Control of Inhibition by the Direct Action of Cannabinoids on GABA
Receptors

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Cannabinoids are known to regulate inhibitory synaptic transmission via activation of presynaptic G protein-coupled cannabinoid CB1 receptors (CB1Rs). Additionally, recent studies suggest that cannabinoids can also directly interact with recombinant GABA receptors (GABAARs), potentiating currents activated by micromolar concentrations of γ-aminobutyric acid (GABA). However, the impact of this direct interaction on GABAergic inhibition in the central nervous system is unknown. Here we report that currents mediated by recombinant GABAARs activated by high (synaptic) concentrations of GABA as well as GABAergic inhibitory postsynaptic currents (IPSCs) at neocortical fast spiking (FS) interneurons to pyramidal neuron synapses are suppressed by exogenous and endogenous cannabinoids in a CB1R-independent manner. This IPSC suppression may account for disruption of inhibitory control of pyramidal neurons by FS interneurons. At FS interneuron to pyramidal neuron synapses, endocannabinoids induce synaptic low-pass filtering of GABAAR-mediated currents evoked by high-frequency stimulation. The CB1R-independent suppression of inhibition is synapse specific. It does not occur in CB1R containing hippocampal cholecystokinin-positive interneuron to pyramidal neuron synapses. Furthermore, in contrast to synaptic receptors, the activity of extrasynaptic GABAARs in neocortical pyramidal neurons is enhanced by cannabinoids in a CB1R-independent manner. Thus, cannabinoids directly interact differentially with synaptic and extrasynaptic GABAARs, providing a potent novel context-dependent mechanism for regulation of inhibition.

Keywords: CB1 receptors, DSI, endocannabinoids, fast-spiking interneurons, neocortex, inhibitory synaptic transmission

Introduction

Most of the effects of cannabinoids on mammalian behavior are attributed to activation of G protein-coupled cannabinoid CB1 receptors (CB1Rs), which are abundantly expressed throughout the brain. It has been postulated that in a number of central nervous system (CNS) synapses activity-dependent synthesis and release of endocannabinoids from the postsynaptic neuron and consequent retrograde activation of presynaptic CB1Rs induces a transient suppression of synaptic efficacy (Kreitzer and Regehr 2001; Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001). However, cannabinoids can still affect behavior both in the presence of CB1R antagonists and in CB1R knockout (CB1R−/−) mice (Di Marzo et al. 2000; Baskfield et al. 2004; Sigel et al. 2011), suggesting the existence of additional brain targets for cannabinoids (Oz 2006).

Several studies indicate that cannabinoids can directly modulate the properties of various ligand-gated ion channels of the Cys-loop receptor superfamily, including nicotinic acetylcholine receptors (nAChRs), serotonin 5HT3 receptors and glycine receptors (GlyRs) (Oz et al. 2004; Lozovaya et al. 2005; Hejazi et al. 2006). The GABA A receptor (GABAAR), the main receptor mediating inhibitory synaptic transmission in the mature mammalian CNS, also belongs to the Cys-loop receptor superfamily. A recent study reported that the endocannabinoid 2-arachidonoylglycerol (2-AG) directly potentiates the function of recombinant GABAARs activated by low micromolar concentrations of γ-aminobutyric acid (GABA) (Sigel et al. 2011). This potentiation is mediated by an interaction of cannabinoids with the β2 subunit of the GABAAR (Baur et al. 2013). However, there is no evidence that such effects occur in neurons and at synapses. Demonstrating the functional implication of modulation is an important prerequisite for postulating a meaningful physiological or pathophysiological role for the interaction of cannabinoids with the GABAAR. In this study we demonstrate that although cannabinoids enhance recombinant GABAAR function at low micromolar concentrations of GABA (Sigel et al. 2011) (i.e., ambient levels sufficient to activate certain extrasynaptic GABAARs), they inhibit GABAAR function at millimolar GABA concentrations (i.e., those achieved in the synaptic cleft during synaptic transmission). Crucially, in neocortical pyramidal neurons, these effects of cannabinoids enhance the function of extrasynaptic GABAARs whilst inhibiting synaptic GABAARs in a synapse-specific manner. Importantly, we provide evidence that endogenously released cannabinoids can mimic these effects and consequently greatly impact upon interneuron–pyramidal cell communication in neuronal networks.

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Materials and Methods

Brain Slices

Wistar rats, Sprague-Dawley rats, wild-type (WT) CD1 mice or CB1R\(^{-/-}\) mice (Ledent et al. 1999), WT C57Bl6 mice or CB2R\(^{-/-}\) mice (Marsicano et al. 2002) (14–17 days old) were anesthetized with ether and killed by decapitation in agreement with the European Directive 86/609/EEC requirements. After that the brain was removed and transverse sagittal neocortical slices (300 \(\mu\)m) of somatosensory cortex were sectioned in ice-cold solution containing (in mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO\(_3\), 1.25 NaHPO\(_4\), 2 CaCl\(_2\), and 1 MgCl\(_2\) (carboxygenated with 5% CO\(_2)/95% O_2\)). For recordings, slices were transferred to a recording chamber containing extracellular solution of the same ionic composition as used for slicing. Neurons were visualized via a \(\times 40\) water immersion objective using infrared differential interference contrast (IR-DIC) video microscopy. Recordings were made at room temperature (20–22 °C) unless otherwise noted. Whole-cell voltage- and/or current-clamp recordings were performed simultaneously from two neurons using borosilicate glass pipettes with a resistance of 5–7 MΩ, filled with intracellular solution containing (in mM): 105 K gluconate, 30 KCl, 4 Mg-ATP, 10 phosphocreatine, 0.3 guanosine triphosphate (GTP), and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.3, KOH). In synapticantly connected neurons, suprathreshold intracellular stimulation of the presynaptic interneurons evoked GABA\(_R\)-mediated inhibitory postsynaptic currents (IPSCs), or IPSPs, in the postsynaptic pyramidal cell. Typically, presynaptic cells were stimulated with a 10 Hz train of 2 or 3 short suprathreshold depolarizing current pulses. Trains were delivered every 10 s. In experiments on IPSPs, the intracellular solution of the pyramidal cell contained a low chloride concentration so that the IPSP was hyperpolarizing. This solution contained (in mM): 130 K gluconate, 10 Na gluconate, 4 NaCl, 4 Mg-ATP, 4 phosphocreatine, 0.3 GTP, and 10 HEPES (pH 7.3, KOH). In experiments with extrasympathetic current recordings, intracellular solution contained (in mM): 140 CsCl, 2 Mg-ATP, 0.3 GTP, 4 phosphocreatine, 10 HEPES (pH 7.3, CsOH). Hippocampal cholecystokinin (CCK)-positive interneurons were identified by morphology, location, characteristic firing pattern and long-lasting asynchronous release evoked by a train of 15 action potentials (Aps) (Földy et al. 2006).

In experiments with endocannabinoid release, the release of endocannabinoids was induced either by a conditioning train of depolarizing current pulses (in voltage-clamp mode) or a single depolarizing current pulse (in current-clamp mode) or a single depolarizing current pulse (in current-clamp mode) in the postsynaptic neuron. Both protocols were able to induce conventional CB1R-dependent depolarization-induced suppression of inhibition (DSI) in hippocampal CCK-positive interneuron to CA1 pyramidal cell pairs. In experiments shown in Figure 3, IPSCs were recorded in pyramidal neurons under voltage clamp (\(V_C = -70\) mV) upon stimulation of the fast spiking (FS) interneuron. After recording 50 baseline IPSCs (control) evoked at 0.1 Hz, IPSCs were recorded at different time intervals (indicated in the text) following a conditioning stimulation (a train of 16 depolarizing pulses, 20 ms to ~30 mV, at 25 Hz) of the postsynaptic pyramidal cell. The sequences of conditioning postsynaptic stimulation and presynaptic stimulation were repeated 50 times at 0.1 Hz and the IPSCs were averaged. In the experiments shown in Figure 5D, each episode of paired pyramidal neuron and FS interneuron stimulation was preceded (1 s) by a conditioning depolarizing current pulse (700 ms, 100–200 pA) in the pyramidal neuron. The sequences of conditioning postsynaptic stimulation and presynaptic stimulation were repeated 30 times at 0.1 Hz. All stimulus delivery and data acquisition was performed using EPC-8, EPC-9 amplifiers with PULSE or Patch Master software (HEKA Elektronik, Lambrecht, Germany).

HEK293 Cells

HEK293 cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/mL streptomycin and 100 U/mL penicillin. Cells were transfected with expression plasmids for GABA\(_A\)R subunits \(\alpha_1/\alpha_2, \beta_2, \gamma_2\) (Pritchett and Seeburg 1990) in the ratios 2:2:1. pEYPF-N1 (Invitrogen) was added to the transfection mixture as a marker for transfected cells, which were maintained in culture for up to 48 h before use. For recordings, cells were transferred to a recording chamber containing (in mM): 126 NaCl, 3 KCl, 2 MgSO\(_4\), 2 CaCl\(_2\), 10 (N+)-glucose, 1.20 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\) (carboxygenated with 5% CO\(_2)/95% O_2\)). Outside-out patch-clamp recordings were made at room temperature (20–22 °C), using 3–4 MΩ borosilicate glass electrodes, filled with intracellular solution containing (in mM): 77 K-glucuronate, 77 KCl, 0.5 ethylene glycol-bis (2-aminoethylether)-N,N,N,N-tetra acetic acid (EGTA), 10 HEPES, 4 Mg-ATP, 4 K-phosphocreatine, 0.4 GTP (pH 7.3 with KOH). Patches were voltage-clamped at −70 mV and placed in front of a piezo-controlled (P 245.70, Physik Instrumente, Walldbronn, Germany) fast application system with a double-barreled application pipette. Reliability of application was checked before experiments as described (Colquhoun et al. 1992). Complete solution exchange time measured by open-tip response was ~100 μs. Current responses were recorded upon application of GABA using either an EPC-8 or EPC-9 amplifiers with PULSE software (HEKA Elektronik, Lambrecht, Germany).

Isolated Hippocampal Pyramidal Neurons

Wistar rats (12–17 days old) were decapitated under ether anesthesia, after which the brain was removed. The hippocampus was sectioned at 4 °C into slices (300–500 μm) in solution containing (in mM): 150 NaCl; 5 KCl; 1.25 NaH\(_2\)PO\(_4\); 26 NaHCO\(_3\); 1.1 MgCl\(_2\); 10 glucose (carboxygenated with 5% CO\(_2)/95% O_2\)). After slicing, slices were incubated for 5–10 min at 32 °C with 0.5 mg/ml of protease (type XXIII) from Aspergillus oryzae. Single hippocampal CA1 and CA3 cells were freed from a slice by the vibrodissociation technique (Vorobjev 1991) using a fire-polished glass stick with tip (diameter 10 μm) located in the stratum pyramidale zone. CA3 and CA1 hippocampal pyramidal cells were identified by their characteristic form and partially preserved dendritic arborization. After isolation, cells were suitable for recordings for 2–4 h. Cells were transferred to a recording chamber containing (in mM): 130 NaCl; 3 CaCl\(_2\); 5 KCl; 2 MgCl\(_2\); 10 HEPES–NaOH; 0.1 μM tetrodotoxin (TTX) (pH 7.4). Whole-cell recordings were made at room temperature (20–22 °C), using 2.5–5 MΩ borosilicate glass electrodes, containing (in mM): 70 Tris–PO\(_4\); 5 EGTA; 40 tetraethyammonium chloride; 30 Tris–Cl; 5 Mg-ATP; 0.5 GTP (pH 7.2). GABA activated currents were induced by step application of agonists in the “concentration clamp” mode (Krishtal et al. 1983), using the computerized “Pharma-Robot” set-up (Pharma-Robot, Kiev). This equipment allows complete solution exchange within 15 ms. Currents were recorded using patch-clamp amplifier (DAGAN, USA). Kinetics of \(I_{GABA}\) in isolated hippocampal neurons was fitted by a single exponential.

Chemicals

WIN55,212–2, CP55,940, Anandamide, 2-AG, nimesulide, capsaicin, and JZL 184 were obtained from Tocris Bioscience (Avonmouth, UK). SR141716A was a gift from Solvay Pharmaceuticals (Weesp, the Netherlands). RHC-80267, tetrahydrodipherstatin (THL), and UB602 were obtained from Sigma-Aldrich. Of these chemicals appropriate stock solutions were prepared in dimethyl sulfoxide (DMSO). Final DMSO concentration in the medium never exceeded 0.1%. Anandamide and 2-AG stock solutions were in anhydrous ethanol (Tocris Bioscience, Avonmouth, UK). In all experiments with application of these drugs control solutions always contained an equal amount of DMSO or ethanol.

Data Analysis

All data were analyzed off-line using IGOR PRO software (WaveMetrics, Lake Oswego, OR). Values shown in bar histograms and text are mean ± standard error of the means (SEM), unless noted otherwise. Data were filtered at 3–5 kHz. Statistical analysis was done using two-tailed t-test (\(P\geq 0.05\), unless noted otherwise. For CV analysis, we calculated CV of the IPSC amplitude under control conditions and in the presence of cannabinoids. An equal number of IPSCs was chosen for both conditions (>40 IPSCs). For details on analysis see Faber and Korn (1991). Because of intrinsic regularity at the onset of pyramidal cell spiking, we only analyzed pyramidal cell APs within the boxed...
Cannabinoids Decrease the Amplitude and Alter the Kinetics of Currents Mediated by Recombinant GABA\textsubscript{A}Rs Activated by a High (Synaptic) GABA Concentration

To test whether cannabinoids directly modulate GABA\textsubscript{A}Rs activated by 1 mM of GABA (mimicking synaptic concentration) we coexpressed recombinant $\alpha_1$, $\beta_2$, and $\gamma_2$ or $\alpha_2$, $\beta_2$, and $\gamma_2$ GABA\textsubscript{A}R subunits in HEK293 cells, since this cell line does not express any known cannabinoid receptors endogenously (Chen et al. 2003; Lauckner et al. 2008) and these GABA\textsubscript{A}R subtypes are predominant at synapses in the adult rat neocortex (Wisden et al. 1992). For both GABA\textsubscript{A}R isoforms, the endogenous CB1R agonist 2-AG (1 $\mu$M) significantly and reversibly reduced the amplitude of GABA\textsubscript{A}R-mediated currents ($I_{\text{GABA}}$) induced by fast application of GABA (1 s; $V_m = -60$ mV, Fig. 1A). In the presence of 2-AG the current amplitude normalized to control ($I_{\text{norm}}$) was 0.52 ± 0.05 ($n = 10$, $P < 10^{-5}$) for $\alpha_1\beta_2\gamma_2$ and 0.44 ± 0.05 ($n = 8$, $P < 10^{-5}$) for $\alpha_2\beta_2\gamma_2$. After washout of 2-AG $I_{\text{norm}}$ recovered to 0.88 ± 0.09 ($n = 3$, washout compared with 2-AG: $P < 0.007$) for $\alpha_1\beta_2\gamma_2$ and 0.81 ± 0.07 ($n = 4$, washout compared with 2-AG: $P < 0.002$) for $\alpha_2\beta_2\gamma_2$ (Fig. 1B).

Additionally, 2-AG increased GABA\textsubscript{A}R desensitization during GABA application. Correspondingly, in the presence of 2-AG the charge transfer of $I_{\text{GABA}}$ normalized by peak amplitude (normalized area), reflecting changes in the rate of desensitization, was $0.49 \pm 0.06$ ($n = 5$, $P < 0.001$) for $\alpha_1\beta_2\gamma_2$ and $0.56 \pm 0.12$ ($n = 5$, $P \leq 0.04$) for $\alpha_2\beta_2\gamma_2$ of respective controls (see Supplementary Fig. 1). The modulation of GABA\textsubscript{A}R-mediated current amplitude and kinetics in the presence of 2-AG cannot be attributed to gradual alterations during prolonged recording, as these parameters stayed constant during a >30 min control experiment without 2-AG application (see Supplementary Fig. 2). Qualitatively, similar results were obtained with other endogenous (anandamide [AEA], 1 $\mu$M) and synthetic (CP 55,940 [CP], 1 $\mu$M) CB1R agonists (Fig. 1B, see Supplementary Fig. 1). Similarly, native GABA\textsubscript{A}Rs in acutely isolated hippocampal pyramidal neurons from rat brain were also sensitive to these direct effects of cannabinoids (see Supplementary Fig. 3).

Cannabinoids Modulate Inhibitory Synaptic Transmission-Independent of CB1R Activation

To test whether the CB1R-independent modulation of GABA\textsubscript{A}Rs by cannabinoids has an effect on GABAergic synaptic transmission, we investigated inhibitory connections formed by neocortical layer 2/3 parvalbumin (PV)-positive FS interneurons and neighboring pyramidal (PYR) neurons. These synapses do not express presynaptic CB1Rs (Bodor et al. 2006). GABA\textsubscript{A}R-mediated currents were induced by fast applications of GABA (1 s; $V_m = -60$ mV) to outside-out patches in the absence (left), presence (middle), and after washout (right) of 1 $\mu$M 2-AG. Each trace is the average of 3–5 individual responses. (B) Summary of the effects of cannabinoid receptor agonists 2-AG, AEA and CP (1 $\mu$M each) on the amplitude (peak) of the currents mediated by activation of GABA\textsubscript{A}Rs composed of either $\alpha_1\beta_2\gamma_2$ or $\alpha_2\beta_2\gamma_2$ subunits. Black circles represent average values from individual experiments indicated by open circles. All values are normalized to control.
However, to exclude any contribution of CB₁R activation recordings were made from rats and WT mice in the presence of the CB₁R antagonist SR141716A (SR) or from CB₁R knockout (CB₁R⁻/⁻) mice. FS interneurons were identified by their characteristic action potential (AP) firing pattern and morphology (Fig. 2A, B) (Reyes et al. 1998). Because endogenous cannabinoids are subject to fast enzymatic degradation in brain slices we used the synthetic CB₁R agonist CP. Evoked GABAergic IPSCs were recorded from pyramidal neurons upon stimulation of neighboring synaptically connected FS interneurons (see Materials and Methods). In rats, SR (5 μM) alone did not cause a significant modulation of the IPSC amplitude ($I_{\text{norm}}: 0.89 ± 0.08; n = 5; P ≥ 0.16$, Fig. 2C, top). However, the subsequent bath application of the synthetic CB₁R agonist CP...
(1 μM) gradually reduced the IPSC amplitude to 0.62 ± 0.06 of the amplitude in SR alone (n = 8; P < 0.001, Fig. 2C, bottom and Fig. 2D, top). Similarly, in CB1R−/− mice the amplitude of IPSCs recorded in FS−PYR cell pairs was reduced by CP (1 μM) to 0.45 ± 0.05 of control (n = 6; P < 0.001; Fig. 2D, bottom). A comparable result was observed in rats after application of the synthetic cannabinoid WIN55,212-2 (5 μM) in the presence of SR (Inorm: 0.51 ± 0.12; n = 5; P < 0.006).

To discover whether the modulation of GABAergic transmission by CP occurred pre- or postsynaptically, we measured the paired pulse ratio (PPR—ratio of amplitudes IPSC2/IPSC1) and analyzed the coefficient of variation (CV) (Faber and Korn 1991) of IPSC amplitudes. Previous studies have shown that presynaptic cannabinoid effects are consistently associated with an increase in PPR (Wilson and Nicoll 2001) or with a change in CV proportional to the change in IPSC amplitude (Min et al. 2010). However, here we observed that the PPR was not significantly changed by CP in rats in the presence of SR or in CB1R−/− mice (rat SR: 0.81 ± 0.03; SR + CP: 0.75 ± 0.04; n = 8; P ≥ 0.15; CB1R−/− control: 0.82 ± 0.03; CP: 0.91 ± 0.10; n = 6; P ≥ 0.25; Fig. 2E). In addition, in both rats and CB1R−/− mice the average CV2 ratio was close to 1 (rat: 1.03 ± 0.21; n = 8; CB1R−/− mice: 1.09 ± 0.10; n = 6), consistent with a postsynaptic locus of IPSC reduction (Fig. 2F). Taken together, these data show that IPSCs at FS–PYR inhibitory connections are modulated postsynaptically by CP, and that this modulation is independent of CB1R activation.

CB1R-Independent Short-Term Plasticity Mediated by Activity-Dependent Release of Endocannabinoids

Next, we tested whether endogenously released cannabinoids can suppress GABAergic IPSCs independent of CB1R activation at FS–PYR inhibitory connections. Endocannabinoid release was induced by a conditioning train of depolarizations in the postsynaptic pyramidal neuron (see Materials and Methods). After recording baseline IPSCs (at stimulation frequency of 0.1 Hz), IPSCs were evoked 1 s after the conditioning depolarization (Fig. 3A). In WT mice, in the presence of SR, the amplitude of IPSCs recorded after the conditioning depolarization was reduced to 0.67 ± 0.06 of the baseline (control) value (n = 5; P < 0.001; Fig. 3B, left panel and D). The reduction in IPSC amplitude was not associated with a change in the PPR (control: 0.79 ± 0.05; conditioning: 0.81 ± 0.02; n = 5; P > 0.65; Fig. 3D), indicative of a postsynaptic locus of IPSC suppression. To confirm that the reduction of the IPSC amplitude was due to the release of endocannabinoids from the postsynaptic neuron, we pharmacologically inhibited endocannabinoid production using THL. THL is known to suppress the activity of diacylglycerol lipase (DAGL), a key enzyme involved in the biosynthesis of the endocannabinoid 2-AG (Bisogno et al. 2003; Melis, Pistis et al. 2004). It has been shown that intracellular application of this compound can block the synthesis of 2-AG from cortical neurons (Martinelli et al. 2008; Min et al. 2010). With THL (5 μM) in the recording pipette of the postsynaptic pyramidal neuron, the conditioning train no longer caused a reduction of the IPSC amplitude (1.06 ± 0.1 of control; n = 4; P > 0.25; Fig. 3B, right panel and D). The same results were obtained in experiments with CB1R−/− mice (Fig. 3C,E). These data show that endocannabinoids mediate a CB1R-independent depolarization-induced suppression of inhibitory synaptic transmission at neocortical FS–PYR cell pairs.

Next, we investigated the duration of the CB1R-independent suppression of IPSC amplitude by varying the time interval (Δt) between the postsynaptic conditioning depolarization and the presynaptic stimulation in FS–PYR cell pairs. In rats, suppression of the IPSC amplitude in the presence of SR was observed as early as 180 ms after postsynaptic depolarization (Inorm: 0.61 ± 0.08; n = 5; P < 0.006), and the IPSC amplitude recovered to the control value within ~5 s after conditioning (Inorm: 0.97 ± 0.09; n = 5; P > 0.75; Fig. 3F). Notably, the results obtained in the absence of SR were not significantly different from those obtained in the presence of SR (Fig. 3F).

When brain slices were incubated with the DAGL inhibitor RHC80267 (30 μM; no SR present), which partially suppresses endocannabinoid synthesis (Melis, Perra et al. 2004; Szabo et al. 2006), the reduction of the IPSC amplitude was less pronounced at 800 ms after postsynaptic depolarization (Inorm in RHC80267: 0.85 ± 0.04 vs. control: 0.59 ± 0.07; n = 5; P < 0.02) and was no longer observed after a 2.6 s delay (Inorm: 1.14 ± 0.29; n = 5; P > 0.35; Fig. 3F).

The opposite effect on endocannabinoid signaling can be achieved by inhibiting the degradation of endocannabinoids. The monoacylglycerol lipase (MAGL) inhibitor URB602 has been shown to prolong the time course of CB1R-dependent DSI in hippocampus (Makara et al. 2005). Therefore, we tested the effects of URB602 on the time course of the CB1R-independent suppression of IPSC. Inhibition of the endocannabinoid degradation by bath application of URB602 (100 μM) increased the time-window for IPSC suppression after conditioning to >20 s (Inorm at 20 s: 0.82 ± 0.02; n = 5; P < 0.01; (Fig. 5G), without affecting the initial level of the IPSC suppression (Inorm at 180 ms: 0.69 ± 0.13; n = 4). Another endocannabinoid metabolizing enzyme involved in 2-AG degradation is cyclooxygenase-2 (COX-2) (Kozak et al. 2000, 2004, for review see Yang and Chen 2008). COX-2 has been shown to be present in postsynaptic dendritic spines (Kaufmann et al. 1996; Sang and Chen 2006). Thus, postsynaptic COX-2 could be effectively involved in termination of the CB1R-independent endocannabinoid-mediated GABAAR inhibition. Indeed, in the presence of nimesulide (30 μM), an inhibitor of the COX-2, the time window for IPSC suppression was also increased (Fig. 3G).

Postsynaptic conditioning depolarization induced a similar reduction of IPSC amplitudes in WT and CB1R−/− mice in the absence of CB1R antagonists. Initial suppression of IPSC amplitude was to 0.56 ± 0.08 (n = 5; P < 0.008) and 0.65 ± 0.07 (n = 5; P < 0.009) of control for WT and CB1R−/− mice, respectively (WT vs. CB1R−/−: P > 0.4). The IPSC amplitudes recovered within ~5 s after postsynaptic depolarization (Inorm: WT: 0.83 ± 0.14; n = 5; P > 0.25; CB1R−/−: 0.96 ± 0.07; n = 6; P > 0.55; Fig. 3H). In CB1R−/− mice, IPSC suppression was reduced by RHC80267, with full recovery occurring after a 2.6 s delay (Inorm: 1.15 ± 0.12; n = 5; P ≥ 0.35) and it was almost completely abolished in experiments where THL (5 μM) was included in the postsynaptic recording pipette (Fig. 3H), confirming that the suppression is mediated by postsynaptic endocannabinoid synthesis.

A previous study has suggested that activation of vanilloid TRPV1 receptors by endocannabinoids (Grueter et al. 2010) might also attenuate synaptic transmission postsynaptically in a CB1R-independent manner. Therefore, we performed
Figure 3. Depolarization-induced mobilization of endocannabinoids causes a CB₁R-independent suppression of inhibitory synaptic transmission. (A) Experimental setup (see Materials and Methods for details). (B) Left, IPSC amplitudes under control conditions, after conditioning (red) and during recovery in WT mice in the presence of SR (5 μM). Each open symbol represents the amplitude of a single IPSC. Closed symbols show the average amplitude of 5 IPSCs recorded at preceding time points. Solid lines show the average of 50 responses. Data from individual example experiments for each condition are shown. Traces above show the average response for each condition. Right, same as left, but with THL (5 μM) in the postsynaptic recording pipette. (C) Same as in panel B, but for CB₁R−/− mice. (D) Left, summary of the IPSC suppression after conditioning for WT mice in the absence and presence of THL in the postsynaptic recording pipette. Middle, example traces of two consecutive averaged IPSCs normalized to the amplitude of the first IPSC during control (black) and after conditioning (red, average of 50 traces for each condition). Right, the summary data on PPR. (E) Same as in panel D, but for CB₁R−/− mice. (F, G) The amplitudes of IPSCs recorded in rat brain slices at various delays (Δt) after conditioning, normalized to the averaged baseline IPSC amplitude. Connected symbols show experiments performed: (F) in the absence (green) or presence of SR (5 μM; black), or after treatment with RHC80267 (30 μM; red); (G) in the presence of SR alone (5 μM; black; same data as in panel F), or in the presence of SR and URB602 (100 μM; red), or SR and nimesulide (30 μM, blue). (H) A comparison of the conditioning-induced suppression of IPSC amplitudes in WT (green) and CB₁R−/− mice (black), either under control conditions, or after treatment with RHC80267 (30 μM, red), or THL (5 μM, blue). SR was not used. Each point shows the average of 4–5 cell pairs.
stimulation of the FS interneuron with a 50 Hz train of 10 short suprathreshold depolarizing current pulses. As before, the release of endocannabinoids was induced by a conditioning train of depolarizing current pulses (in voltage-clamp mode) applied to postsynaptic pyramidal neuron (Fig. 4B). In control conditions, the train of presynaptic repetitive stimuli induced short-term depression of the IPSCs (Fig. 4C, black). Similar to the observation illustrated in Figure 3, after conditioning the peak amplitude of the first IPSC in the train was reduced to 0.72 ± 0.07 (n = 7, P < 0.006, Fig. 4C, red) of the control value. Additionally, the extent of the synaptic depression during the train significantly increased (Fig. 4C, red). Correspondingly, normalized charge transfer in the train significantly decreased to 0.73 ± 0.04 after conditioning (n = 7, P < 0.0006, Fig. 4D,F). Thus, the total non-normalized charge transfer was reduced to 0.61 ± 0.07 after conditioning (n = 7, P < 0.001, Fig. 4G). However, in the presence of THL (10 μM) in the bath solution (after 20 min preincubation) both the peak amplitude of the first IPSC and the normalized charge transfer remained unaltered (Fig. 4F). These results suggest that endogenously released endocannabinoids do not only suppress inhibitory synaptic transmission, but also modulate temporal summation of GABA-mediated responses upon high-frequency stimulation, resulting in synaptic low-pass filtering.

Exogenous and Endogenous Cannabinoids Reduce FS–Pyramidal Cell Interactions in a CB1R-Independent Manner

The impact of CB1R-independent modulation of GABAARs by cannabinoids on local neuronal microcircuit activity was assessed in unidirectionally connected FS–PYR pairs in rats in the presence of SR (5 μM). Pyramidal neuron firing was evoked by constant current injection, while the connected FS interneuron was rhythmically stimulated at 20 Hz (Fig. 5A). Under control conditions, FS interneuron AP firing effectively controlled the pattern of AP firing in the postsynaptic pyramidal cell. Application of CP (1 μM) greatly reduced the efficacy of the FS input, resulting in a decreased synchrony of firing between the FS interneuron and the pyramidal neuron (Fig. 5B). Under control conditions, the probability of AP appearance in the pyramidal neuron within the 52 ms window between two consecutive FS interneuron stimulations showed a minimum at <20 ms after the onset of FS interneuron stimulation (Fig. 5C, left; pooled data, n = 8). This approximately corresponds to the peak of the IPSP (Fig. 5C, inset). However, in the presence of CP, the AP probability distribution was more uniform (Fig. 5C, right; pooled data, n = 8). The decrease in the AP probability in pyramidal neurons caused by FS interneuron stimulation was significantly less pronounced in the presence of CP when compared with control (Kuiper’s test: P < 0.001; Fig. 5C,E). Without FS cell stimulation, AP probability in pyramidal neurons remained uniformly distributed both under control conditions and in the presence of CP (see Supplementary Fig. 5A). Importantly, the AP shapes of both FS interneurons and pyramidal neurons were unaltered in CP (see Supplementary Fig. 5B), indicating that CP by itself does not change the intrinsic pyramidal and FS cells firing patterns.

Inspection of Figure 5B (upper panel) reveals that APs are more scattered towards the end of the depolarizing pulses. This phenomenon might indicate that the depolarizing step in the pyramidal cell itself could lead to synthesis of Exocannabinoids Induce Low-Pass Filtering of GABAAR-Mediated Currents Evoked by High-Frequency Stimulation

So far we have shown that endocannabinoids can directly modulate the amplitude of inhibitory synaptic currents at FS–PYR synapses independent of CB1R activation. Additionally, the direct modulation of GABAAR desensitization by endocannabinoids may alter temporal summation of GABA-mediated responses upon high-frequency stimulation. To verify this hypothesis, we first tested effects of 2-AG on I\textsubscript{GABA} evoked by a train of short GABA pulses applied to outside-out patches from HEK293 cells expressing recombinant GABA\textsubscript{A}Rs using ultrafast agonist application techniques. Under control conditions, the amplitude of the currents evoked by repetitive applications (10 Hz) of 10 short (2 ms) pulses of a saturating GABA concentration (1 mM) progressively declined due to receptor desensitization (Fig. 4A, left). This depression of I\textsubscript{GABA} during repetitive GABA application was strongly enhanced in the presence of 2-AG (1 μM; Fig. 4A, middle). While the first peak in the train was reduced on average to 0.54 ± 0.12 (n = 4), the effect of 2-AG further developed towards the end of the application train. Correspondingly, when we normalized responses in the train to the amplitude of the first peak, the charge transfer in the train was significantly decreased in the presence of 2-AG to 0.64 ± 0.151 (n = 4, P ≤ 0.05, Fig. 4A, right). This observation indicates that 2-AG can be a powerful modulator of GABA\textsubscript{A}R-mediated responses at high-frequency stimulation.

To test whether the direct modulation of GABA\textsubscript{A}R desensitization by endocannabinoids contributes to the activity-dependent changes in GABAergic synaptic transmission we performed experiments in FS–PYR synapses in neocortical slices of the CB1R\textsuperscript{−/−} mice. Train-IPSCs were recorded in pyramidal neurons under voltage clamp (V\textsubscript{m} = −70 mV) upon experiments in CB1R\textsuperscript{−/−} mice with a conditioning protocol as shown in Figure 3, but with the TRPV1 receptor antagonist capsazepine in the bath solution. In the presence of 10 μM capsazepine, suppression of IPSCs 1 s after conditioning was not significantly different from suppression in the absence of the TRPV1 antagonist (see Supplementary Fig. 4). Normalized IPSC amplitude (IPSC [norm]) was 0.66 ± 0.03 in capsazepine, (n = 6), vs. 0.67 ± 0.05 in control (n = 5, P > 0.85, two sample, two-tailed t-test). This result excludes the involvement of TRPV1 receptors in the observed effects on inhibitory synaptic transmission.

Previous studies have suggested that synaptic effects of the endocannabinoid 2-AG are strongly temperature dependent (Hájos et al. 2004). To see whether CB1R-independent synaptic effects of 2-AG were also temperature dependent, we repeated the experiment with conditioning at a more physiological temperature (33 °C). The maximal initial suppression of IPSC amplitude measured in CB1R\textsuperscript{−/−} mice 180 ms after conditioning was not significantly different from that recorded at room temperature, thereby confirming that our findings are also preserved at physiological temperature (IPSC [norm] was 0.73 ± 0.03 at 33 °C n = 6 vs. 0.65 ± 0.07 at 22 °C, n = 5; P > 0.3).

Taken together, these results show that conditioning depolarization of the postsynaptic pyramidal neuron triggers DAGL-dependent synthesis of the endocannabinoid 2-AG, resulting in a transient CB1R-independent suppression of inhibition at neocortical FS–PYR synapses.
Figure 4. Endocannabinoids induce low-pass filtering of GABA<sub>R</sub>-mediated currents evoked by high-frequency stimulation. (A) Frequency-dependent modulation of \( I_{\text{GABA}} \) by 2-AG in outside-out patches. Representative traces of the currents mediated by recombinant GABA<sub>R</sub>s (composed of \( \alpha_1\beta_2\gamma_2 \) subunits coexpressed in HEK293 cells) induced by the train (10 Hz, 10 pulses) of short (2 ms) applications of GABA (1 mM) in control (left), and in the presence 2-AG (1 \( \mu \text{M} \); middle). (Right), the same traces normalized to the peak amplitude of the first response and superimposed, \( V_m = -60 \text{ mV} \). Dots indicate the GABA applications. Note a stronger reduction of the current amplitudes in the train in the presence of 2-AG. (B–G) Frequency-dependent conditioning-induced modulation of GABAergic IPSCs recorded from neocortical FS–PYR pairs in brain slices of CB<sub>1</sub>R<sup>−/−</sup> mice. (B) Experimental setup. Train-IPSCs were recorded in pyramidal neurons under voltage clamp (\( V_m = -70 \text{ mV} \)) upon stimulation of the FS interneuron with a 50 Hz train of 10 short suprathreshold depolarizing current pulses. The release of endocannabinoids was induced by a conditioning train of depolarizing current pulses in the postsynaptic pyramidal neuron. (C) Representative averaged traces of 30 individual sweeps of train-IPSCs induced by presynaptic stimulation in control (black) and 0.5 s after conditioning depolarization of pyramidal cell (red). Traces above represent averaged superimposed presynaptic FS interneuron action potentials in control and after conditioning depolarization of pyramidal cell (red). (D) The same IPSCs traces normalized to the peak amplitude of the first IPSC. Traces above represent averaged superimposed FS interneuron action potentials in control and after conditioning depolarization of pyramidal cell (shown in box in panel C) at extended timescale. (E) Representative averaged IPSCs traces with 10 \( \mu \text{M} \) THL in the bath (blue) in control and after conditioning. Data from a different experiment. Traces above represent averaged superimposed FS interneuron action potentials with 10 \( \mu \text{M} \) THL in the bath in control (without conditioning) and after conditioning. (F) Normalized charge transfer of the train-IPSCs estimated from normalized by the amplitude of the first peak currents (as shown in panel D) is reduced after conditioning (control vs. conditioning) and is unaltered after conditioning in the presence of 10 \( \mu \text{M} \) THL (THL vs. conditioning THL). (G) Integral charge transfer of the IPSCs in train estimated from non-normalized currents (as shown in panel C) is reduced to a greater extent.
Figure 5. Exogenous and endogenous cannabinoids reduce FS–pyramidal cell interaction in a CB1R-independent manner. (A) Experimental setup. A FS interneuron (FS) and a connected pyramidal neuron (P) were kept in current-clamp mode. The postsynaptic pyramidal neuron was injected with a 700 ms depolarizing current pulse to trigger AP firing, while the presynaptic FS interneuron was stimulated with a train (20 Hz) of stimuli. Pyramidal neuron APs within the area marked by dashed lines were subjected to further analysis. All experiments were performed in rat neocortical slices in the presence of SR (5 μM). (B) A representative example showing the firing of a pyramidal neuron [overlay of 30 traces] during simultaneous stimulation of the presynaptic FS interneuron under control conditions (top) and in the presence of CP (1 μM; bottom). Lines under the traces represent FS interneuron stimulation pulses. (C) The AP distribution of a pyramidal cell under control conditions (left; n = 8) and in the presence of CP (1 μM, right; n = 8). The inset shows an example of the IPSP [response of a pyramidal neuron at resting potential (bottom) to one pulse of FS interneuron stimulation (top). (D) AP distribution in the pyramidal neurons after conditioning train (n = 6). Experimental setup was the same as in panel (A), but instead of CP administration, each episode of paired pyramidal neuron and FS interneuron stimulation was preceded (1 s) by a conditioning depolarizing current pulse (700 ms) in the pyramidal neuron to release endocannabinoids. (E) Cumulative spike probability plot in control, after CP application and after conditioning. Distributions differ significantly (Kuiper’s test: P < 0.001).
endocannabinoids, which in turn would lead to suppression of postsynaptic GABA_2Rs which becomes more apparent at the later phases of the prolonged depolarization. Indeed, when current injection to pyramidal cells synchronized with pulsed FS interneuron stimulation was preceded (1 s) by a conditioning depolarizing current injection (700 ms) to the postsynaptic pyramidal cell to mobilize endocannabinoids the AP probability distribution in pyramidal neuron (as for CP) was more uniform (Fig. 5D,E, n = 6). Thus, both exogenously applied and endogenously released cannabinoids desynchronize neocortical FS–pyramidal neuron interactions independent of CB_1R activation.

CB_1R-Independent Modulation of Inhibitory Synaptic Transmission is Synapse Specific

It is surprising that direct CB_1R-independent modulation of inhibitory synaptic transmission by cannabinoids has not been observed previously, especially since the effect of cannabinoids on inhibitory synaptic transmission has been well studied (Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001; Földy et al. 2006). One possible explanation for this apparent discrepancy posits that the CB_1R expressing synapses made by Földy et al. (2006). Importantly, these data are in agreement with the high sensitivity of CB_1Rs (Katona et al. 1999). Importantly, these data are in sharp contrast to those obtained in neocortical FS–PYR synapses (which are known not to express CB_1Rs; Bodor et al. 2005) and indicate that sensitivity of postsynaptic GABA_2Rs to direct modulation by endocannabinoids is synapse specific.

In the hippocampus, inhibitory synaptic transmission at CB_1R-positive synapses is mainly mediated by α_2 subunit-containing GABA_2Rs (Nyiri et al. 2001; Panzanelli et al. 2011). By contrast, at parvalbumin-containing FS basket cell to pyramidal neuron synapses, inhibitory transmission is mediated primarily by α_1 subunit-containing GABA_2Rs (Klausberger et al. 2002). To test whether differences in the subunit expression in the two types of synapses may account for the differential sensitivity of GABA_2Rs to CP, we utilized α_2−/− mice. Surprisingly, for CCK-positive interneuron to CA1 pyramidal neuron synapses in α_2−/− mice (in the presence of SR), CP (1 μM) significantly reduced IPSCs to (0.47 ± 0.12; n = 5, P < 0.05, Fig. 6C,D). Since α_1 and α_2 subunit-containing GABA_2Rs expressed in HEK293 cells display similar sensitivity to cannabinoids (Fig. 1B), these data suggest that additional factors specific to synaptic expression of selected GABA_2R subtypes may contribute to their differential sensitivity to cannabinoids (see Discussion).

CB_1R-Independent Modulation by Cannabinoids of Extrasynaptic GABA_2R-Mediated Conductance

A recent study has reported that currents mediated by recombinant GABA_2Rs expressed in oocytes and activated by low concentrations of GABA can be directly potentiated by the endocannabinoid 2-AG (Sigel et al. 2011). This finding implies that the polarity of CB_1R-independent GABA_2R modulation strongly depends on GABA concentration. In agreement, we found that 2-AG increased currents mediated by native GABA_2Rs evoked by the application of a low GABA concentration (1 μM) to nucleated patches pulled from neocortical layer 2/3 pyramidal neurons (Fig. 7A). Based on this observation, we hypothesized that the activity of extrasynaptic GABA_2Rs, which are naturally exposed to relatively low GABA concentrations, might be enhanced by cannabinoids. To test whether extrasynaptic tonically active GABA_2Rs are modulated by endocannabinoids, we monitored holding currents in voltage-clamp mode (V_h = −80 mV) in pyramidal neurons of layer 2/3 in slices from WT mouse somatosensory cortex before and after a conditioning train of depolarization. The extracellular solution contained 0.1 μM TTX and selective antagonists of CB_1Rs (SR, 5 μM), AMPARs (NBQX, 10 μM) and NMDARs (APV, 20 μM). Experiments were performed with a symmetrical chloride concentration (see Materials and Methods). To assess the contribution of the GABA_2R-mediated tonic current to the total holding current, experiments were performed under control conditions and in the presence of GABA_2R selective antagonist picrotoxin (PTX, 50 μM). The GABA_2R-mediated tonic current was calculated as the difference between the holding current in control and in the presence of PTX. On average GABA_2R-mediated tonic current measured under control conditions (I_tonic) was 14.2 ± 2.6 pA, (n = 6, Fig. 7B,C). When we evoked endocannabinoid release by a conditioning depolarization (20 ms pulses to −10 mV, at 10 Hz for 5 s) we observed a transient increase in the holding current, with the PTX-sensitive current mediated by extrasynaptic GABA_2Rs measured immediately after the end of a conditioning train (after depolarization [AD] current (I_AD) being 40 ± 8.7 pA (n = 6, Fig. 7B,C). A significant increase of the PTX-sensitive current after conditioning (I_AD/I_tonic = 2.8 ± 0.6; n = 6, P < 0.02) suggests a potentiation of extrasynaptic GABA_2R function. The effect of conditioning was completely prevented when slices were incubated (for 20 min) with the 2-AG synthesis inhibitor THL (10 μM, Fig. 7B, lower trace) indicating that this potentiation is mediated by endogenously released 2-AG (in THL: I_AD/I_tonic = 0.8 ± 1.0, n = 5, P ≥ 0.8). Interestingly, in the presence of THL, I_tonic was significantly reduced when compared with control conditions, suggesting that the ambient endocannabinoid tone already increases tonic extrasynaptic GABA_2R function. In agreement, I_tonic was dramatically enhanced when the ambient endocannabinoid tone was increased by preincubating slices (40 min) with JZL 184 (100 nM), an inhibitor of MAGL, the primary enzyme responsible for degrading 2-AG (Long et al. 2009) (Fig. 7C). Thus, 2-AG, either ambient or mobilized by conditional depolarization, enhance the activity of pyramidal neuron extrasynaptic GABA_2Rs in a CB_1R-independent manner. This enhancement of extrasynaptic GABA_2R function by cannabinoids is in stark...
contrast to the effect on FS–PYR synaptic GABA<sub>A</sub>Rs, which are suppressed by cannabinoids.

**Discussion**

In this study we describe several fundamentally novel aspects of modulation of GABAergic inhibition by cannabinoids: (1) GABAergic inhibitory synaptic transmission at FS interneuron to pyramidal neuron synapses in layer 2/3 of somatosensory cortex, which do not contain CB<sub>1</sub>Rs, can be suppressed by (endo)cannabinoids. (2) At the neuronal network level, CB<sub>1</sub>R-independent cannabinoid suppression of GABAergic synaptic transmission may have dramatic consequences for inhibitory control in layers 2/3 of the somatosensory cortex, by

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**Figure 6.** CB<sub>1</sub>R-independent cannabinoid suppression of GABAergic synaptic transmission is synapse-specific. (A) The time course of IPSC amplitude changes recorded in CA1 pyramidal neurons upon stimulation of synaptically connected CCK-positive interneurons in WT mice during CP (1 μM) application in the presence of SR (5 μM). For analysis, 5 subsequent responses were averaged and normalized to the mean IPSC amplitude obtained before CP application (100 sweeps). The plot shows the cumulative data from 5 experiments. On the right are averaged example traces before (black) and after CP (red) application. (B) The same as in (A), but for CB<sub>1</sub>R<sup>−/−</sup> mice. SR was not added. (C) The same as in (A), but for α<sub>2</sub>β<sub>3</sub><sup>−/−</sup> mice in the presence of SR. Error bars in (A–C): ± SD. (D) Summary data of the CB<sub>1</sub>R-independent IPSC suppression by CP. IPSC<sub>norm</sub>—ratio of IPSC amplitudes measured in 1 μM CP and in respective control.
greatly reducing the ability of FS interneurons to control pyramidal neuron firing. (3) At FS interneuron to pyramidal neuron synapses, endocannabinoids induce synaptic low-pass filtering of GABAAR-mediated currents evoked by high-frequency stimulation. (4) CB1R-independent cannabinoid suppression of GABAergic synaptic transmission is synapse-specific, and does not occur at hippocampal CCK-positive interneuron to pyramidal neuron synapses. (5) In pyramidal neurons of layer 2/3 of somatosensory cortex endocannabinoids potentiate extrasynaptic GABAAR-mediated currents.

Collectively, our data reveal that cannabinoids have a dual direct effect on neuronal GABAARs: inhibiting at high (synaptic) GABA concentrations and potentiating at low (extrasynaptic) GABA concentrations. Exogenously applied, as well as endogenously produced cannabinoids can suppress GABAergic inhibitory synaptic transmission in cortex in a synapse-specific manner, but they can also increase the extrasynaptic tonic GABAAR-mediated conductance. Both CB1R-independent effects would favor loss of control of interneurons over pyramidal neurons (see Supplementary Fig. 6). In contrast to the retrograde CB1R-dependent mode of endocannabinoid action on GABAergic inhibition, the direct modulation of GABAARs does not require additional mediators. Hence, endocannabinoids may act in an autocrine fashion as intracellular or membrane-delimited messengers.

**Postsynaptic and Extrasynaptic GABAARs as Novel Targets for Cannabinoids**

Thus far, most of the effects of cannabinoids on mammalian brain function and behavior have been attributed to activation of the CB1R—a G protein-coupled receptor. However, it is unlikely that the suppression of synaptic GABAARs in neocortical FS–PYR synapses observed here can be attributed to the activation of cannabinoid receptors or vanilloid TRPV1 receptors. First, effects on synaptically evoked responses persisted in the presence of a CB1R antagonist as well as in CB1R−/− mice.
Second, the FS-PYR synapses do not contain presynaptic CB$_1$Rs (Bodor et al. 2005). Moreover, in our experiments with CP, PPR remained unchanged, which together with the results of the CV analysis (Fig. 2) indicate that this CB$_1$R agonist acts postsynaptically. Third, at high (mimicking synaptic) GABA concentrations, cannabinoids inhibited currents mediated by recombinant GABA$_A$Rs expressed in HEK293 cells, which do not express any known cannabinoid receptors (Griffin et al. 2000).

In a recently published study, Sigel et al. (2011) reported only direct potentiation of recombinant GABA$_A$Rs by cannabinoids at low GABA concentrations, but did not observe the inhibition of GABA$_A$Rs at high GABA concentrations in an oocyte expression system. This apparent discrepancy with our data might be explained by differences in the experimental setup and limitations of two electrode voltage-clamp recordings from whole oocyte. Due to oocyte geometry, a relatively slow rate of bath GABA application (rise time of the current is ~1 s) may mask the effect of cannabinoids on desensitization and thus on amplitude which was apparent in our experiments, which utilized HEK293 cells and a fast agonist application system.

Although our results indicate that cannabinoids can bidirectionally modulate neuronal GABA$_A$Rs without activating the CB$_1$R, the mechanism underlying this modulation remains unclear. Mechanistically, by adapting a previously published model of GABA$_A$R activation (Jones and Westbrook 1995), we show (see Supplementary Text) that simultaneous alteration of two parameters in the model (slower GABA unbinding and more rapid desensitization) can explain both opposite changes of GABA$_A$R-mediated responses by cannabinoids: enhancement of $I_{\text{GABA}}$ at low GABA concentration and inhibition of $I_{\text{GABA}}$ at high concentration of GABA, respectively. In other words, the functional impact of endocannabinoids on the GABA$_A$R depends upon the physiological context of GABA$_A$R activation.

Dual concentration-dependent effects on GABA$_A$Rs are also known for benzodiazepines (Mozrzymas et al. 2007; Bianchi 2010; Barberis et al. 2011) and neurosteroids (Haage and Johansson 1999) yet without a clear understanding of the underlying mechanism. It is important to note that these compounds, which act on modulatory sites of the GABA$_A$R, have similar therapeutic properties as cannabinoids, inducing anxiolytic, sedative, hypnotic, and anticonvulsant effects (Franks and Lieb 2001). Hence, some therapeutic effects of cannabinoids might not be due to CB$_1$R activation, but could be caused by a direct interaction with the GABA$_A$R.

In agreement with cannabinoids directly interacting with a site on the GABA$_A$R, it has recently been shown that the $\beta_2$ subunit of the GABA$_A$R plays a crucial role in the direct modulation by cannabinoids (Sigel et al. 2011; Baur et al. 2013). It is well known that in addition to subunit composition, GABA$_A$R function (gating properties and sensitivity to other compounds) depends on many other factors such as the channel density, clustering, binding to other proteins etc. All these factors may also interfere with cannabinoid sensitivity. Indeed, we showed that $\alpha_2$-containing GABA$_A$Rs in recombinant systems have the same sensitivity as those containing the $\alpha_1$ subunit (Fig. 1). However, GABA$_A$Rs in hippocampal CCK-positive interneurons to CA1 pyramidal neuron synapses become sensitive to cannabinoids only after deletion of $\alpha_2$ subunit (Fig. 6).

These data suggest that additional factors specific to synaptic expression of selected GABA$_A$R subtypes may contribute to their differential sensitivity to cannabinoids. For example, the hippocampal CA1 neurons of $\alpha_2^{-/-}$ mice exhibit considerable reorganization of inhibitory synapses (e.g., a loss of gephyrin clusters) (Panzanelli et al. 2011). Nevertheless, these observations indicate that cannabinoid–GABA$_A$R interactions have a key role in the observed effects and further emphasize caution in translating the results of recombinant receptors to native neuronal receptors.

**Endocannabinoid-Mediated DSI Versus CB$_1$R-Independent Suppression of Synaptic Inhibition**

Classical retrograde CB$_1$R-dependent suppression of inhibitory synapses by endocannabinoids (e.g., DSI), where the endocannabinoids are produced postsynaptically, but act presynaptically, has been extensively studied in a variety of preparations (Kreitzer and Regehr 2001; Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001). Therefore, a major question is why previous studies did not observe direct CB$_1$R-independent effects of cannabinoids on GABAergic synaptic transmission. A possible explanation for this is the fact that direct modulation of GABA$_A$Rs by cannabinoids is synapse specific. Many studies directly looking at CB$_1$R mediated modulation of inhibitory synapses have relied on measuring spontaneous synaptic transmission or used extracellular stimulation of inhibitory synapses (see for example Pillet and Alger 1992; Wilson and Nicoll 2001). In both cases, the contribution of different presynaptic interneuron types to measured activity is unclear. Therefore, it could be that such an approach favors the recruitment of synapses that are insensitive to direct cannabinoid effects. In this respect, studies using paired recordings from interneurons and pyramidal neurons allow a better insight into direct cannabinoid modulation. Most studies using paired recordings to study cannabinoid modulation have focused on CCK-positive interneuron to pyramidal neuron synapses in the hippocampus (see for example Földy et al. 2006; Neu et al. 2007). These interneurons are well known to express large quantities of CB$_1$Rs on their presynaptic axon terminals (Katona et al. 1999), making them very sensitive to CB$_1$R mediated inhibition of presynaptic release. We found that these synapses are insensitive to direct GABA$_A$R modulation by cannabinoids, which explains why this phenomenon was not observed in earlier studies. In contrast, we observed direct CB$_1$R-independent modulation of synaptic transmission at neocortical FS-interneuron to pyramidal neuron synapses. These synapses do not express CB$_1$Rs (Bodor et al. 2005). However, in earlier studies on FS-interneuron to pyramidal neuron synapses CB$_1$R-independent cannabinoid modulation was not observed (Glickfeld and Scanziani 2006; Galarreta et al. 2008). In these two studies the effect of the synthetic cannabinoid WIN55,212-2 was tested, and no synaptic suppression was seen. However, it could be that the concentration of WIN55,212-2 used in these two studies (1 μM) was not high enough to affect GABA$_A$Rs. Indeed, we have found that a higher concentration of WIN55,212-2 (5 μM) does affect FS-interneuron to pyramidal neuron synapses. Also effects of endogenously released cannabinoids were not observed earlier. A possible explanation for this discrepancy might be the relatively short duration of direct modulation by endogenously released cannabinoids (only lasting several seconds,
compared with suppression for tens of seconds by classical CB$_1$R-dependent DSI). This could make direct effects mediated by endogenously released cannabinoids easy to overlook. Thus, the synapse specificity and the short time domain of direct endocannabinoid effects could be key in explaining the discrepancy with earlier studies.

Our findings show that postsynaptic depolarization can lead to endocannabinoid-mediated IPSC suppression at FS–PYR synapses in layer 2/3 of somatosensory cortex in the presence of a CB$_1$R antagonist and in CB$_3$R$^{-/-}$ mice. This process seems to be mediated by 2-AG, as treatment with the DAG lipase inhibitors THL or RHC80267 significantly reduced IPSC suppression. The 2-AG is responsible for most forms of endocannabinoid-dependent short- and long-term plasticity in the brain. In agreement, endocannabinoid-mediated synaptic plasticity such as DSI is no longer observed in DAG lipase knockout animals (Gao et al. 2010; Tanimura et al. 2010) However, use of the inhibitors of DAG lipase to block 2-AG synthesis has led to ambivalent results. Many forms of endocannabinoid-mediated long-term depression and associative short-term plasticity are readily suppressed by such inhibitors (Chevaleyre and Castillo 2003; Melis, Perra et al. 2004). However, the hippocampal DSI in some studies was reported to be insensitive (Chevaleyre and Castillo 2003; Min et al. 2010), while in others it was shown to be sensitive to DAG lipase inhibitors (Zhang et al. 2011; Hashimotodani et al. 2013).

Despite this controversy, the fact that DAG lipase inhibitors readily reduce direct CB$_1$R-independent modulation of FS–Pyr synapses in neocortex clearly suggests that 2-AG is involved in this process. Accordingly, the MAGL inhibitor URB602, which has previously been shown to prolong the duration of conventional hippocampal DSI (Makara et al. 2005) and nimesulide, an inhibitor of the endocannabinoid metabolizing enzyme COX-2, both prolong the duration of CB$_1$R-independent IPSC suppression. Both MAGL and COX-2 are involved in the breakdown of 2-AG, confirming that 2-AG is responsible for CB$_1$R-independent depolarization-induced IPSC suppression. Interestingly, while MAGL seems to be preferentially located in presynaptic axon terminals (Gulyas et al. 2004), COX-2 is localized mostly postsynaptically (Kaufmann et al. 1996; Sang and Chen 2006). This would place COX-2 in a better position to degrade 2-AG close to postsynaptic GABA$_A$Rs. However, our results suggest that both these enzymes are important for the termination of CB$_1$R-independent IPSC inhibition by 2-AG.

CB$_1$R-dependent and -independent suppression of inhibition have distinct implications for synaptic functioning. The CB$_1$R signaling pathway powerfully inhibits neurotransmitter release from CB$_1$R containing interneuron terminals, thereby affecting the reliability of synaptic inhibition. In contrast, CB$_1$R-independent postsynaptic GABA$_A$R modulation by endocannabinoids affects synaptic gain without changing the reliability of synaptic input. These two modes of endocannabinoid action may have dramatically different effects on inhibitory synaptic transmission. The difference is particularly prominent during high-frequency presynaptic stimulation. In CCK-positive interneuron to pyramidal neuron synapses, increased presynaptic firing (>20 Hz) can overcome CB$_1$R-dependent DSI due to relief from G-protein inhibition of voltage-gated Ca$^{2+}$ channels (Losonczy et al. 2004; Földy et al. 2006). Thus, CB$_1$R-dependent DSI affects only low frequency input, leaving high-frequency signals unaffected (i.e., it operates as a high-pass frequency filter). By contrast, CB$_1$-dependent suppression of inhibition still remains potent and even increases at high frequencies (Fig. 4, i.e., operates at broad band of frequencies). Given the synapse-specificity observed in this study, one can speculate that the mode of synaptic filtering of inhibitory synaptic transmission is specific for particular synapse and is determined by expression or the absence of presynaptic CB$_1$Rs.

PV-positive FS interneurons represent one of the two major types of perisomatic inhibitory interneurons in neocortex. Perisomatic inhibition provided by FS interneurons is considered critical for the emergence and control of synchronous population activity (Cobb et al. 1995). Recently it has been found that in mice with spontaneous seizures in a model of temporal lobe epilepsy, perisomatic pyramidal cell innervation from CCK-expressing basket cells and CB$_1$R-immunoreactive boutons is selectively reduced, while perisomatic innervation from PV FS interneurons persists (Wyeth et al. 2010). Preserved perisomatic inhibition provided by PV containing FS cells, therefore, may contribute to pathological activity in some intractable epilepsies and thus could represent a future therapeutic target. Intrinsic spike-generating properties of FS neurons ensure rapid and precise hypersynchronization associated with epilepsy. Cannabinoids are known as protective against neurodegeneration and brain damage and exhibit anticonvulsant activity (Irving et al. 2002). Given that in conditions of high-frequency bursting activity (>20 Hz) the impact of CB$_1$R-dependent inhibition is greatly reduced (Földy et al. 2006) it is possible that the CB$_1$-R-independent modulation of GABA$_A$Rs accounts for the anticonvulsant activity of cannabinoids.

In conclusion, we describe a novel CB$_1$R-independent mechanism by which both exogenously applied as well as endogenously released cannabinoids can control GABAergic inhibition. Given the ubiquitous nature of endocannabinoid signaling in the brain, this mechanism could have important implications for synaptic transmission, plasticity, and microcircuit processing in cortical and subcortical regions under normal and pathological conditions. Collectively, these findings provide a new dimension in cannabinoid signaling in the brain, and suggest a novel mechanism for synapse-specific, tonic, and activity-dependent regulation of synaptic inhibition. In addition, our findings underline the importance of studying endocannabinoid signaling beyond actions on CB$_1$Rs.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

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References


