

Cannabidiol Inhibits Endocannabinoid Signaling in Autaptic Hippocampal Neurons

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Received January 23, 2018; accepted April 16, 2018

ABSTRACT

Δ^9 -Tetrahydrocannabinol (THC) and cannabidiol (CBD) are two main cannabinoid constituents of marijuana and hashish. The pharmacology of Δ^9 -THC has been extensively studied, whereas our understanding of the pharmacology of CBD has remained limited, despite excitement in CBD's potential role in treating certain pediatric epilepsies and its reputation for attenuating some Δ^9 -THC-induced effects. It was established early on that CBD binds poorly to the orthosteric site of CB₁ or CB₂ cannabinoid receptors, and its actions were commonly attributed to other noncannabinoid receptor mechanisms. However, recent evidence suggests that CBD does indeed act at cannabinoid CB₁ receptors as a negative allosteric modulator (NAM) of CB₁ signaling. By altering the orthosteric signaling of a G protein-coupled receptor,

allosteric modulators greatly increase the richness of G protein-coupled receptor pharmacology. We have recently surveyed candidate CB₁ NAMs in autaptic hippocampal neurons, a well-characterized neuronal model of endogenous cannabinoid signaling, and have now tested CBD in this model. We find that although CBD has no direct effect on excitatory transmission, it does inhibit two forms of endogenous cannabinoid-mediated retrograde synaptic plasticity: depolarization-induced suppression of excitation and metabotropic suppression of excitation, while not affecting signaling via GABA-B receptors. These results are consistent with the recently described NAM activity of CBD and suggest interesting possible mechanisms for CBD's therapeutic actions.

Introduction

Although there are many cannabinoids present in marijuana and hashish, two components found in greatest abundance are Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD) (Elsohly and Slade, 2005). Δ^9 -THC was identified as the chief psychoactive ingredient of marijuana (Gaoni and Mechoulam, 1964) and has been the subject of thousands of studies in the intervening years. Although isolated earlier (Adams et al., 1940), CBD by contrast has remained a more elusive and poorly studied target, partly because CBD does not compete with conventional orthosteric radioligands for binding at the CB₁ cannabinoid receptor (Thomas et al., 1998), but also because CBD itself does not elicit distinctive psychoactive effects (Mechoulam et al., 1970). This picture has gradually changed; varying the ratio of Δ^9 -THC and CBD in cannabis and cannabinoid preparations has been reported to yield differential effects (Karniol and Carlini, 1973; Hiltunen and Jarbe, 1986; Petit et al., 1998; Russo and Guy, 2006), and much effort has gone into development of marijuana strains with different proportions of these two components. More recently, CBD has generated considerable interest as an antiepileptic (reviewed in O'Connell et al., 2017) and for other

potential therapeutic properties (reviewed in Maccarrone et al., 2017).

A recent study by Laprairie et al. (2015) revisited the hypothesis that CBD may interfere with CB₁ activation (Petitet et al., 1998) but from the perspective of allosteric modulation. The premise of allosteric modulation is that many receptors have not only a primary orthosteric site of activation, but also one or more allosteric sites. Ligands that bind these sites are termed allosteric ligands and can exert a variety of actions ranging from potentiation/inhibition of the orthosteric signal to agonism/antagonism independent of orthosteric activation. Some major classes of drugs such as the benzodiazepines are well known to act as allosteric modulators, and two allosteric modulators of G protein-coupled receptors, cinacalcet and maraviroc, have been approved for clinical use in the United States. Allosterism at CB₁ has seen a flurry of recent interest. A negative allosteric modulator, or negative allosteric modulator (NAM), would bind to CB₁ at a secondary site and presumably act either by interfering with the kinetics of orthosteric ligand binding and/or receptor activation/signaling. Such a NAM may lack the side effects seen with orthosteric antagonism (or orthosteric inverse agonism). We have recently surveyed the function of the first-generation NAMs at CB₁ using autaptic hippocampal neurons (Straiker et al., 2015). These neurons express CB₁ cannabinoid receptors and a form of retrograde signaling known as depolarization-induced suppression of

This work was supported by National Institutes of Health [Grants EY24625, EY24717, DA009158, and DA021696].
<https://doi.org/10.1124/mol.118.111864>.

ABBREVIATIONS: 2-AG, 2-arachidonoyl glycerol; CBD, cannabidiol; CI, confidence interval; DHPG, dihydrophenylglycine; DSE, depolarization-induced suppression of excitation; EPSC, excitatory postsynaptic current; MSE, metabotropic suppression of excitation; NAM, negative allosteric modulator; THC, tetrahydrocannabinol.

excitation (DSE) (Straiker and Mackie, 2005). We have extensively characterized this form of synaptic plasticity, which we find to be 2-arachidonoyl glycerol (2-AG) mediated, and have employed DSE as a tool to explore the pharmacology of endogenous neuronal cannabinoid signaling (e.g., Straiker et al., 2009, 2011a; Jain et al., 2013). We have now tested CBD in this model and report in this work that CBD interferes with endogenous cannabinoid/CB₁-mediated signaling in a concentration-dependent manner and in a fashion consistent with negative allosteric modulation.

Materials and Methods

Animals and Cell Culture. All animal care and experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Indiana University and conform to the National Institutes of Health *Guidelines on the Care and Use of Animals*. Mouse hippocampal neurons isolated from the CA1–CA3 region were cultured on microislands, as previously described (Furshpan et al., 1976; Bekkers and Stevens, 1991). Briefly, neurons were obtained from animals (at postnatal day 0–2, killed via rapid decapitation without anesthesia) and plated onto a feeder layer of hippocampal astrocytes that had been laid down previously (Levison and McCarthy, 1991). Cultures were grown in high-glucose (20 mM) minimum essential media containing 10% horse serum, without mitotic inhibitors, and used for recordings after 8 days in culture and for no more than 3 hours after removal from culture medium (Straiker and Mackie, 2005). All electrophysiological experiments were performed exclusively on excitatory neurons. All tests were made on neurons from at least two different preparations.

Electrophysiology. When a single neuron is grown on a small island of permissive substrate, it forms synapses—or autapses—onto itself. All experiments were performed on isolated autaptic neurons. Whole-cell, voltage-clamp recordings from autaptic neurons were carried out at room temperature using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The extracellular solution contained (millimolars) NaCl 119, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 30, and HEPES 20. Continuous flow of solution through the bath chamber (~2 ml × min⁻¹) ensured rapid drug application and clearance. Drugs were typically prepared as a stock and then diluted into extracellular solution at their final concentration and used on the same day. Recording pipettes of 1.8–3 MΩ were filled with solution containing the following (millimolars): potassium gluconate 121.5, KCl 17.5, NaCl 9, MgCl₂ 1, HEPES 10, EGTA 0.2, MgATP 2, and lithium guanosine triphosphate 0.5. Excitatory postsynaptic currents (EPSCs) were elicited by a brief (1 millisecond) depolarization to 0 mV. DSE was elicited by longer depolarizations (50 milliseconds–10 seconds) also to 0 mV. Access resistance was monitored, and only cells with a stable access resistance were included for data analysis.

Materials. Baclofen was purchased from Sigma-Aldrich (St. Louis, MO). CBD was obtained from National Institute on Drug Abuse Drug Supply Program (Research Triangle Park, NC). CBD was stored at –80°C and diluted shortly before use.

Statistical Analysis. For electrophysiology analyses, depolarization response curves were obtained to determine inhibition of excitatory synaptic transmission by endogenous 2-AG by depolarizing neurons for progressively longer durations (50, 100, 300, and 500 milliseconds; 1, 3, and 10 seconds). The data were fitted with a nonlinear regression (Sigmoidal dose response; GraphPad Prism 6, La Jolla, CA), allowing calculation of an ED₅₀, the effective dose or duration of depolarization at which a 50% inhibition is achieved. Statistically significant differences in these curves were taken as nonoverlapping 95% confidence intervals (CIs). Values on graphs are presented ± S.E.M.

Results

CBD Suppresses CB₁ Receptor-Mediated DSE. We tested the ability of CBD to suppress synaptic transmission in autaptic hippocampal neurons, first recording from cells to obtain baseline EPSCs, and then treating cells for 5 minutes with CBD (500 nM). As shown in Fig. 1A, CBD did not inhibit EPSCs, indicating that at 500 nM CBD does not directly inhibit (or activate) neurotransmission in these neurons [Fig. 1A; relative EPSC charge (500 nM CBD): 1.03 ± 0.03, *n* = 5].

Depolarization of excitatory autaptic hippocampal neurons elicits a form of endocannabinoid (2-AG) and CB₁-mediated retrograde inhibition termed DSE (Straiker and Mackie, 2005). This can be quantified by subjecting the neuron to a series of successively longer depolarizations (50, 100, 300, and 500 milliseconds; 1, 3, and 10 seconds), resulting in progressively greater inhibition of neurotransmission (Straiker et al., 2011b). A sample time course from such a depolarization series is shown in Fig. 1D. Plotting maximal inhibition as a result of each duration of depolarization yields a depolarization-response curve that permits the characterization of some pharmacological properties of endocannabinoid signaling, including the calculation of an ED₅₀ (depolarization). This ED₅₀ corresponds to the duration of depolarization that results in 50% of the maximal inhibition. A NAM would be expected to shift a depolarization-response curve up and to the right (i.e., less DSE in a noncompetitive fashion). We tested CBD at 100 and 500 nM and 2 μM, finding that 500 nM and 2 μM CBD substantially shifted the ED₅₀ [Fig. 1, B and C; baseline ED₅₀ (duration of depolarization [95% CI]): 1.0 second (0.6–1.5); ED₅₀ (100 nM CBD): 1.6 seconds (1.1–2.2); *n* = 7; ED₅₀ (500 nM CBD): 4.9 seconds (2.4–10.2); *n* = 5; ED₅₀ (2 μM CBD): 2.8 seconds (1.6–4.8); *n* = 5; 95% CI nonoverlapping for 500 nM and 2 μM CBD versus baseline]. Maximal inhibition was significantly decreased after treatment with either 500 nM or 2 μM CBD [baseline E_{max} (relative EPSC charge [95% CI]): 0.37 (0.28–0.47); E_{max} in presence of 100 nM CBD: 0.52 (0.47–0.58); 500 nM CBD: 0.67 (0.56–0.78); 2 μM CBD: 0.77 (0.71–0.82); 95% CI nonoverlapping for 500 nM and 2 μM versus baseline]. Averaged time courses of DSE in response to a 3-second depolarization before and after treatment with 500 nM CBD are shown in Fig. 1E. We additionally tested whether CBD would interfere with exogenously applied 2-AG to confirm that the effect of CBD was not due to a postsynaptic alteration of 2-AG synthesis and/or release. We used a half-maximal concentration of 2-AG, 500 nM, and found that 500 nM CBD diminished EPSC inhibition by 2-AG on reapplication in the same cells [Fig. 1, F and G; relative EPSC charge after 500 nM 2-AG: 0.63 ± 0.03; relative charge with 2-AG and CBD (both 500 nM): 0.81 ± 0.04, *n* = 7; *P* < 0.05 paired *t* test].

If the effect of CBD was due to a general inhibition of presynaptic G protein-coupled receptor signaling, then one would expect CBD to also suppress the activity of other G_{1/6}-coupled receptors. GABA-B receptors also presynaptically inhibit neurotransmitter release in autaptic hippocampal neurons (Straiker et al., 2002). We found that inhibition of neurotransmitter release by GABA-B activation with baclofen (25 μM) remained intact in the presence of 500 nM CBD [Fig. 1H; relative EPSC charge after 25 μM baclofen (±S.E.M.): 0.30 ± 0.07; baclofen + CBD (500 nM): 0.26 ± 0.04; *n* = 5; *P* > 0.05, paired *t* test].

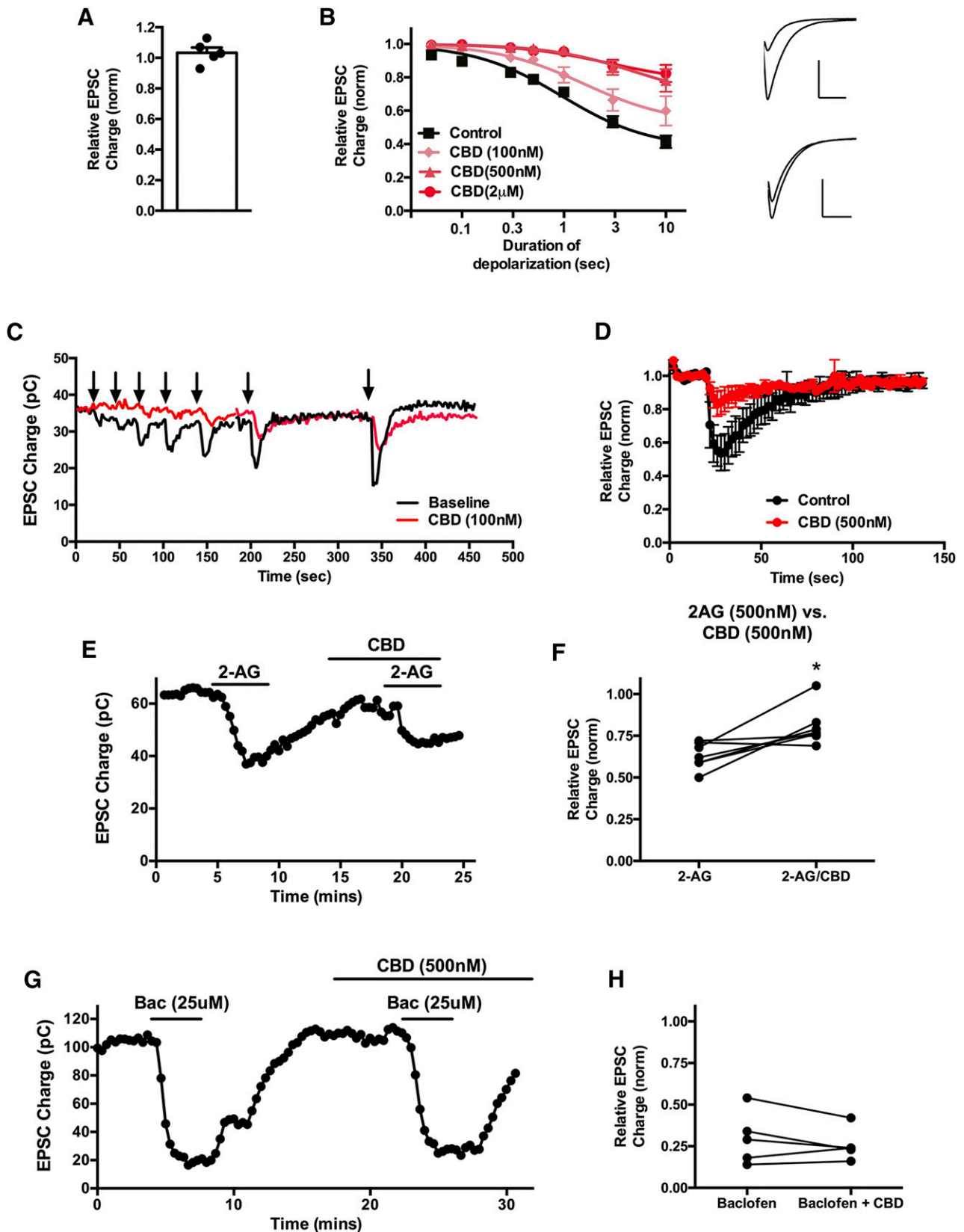


Fig. 1. CBD suppresses CB₁ receptor–mediated DSE. (A) CBD (500 nM) does not directly inhibit excitatory postsynaptic currents. (B) The 100 or 500 nM CBD treatment (5 minutes) shifts the response curve for DSE in a concentration-dependent manner. Inset shows sample EPSCs before and after DSE under baseline conditions (top) and after CBD (500 nM) treatment (bottom). (C) Sample time course showing DSE responses to progressively longer depolarizations before and after 100 nM CBD treatment. (D) Averaged DSE time course in response to 3-second depolarization before and after 500 nM CBD treatment. (E) Representative experiment showing that half-maximal 2-AG responses (500 nM) are diminished by 500 nM CBD. (F) Inhibition by 2-AG (500 nM) alone gives a stronger response

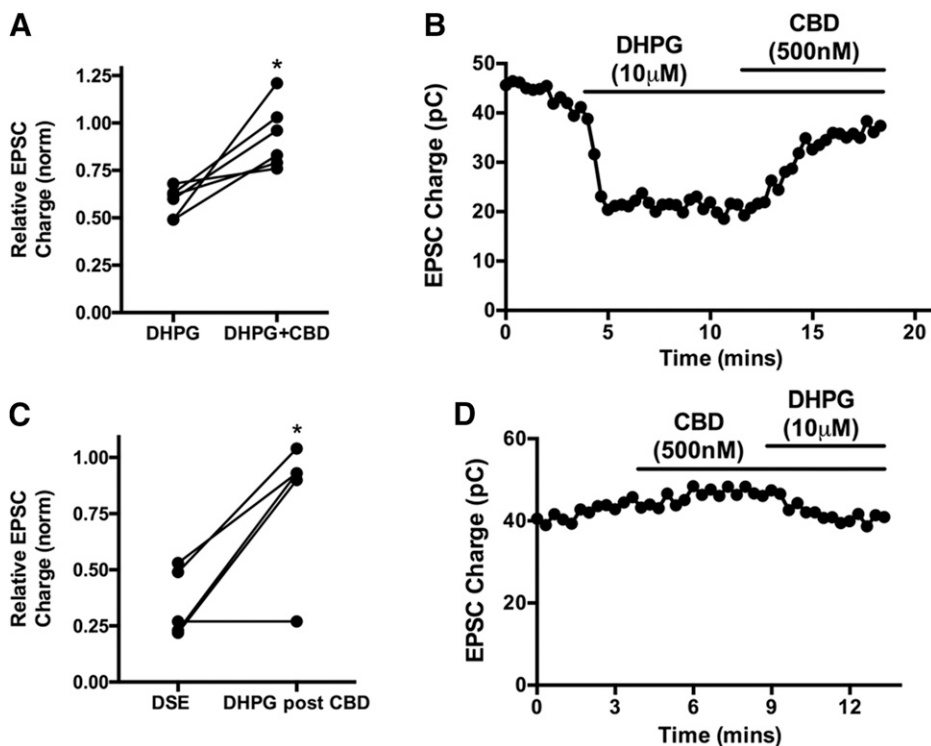


Fig. 2. CBD suppresses endogenous CB₁ receptor-mediated metabotropic suppression of excitation. (A) mGluR group I agonist DHPG (10 μ M) suppresses EPSCs, but this is reversed by treatment with 500 nM CBD. (B) Sample time course showing DHPG response reversed by subsequent cotreatment with CBD. (C) Bar graph shows maximal inhibition due to DSE before CBD and the effect of DHPG (10 μ M) when applied after/with CBD (500 nM). (D) Sample time course showing CBD treatment, followed by copapplication of CBD and DHPG. * $P < 0.05$ by paired t test.

CBD Also Suppresses CB₁ Receptor-Mediated Metabotropic Suppression of Excitation. We additionally tested whether CBD has a similar effect on a related form of cannabinoid-mediated retrograde synaptic plasticity known as metabotropic suppression of excitation (MSE). First described in the cerebellum (Maejima et al., 2001), we have characterized MSE in autaptic hippocampal neurons and shown it to be CB₁ receptor dependent (Straiker and Mackie, 2007). We have additionally shown that MSE (like DSE) involves 2-AG rather than anandamide because 2-AG, but not anandamide, fits the kinetic profile of MSE and because inhibition or knockdown of diacylglycerol lipases eliminates MSE (Jain et al., 2013).

Briefly, postsynaptic G_q-coupled receptors have been found to activate a signaling cascade, involving G_q, phospholipase C, and diacylglycerol lipase, which results in the production of 2-AG that, like the 2-AG, produced as a result of DSE, retrogradely crosses the synapse, where it activates presynaptic CB₁ receptors and thereby inhibits neurotransmission (reviewed in Kano et al., 2009). In addition to metabotropic mGluR group I receptors (Maejima et al., 2001), several other G_q-coupled receptors have been found capable of inducing MSE, including muscarinic (Kim et al., 2002), orexin (Haj-Dahmane and Shen, 2005), and substance P (Drew et al., 2009) receptors. We first treated neurons with the mGluR group I agonist dihydroxyphenylglycine (DHPG) (10 μ M) to transient MSE, then copplied DHPG with CBD (500 nM), finding that this substantially reversed DHPG-induced MSE (Fig. 2, A and B; inhibition with 10 μ M DHPG: 0.59 ± 0.07 ; DHPG + CBD (500 nM): 0.93 ± 0.16 , $n = 6$, $P < 0.05$, paired t test).

Similarly, application of CBD before DHPG prevented the full effect of DHPG-induced MSE [Fig. 2, C and D; inhibition with 10 μ M DHPG in presence of CBD (500 nM): 0.81 ± 0.14 , $n = 5$]. For these experiments, responses are compared with DSE in the same cells elicited before treatment with either drug (Fig. 2C; DSE inhibition: 0.36 ± 0.07 , $n = 5$, $P < 0.05$ paired t test versus DHPG + CBD). DSE and MSE induce the same degree of CB₁-dependent inhibition in a given cell (Straiker and Mackie, 2007).

Discussion

We have tested the important nonpsychoactive component of marijuana, CBD, in a well-characterized model of endogenous cannabinoid signaling. We find that whereas CBD does not directly modulate neurotransmission in this model, it does suppress two forms of endogenous cannabinoid-mediated synaptic plasticity, while not affecting inhibition of synaptic transmission by GABA-B receptors. This finding extends a recent report that CBD acts as a NAM at CB₁ receptors (Laprairie et al., 2015) to models of endocannabinoid-mediated synaptic plasticity.

In considering the role and therapeutic potential of cannabinoids, for many years Δ^9 -THC has received the greatest attention. As the chief psychoactive ingredient of cannabis and as an established (albeit low efficacy) agonist at both of the canonical cannabinoid receptors, THC has been a natural object of study. The pharmacology of CBD, in contrast, has been difficult to sort out. CBD competes poorly with

cannabinoid receptor ligand binding to cannabinoid receptors (Thomas et al., 1998), leading to an initial (and persistent) conclusion that any action of CBD necessarily occurs through noncannabinoid receptors. It may seem surprising that CBD and THC are structurally similar (and with the same molecular weight) yet exhibit different orthosteric binding to CB₁ receptors, but this may be accounted for by steric differences between these molecules as explored in structure–activity studies (e.g., Papahatjis et al., 2002). However, it subsequently became clear that CBD does interfere with cannabinoid function at a potency greater than that expected based on the earlier orthosteric binding data (Thomas et al., 2007). This raised the possibility that CBD acts noncompetitively relative to the orthosteric site at cannabinoid receptors, although this still left open numerous potential mechanisms of action, including action via unrelated receptors or signaling pathways. The recent finding that CBD has a profile consistent with potent negative allosteric modulation of CB₁ signaling stimulated by 2-AG and THC in a broad range of signaling assays (Laprairie et al., 2015) therefore offered a significant and welcome insight. However, awareness of this study and its implications for CBD function are still limited, and CBD is still commonly assumed by many to be inactive at CB₁ receptors (e.g., Devinsky et al., 2017).

Cannabinoid CB₁ receptors are abundant and widely distributed in the central nervous system (Herkenham et al., 1990). In CB₁-expressing neurons, they are highly enriched on presynaptic terminals and have been shown to mediate several forms of neuronal plasticity (reviewed in Kano et al., 2009), among them DSE (Kreitzer and Regehr, 2001), the related depolarization-induced suppression of inhibition (Wilson and Nicoll, 2001), and metabotropic suppression of excitation (MSE) (Maejima et al., 2001). It is therefore important to determine in a well-characterized and tractable model the extent to which CBD alters CB₁-mediated plasticity. Excitatory autaptic hippocampal neurons express DSE and MSE in an architecturally simple system consisting of a single neuron synapsing onto itself, an arrangement free of network-signaling complications that arise in more elaborate preparations. Moreover, the model can be said to be well characterized insofar as it has been used for studies of cannabinoid signaling in ~25 publications to date. Our observations clearly establish that CBD inhibits two forms of endocannabinoid-mediated synaptic plasticity. However, whereas our findings buttress and extend those of Laprairie et al. (2015), the experiments were designed to qualitatively demonstrate antagonism, not allosterism per se. The autaptic neuronal model is unsuited to Schild analyses, which require steady state receptor activation, due to the transient nature of 2-AG engagement of the CB₁ receptor following its release. Thus, the strongest conclusion of our studies is that CBD noncompetitively inhibits CB₁ receptor activation by 2-AG in a model of endogenous cannabinoid signaling, consistent with negative allosteric modulation.

In principle, CBD might be acting at other components of the machinery that regulates eCB synthesis, transport, or metabolism. Our experiments used bath-applied 2-AG argue against an effect on the 2-AG synthesizing or release machinery. On the presynaptic side, we have shown that the time course of DSE is determined by monoacylglycerol lipase metabolism (Straiker et al., 2009) and that fatty acid amide hydrolase only plays a role when overexpressed (Straiker

et al., 2011a). Diminishment of DSE responses via a pre-synaptic mechanism would require enhanced 2-AG metabolism or transport, which would be seen not as diminished maximal inhibition, but rather as a more rapid DSE decay. Indeed, we have previously shown that the more rapid depolarization-induced suppression of inhibition time course in autaptic neurons is a function of more rapid metabolism by a combination of monoacylglycerol lipase and cyclooxygenase 2 (Straiker and Mackie, 2009). CBD is therefore unlikely to be acting on the synthetic, transport, or metabolizing machinery for 2-AG in this system.

Having said this, does the observed antagonism occur at relevant concentrations? Do the concentrations of CBD in the body after marijuana use or likely therapeutic treatments approach a level at which they could be expected to antagonize CB₁ receptor signaling, as we have seen in this work? In a related question, is negative allosteric modulation of CB₁ by CBD a bug or a feature? Is CB₁ antagonism the basis for physiologic consequences that have been noted for CBD treatment? Several ongoing clinical trials are evaluating CBD for epilepsy treatment, particularly for forms of childhood epilepsy, including Dravet's Syndrome and Lennox-Gastaut Syndrome (O'Connell et al., 2017). A recent clinical trial testing CBD at 20 mg/kg per day for several weeks found that CBD reduced tonic/clonic seizures in a significant fraction of children with Dravet's Syndrome (Devinsky et al., 2017, 2018). The same group determined that 20 mg/kg daily treatment yields peak plasma levels of ~1 μ M after 22 days of treatment, i.e., above the concentrations tested in this study (100, 500 nM) that substantially inhibit endocannabinoid signaling (Devinsky et al., 2018). Plasma levels of CBD obtained during recreational consumption are more difficult to quantify because of the considerable variety of strains, formulations, and modes of consumption. Over the past 20 years, most marijuana consumption involved low-CBD strains, but several high-CBD strains have been developed. A hypothetical cigarette containing 0.75 g marijuana, with 5% CBD, would contain 30 mg CBD. A study involving the smoking of a cigarette containing 19 mg CBD was found to result in blood plasma levels of 110 ng/ml (~340 nM) (Ohlsson et al., 1986) at 3 minutes post-treatment, but declined to 10 ng/ml at 1 hour. A smoker might therefore experience a brief NAM effect from smoking, which likely corresponds to the time of maximal THC brain levels. Interestingly, whereas simultaneous inhalation of THC and CBD attenuates some aspects of THC psychoactivity, sequential (separated by 30 minutes) inhalation of CBD and then THC did not (Dalton et al., 1976). CBD varieties with still higher concentrations of CBD, in excess of 15%, have been developed [e.g., Charlotte's Web (Maa and Figi, 2014)]. The same hypothetical cigarette containing this high CBD cannabis would presumably transiently increase CBD in excess of 1 μ M plasma. However, the effect would likely last only a few minutes. Studies of oral consumption of CBD in doses likely encountered in recreational cannabis use found relatively low CBD plasma concentrations. For instance, Agurell et al. (1981) reported that 40 mg CBD ingested in cookies yielded only an average of 5.5 ng/ml (~20 nM) of CBD in plasma.

Cannabis containing substantial CBD has been reported to yield fewer adverse psychologic side effects than THC alone, raising the question of whether a component of cannabis such as CBD might actively moderate the effects of THC (reviewed

in McPartland et al., 2015). The inhibition of the CB₁ signaling we have observed in this study offers one potential mechanism for such an attenuation of selected THC effects. A related question is whether negative allosteric modulation of CB₁ by CBD might contribute to its proposed antiepileptic properties, or alternatively contribute to the reported side effects? The identification of CBD as a NAM is recent enough that this remains a largely unexplored question.

In summary, we find that CBD antagonizes two forms of endogenous cannabinoid CB₁ receptor-mediated synaptic plasticity in autaptic hippocampal neurons. The inhibition profile of CBD is consistent with the recently reported negative allosteric modulation at CB₁ receptors and occurs at or below concentrations that are likely to be encountered in the body in the course of therapeutic CBD consumption.

Authorship Contributions

Participated in research design: Straiker, Mackie.

Conducted experiments: Straiker, Dvorakova, Zimmowitch.

Performed data analysis: Straiker, Dvorakova.

Wrote or contributed to the writing of the manuscript: Straiker, Mackie.

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