects of the cannabinoid agonist WIN55,212-2 (dissolved in Tween-80/DMSO) on

the mean time course of $GABA_A$ currents elicited through electrical stimulation or CNB-GABA photolysis in the same CA1 pyramidal neurons (n = 6 neurons). C. Mean time course of the effect Δ^9-THC (dissolved in Tween-80/DMSO) on photolysis- and synaptically-evoked $GABA_A$ currents (n = 5 pyramidal neurons). Note the absence of an effect of Δ^9-THC on IPSCs or photolysis-evoked currents in these experiments. A gluconic acid-based intracellular solution was used in these experiments.

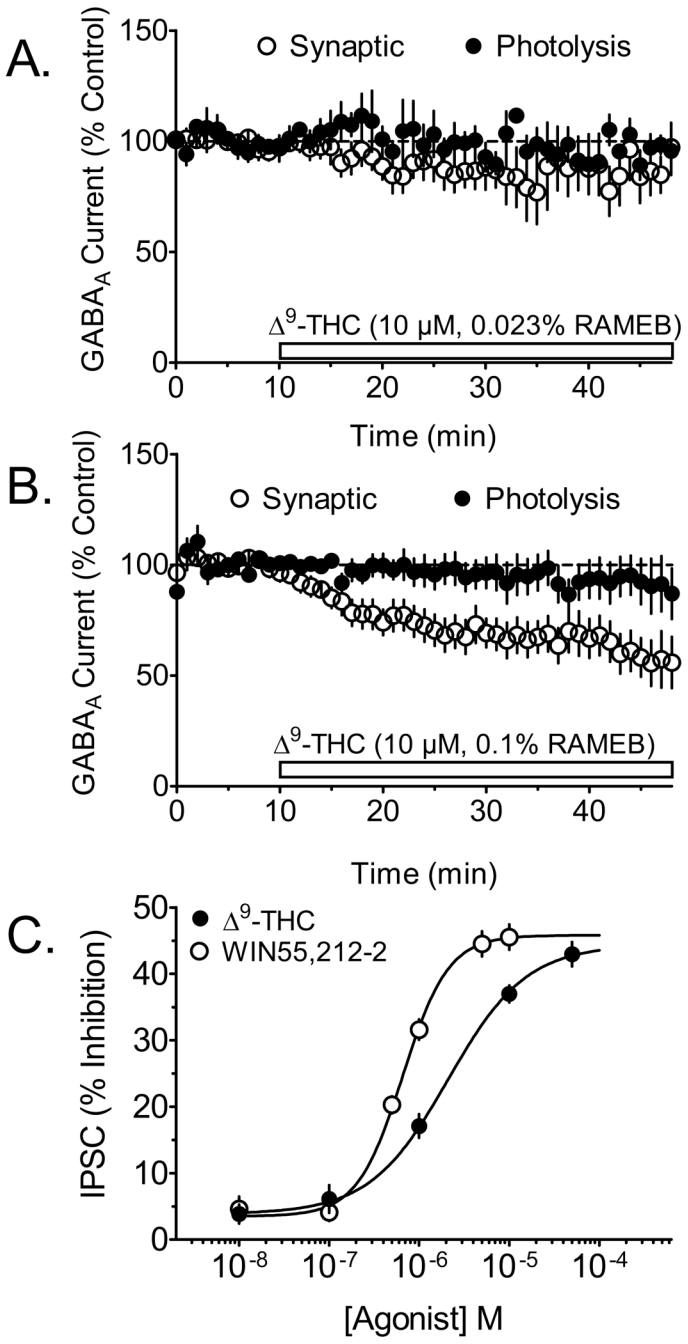

Figure 2.

Effects of different concentrations of RAMEB on Δ^9-THC inhibition of IPSCs, and concentration response comparison with WIN55,212-2. A. Mean time course of the effect of Δ^9-THC (10 μM) dissolved in 0.023% RAMEB solution on synaptic- and photolysis-evoked $GABA_A$-mediated currents recorded in the same CA1 pyramidal neurons (n = 9 neurons). Note the absence of a significant effect of Δ^9-THC on GABA currents in this concentration of the vehicle. B. Mean time course of the effect of Δ^9-THC (10 μM) on photolysis- and synaptically-evoked $GABA_A$ currents (n = 11 neurons), using a higher concentration of the RAMEB vehicle (0.1%). Note the significant inhibition of the synaptically-evoked IPSCs by Δ^9-THC using the 0.1% RAMEB solution, and the absence of effects on the photolysis-evoked currents. C.

Concentration-dependent effect of Δ^9-THC, suspended in 0.1% RAMEB, and WIN55,212-2 in Tween-80-DMSO, on synaptically evoked IPSCs. The EC_{50} for Δ^9-THC was 1.22 μM (95% C.I. = 0.86 μM to 1.73 μM). Note that the maximal effects of Δ^9-THC and WIN55,212-2 on IPSCs are comparable ($p > 0.05$, t-test). The number of neurons used for each point on the concentration-response curve was 5-7. The data shown in all subsequent figures used Δ^9-THC in the 0.1 % concentration of RAMEB.

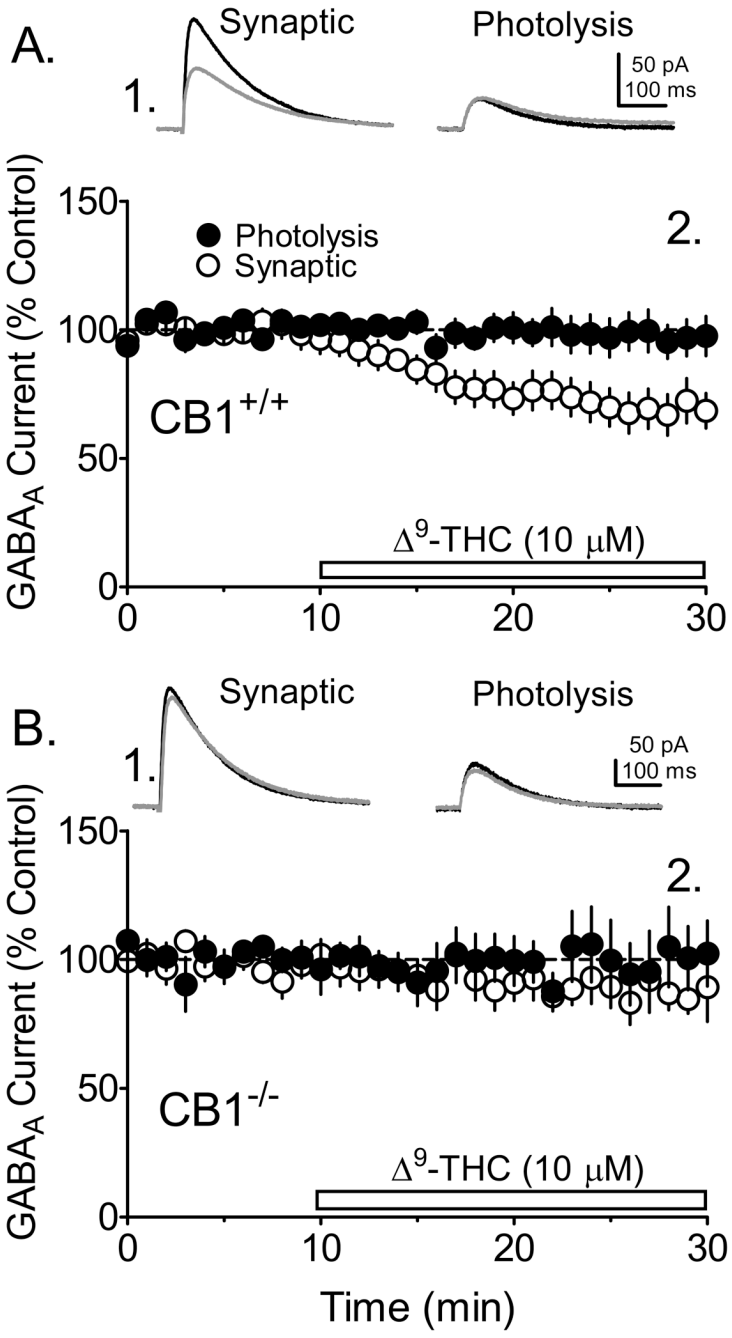

Figure 3.

Effects of Δ^9-THC on photolysis-evoked and synaptic $GABA_A$ currents in WT ($CB1^{+/+}$) and KO($CB1^{-/-}$) mice. The $GABA_A$ receptor-mediated currents were evoked by alternating electrical stimulation of the hippocampal slice (synaptic) with *uv* laser photolysis of caged GABA in the same pyramidal neurons. A1. Signal averages of synaptic and photolysis-evoked GABA currents obtained prior to (black lines), and during Δ^9-THC (10 μM) application (gray lines) in a CA1 pyramidal neuron from a $CB1^{+/+}$ mouse. A2. The mean time course of the effect of Δ^9-THC on photolysis-evoked and synaptic $GABA_A$ currents (n = 11 CA1 pyramidal neurons). Note the significant effect of Δ^9-THC on synaptic IPSCs and the absence of an effect on photolysis-evoked currents. B1. Representative signal averages demonstrating the lack of

Δ^9-THC (gray lines) effect on synaptic and photolysis-evoked responses in CA1 pyramidal neuron obtained from $CB1^{-/-}$ mice (n = 6). B2. Mean time course demonstrating the lack of Δ^9-THC effect on the $GABA_A$ currents. A gluconic acid-based intracellular solution was used in these experiments.

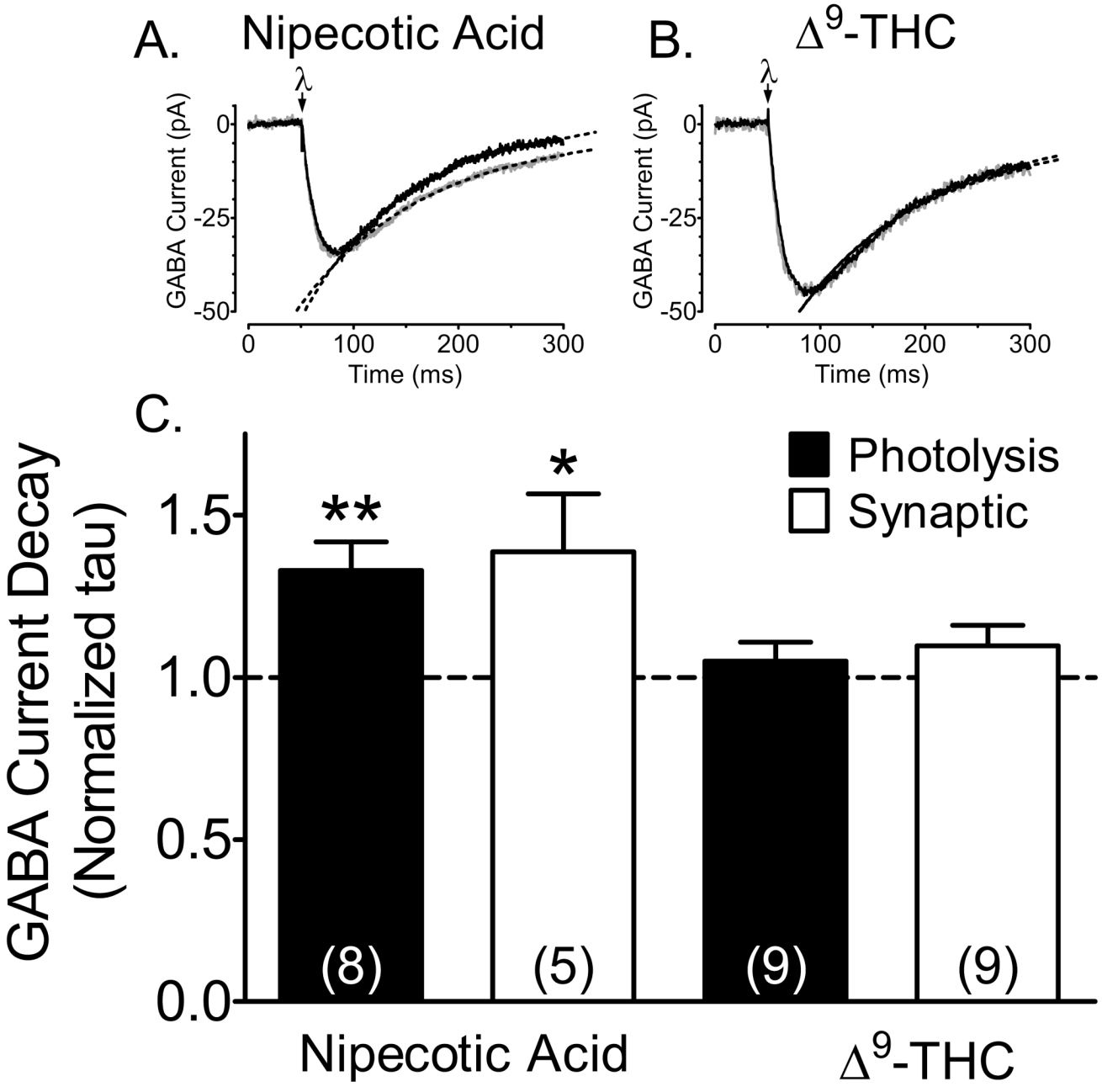

Figure 4.

Δ^9-THC acts presynaptically to reduce GABAergic neurotransmission in the hippocampus. A. Effect of the competitive GABA uptake inhibitor nipecotic acid (10 μM, gray line) on averaged (n = 6-9 individual sweeps) inward currents evoked by CNB-GABA photolysis (uv laser flash applied at λ) in a CA1 pyramidal neuron. The control sweep is shown in black. Also shown (dashed lines) are single exponential curves fitted to the decay phase of the currents that were used to calculate the decay time constants (tau). Note that nipecotic acid significantly increased the tau value in this cell (control tau =100.8 ms, 95% C.I. = 99.0-102.7 ms; nipecotic acid tau =120.9 ms, 95% C.I. = 118.3-123.5 ms). Currents shown here and in B are scaled to the same amplitude. B. Lack of an effect of Δ^9-THC (10 μM, gray line) on photolysis-evoked GABA current decay in a CA1 pyramidal neuron (control tau = 116.8 ms, 95% C.I. = 115.0-118.7 ms;

Δ^9-THC tau = 116.9 ms, 95% C.I. = 114.4-119.5 ms). C. Summary of the effect of nipecotic acid and Δ^9-THC on decay tau values for GABA currents evoked by synaptic stimulation and photolysis of CNB caged GABA. Values represent ratios of the tau value acquired after drug application to that obtained during the control period. Note that Δ^9-THC did not significantly affect synaptic or photolysis-evoked GABA current decay, whereas nipecotic acid prolonged the tau values for each of these currents. *, $p < 0.01$; **, $p < 0.001$, paired t-test. These data indicate that GABA uptake was unaffected by Δ^9-THC under the present recording conditions.