Cannabinoid receptor function in the medial prefrontal cortex

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Citation for published version (APA):
den Boon, F. S. (2013). Cannabinoid receptor function in the medial prefrontal cortex

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Chapter 4

Endocannabinoids produced upon action potential firing evoke a Cl⁻ current via type-2 cannabinoid receptors in the medial prefrontal cortex

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Submitted to Cerebral Cortex
Abstract

The presence of type-2 cannabinoid receptors (CB₂Rs) in neurons of the central nervous system (CNS) has long been controversial. We recently demonstrated their functional presence in layer II/III pyramidal neurons of the rat medial prefrontal cortex (mPFC), by using synthetic cannabinoids. In the present study, we show that the application of the endocannabinoids (eCBs) 2-arachidonoylglycerol (2-AG) and methanandamide (a stable analog of the eCB anandamide (AEA)) can activate CB₂Rs of mPFC layer II/III pyramidal neurons, which subsequently induces a Cl⁻ current. In addition, we show that action potential (AP) firing evoked by 20-Hz current injections results in an eCB-mediated opening of Cl⁻ channels via CB₂R activation. This AP-evoked synthesis of eCBs is dependent on the Ca²⁺ influx through N-type voltage-gated calcium currents. Our results indicate that 2-AG is the main eCB involved in this process. Finally, we demonstrate that under physiologically relevant intracellular Cl⁻ conditions, 20-Hz AP firing leads to a CB₂R-dependent reduction in neuronal excitability. Altogether, our data suggest that the eCB system plays a modulatory role in the mPFC in the prevention of excessive neuronal firing via the activation of CB₂Rs after an initial period of high activity.
Introduction

The endocannabinoid (eCB) system comprises at least two G protein coupled receptors (GPCRs), type-1 and type-2 cannabinoid receptors (CB1Rs and CB2Rs), various lipid endogenous cannabinoids (eCBs), eCB transporters and enzymes responsible for the synthesis and degradation of eCBs (Kano et al. 2009). The eCB system is mostly known for mediating the effects of cannabinoids from the marijuana plant (cannabis sativa). Cannabinoid receptors are activated by phytocannabinoids found in marijuana, synthetic cannabinoids and eCBs, such as 2-arachidonoylglycerol (2-AG) and anandamide (AEA). AEA was the first eCB to be isolated (Devane et al. 1992) and it is thought to be synthesized from its membrane precursor N-acylphosphatidylethanolamine (NAPE) by a specific phospholipase D (NAPE-PLD) and for the most part hydrolyzed by fatty acid amide hydrolase (FAAH). 2-AG, identified a few years later, is synthesized by diacylglycerol lipase α (DGL α) and β (DGL β) from DAG-containing phospholipids and is principally hydrolyzed by monoaclglycerol (MGL) (Mechoulam et al. 1995; Sugiura et al. 1995; Dinh et al. 2002, 2004). The eCB system is involved in many cognitive functions that are associated with the medial prefrontal cortex (mPFC) (Pattij et al. 2008). The expression pattern and functional role of CB1Rs as presynaptic receptors modulating neurotransmitter release in the central nervous system (CNS) are well described (Herkenham et al. 1991; Wilson and Nicoll 2001; Castillo et al. 2012; Katona and Freund 2012). However, there is relatively little knowledge about the functionality of CB2Rs in the CNS. Initially, CB2Rs were not detected in the brain, but their presence in microglia cells and other brain immune cells is now generally accepted (Stella 2010). We have recently shown, using a combination of biochemical and electrophysiological techniques, that CB2Rs are present intracellularly in layer II/III pyramidal neurons of the rat mPFC (Den Boon et al. 2012). Furthermore, we demonstrated that their activation with synthetic, selective agonists leads to an IP3R-dependent opening of Ca2+-activated Cl⁻ channels (CaCCs) located in the plasma membrane. Under physiologically relevant intracellular Cl⁻ conditions, the opening of Cl⁻ channels will stabilize the membrane potential around resting membrane potential, thereby reducing neuronal excitability (Frings et al. 2000; Hartzell et al. 2005). Although we have shown that CB2Rs are involved in the modulation of mPFC neuronal excitability, presumably through the opening of CaCCs, these findings were obtained with synthetic cannabinoids (Den Boon et al. 2012). The questions how and when CB2Rs are activated by endogenously released eCBs in mPFC layer II/III neurons remain to be answered. The downstream signalling pathway following CB2R activation suggests a modulatory role of neuronal excitability for CB2Rs. We hypothesize that in response to increased AP firing, mPFC layer II/III neurons synthesize and release eCBs to prevent excessive neuronal activity via a CB2R-mediated feedback system.

Endogenous ligands for cannabinoid receptors are synthesized ‘upon demand’ from membrane phospholipids following an increase in [Ca2+]i, and/or activation of GPCRs such as metabotropic glutamate receptors or M1/M3 muscarinic acetylcholine receptors (Kano et al. 2009). Aside from the ‘on demand’ pool, eCBs are believed to be present in a ‘basal pool’, being constantly produced, accumulated and released (Maccarrone et al. 2010; Alger and Kim 2011; Di Marzo 2011). It is known that adequate
stimulation (10-50 Hz) can lead to eCB synthesis and subsequent CB2R-mediated plasticity (Brown et al. 2003; Lafourcade et al. 2007). Furthermore, in the mouse PFC, repetitive AP firing at an intermediate frequency (20 Hz) was also reported to evoke eCB synthesis and resulted in 2-AG-dependent CB2R-mediated depression of synaptic transmission (Yoshino et al. 2011). Other studies have also demonstrated CB2R-mediated effects in cortical areas following brief trains of APs (Fortin et al. 2004; Lemtiri-Chlieh and Levine 2007). We propose that stimulation protocols that are able to induce synthesis and release of eCBs which induce CB2R-mediated effects, could also lead to CB2R-mediated effects.

To assess the physiological role of CB2Rs, we proceeded to investigate the eCB-mediated activation of CB2Rs and the subsequent opening of CaCCs, which in turn will modulate neuronal excitability. We demonstrate how the exogenous application of 2-AG and mAEA concentration-dependently evokes a Cl− current in layer II/III pyramidal neurons of the rat mPFC. Furthermore, we reveal the activation of CB2Rs by eCBs following 20-Hz suprathreshold current injections, mediated – at least for the most part – by 2-AG. Finally, we show that 20-Hz AP firing can reduce neuronal excitability through the activation of CB2Rs.

Materials and Methods

Electrophysiology
Coronal slices (300 µm) of the mPFC were obtained from male Wistar rats (Harlan, the Netherlands) aged 14-19 days postnatal and male C57BL/6 Wt mice or male C57BL/6 CB2R KO mice (The Jackson Laboratory, USA) aged 14-19 days postnatal. Animals were killed by decapitation, their brains rapidly removed and placed in oxygenated (95% O2 – 5% CO2) ice cold (4 °C) adapted artificial cerebrospinal fluid (aACSF, containing in mM: 120 choline chloride, 3.5 KCl, 0.5 CaCl2, 6 MgSO4, 1.25 NaH2PO4, 25 D-glucose, 25 NaHCO3). Slices were cut in aACSF on a vibratome (VT1200S, Leica, Germany) and placed for 30 min in ACSF (containing in mM: 120 NaCl, 3.5 KCl, 25 NaHCO3, 25 D-glucose, 2.5 CaCl2, 1.3 MgSO4, 1.25 NaH2PO4; [Cl−]out = 128.5 mM) at 32 °C. Slices were kept at room temperature for at least 1 h prior to recording. Glass recording pipettes were pulled from borosilicate glass (Science Products, Germany) and had a resistance of 2-3 MΩ when filled with pipette solution (containing in mM: 110 KGluconate, 30 KCl, 0.5 EGTA, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 Mg-ATP, 0.5 Na-GTP). Modified ACSF (mACSF) with K+-, Ca2+-, and Na+-channel blockers contained (in mM): 98 NaCl, 3.5 KCl, 25 NaHCO3, 25 D-glucose, 2.5 CaCl2, 1.3 MgSO4, 1.25 NaH2PO4, 25 TEACl, 5 4-AP, 0.2 CdCl2, 0.0005 TTX; [Cl−]out = 131.7 mM. Modified pipette solution with high [Cl−]i (30 mM) contained (in mM): 110 CH3O3SCs, 30 CsCl, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, 0.5 Na-GTP). Modified pipette solution with low [Cl−]i (8.75 mM) contained (in mM): 131.25 KGluconate, 8.75 KCl, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, 0.5 Na-GTP. For recordings of Ca2+ currents carried by voltage-dependent Ca2+ channels (VDCCs), a VDCC ACSF was used, containing in mM: 105 NaCl, 3.5 KCl, 2.5 CaCl2, 1.3 MgSO4, 1.25 NaH2PO4, 25 D-glucose, 25 NaHCO3, 10 TEA-Cl, 5 4-AP, 0.005 TTX. A VDCC pipette solution was used for these experiments, containing in mM: 135
CsMeSO₄, 10 1,2-Bis(2-Aminophenoxy)ethane-\(N,N',N',N''\)-tetraacetic acid, 10 Hepes, 4 MgATP, 0.4 NaGTP. Whole-cell current and voltage clamp recordings were made at 32 °C from the soma of layer II/III pyramidal neurons. Currents were evoked by 200 ms voltage steps (2 s interval) from a hyperpolarizing prepulse potential (400 ms, -120 mV) to test pulses potentials ranging from -90 mV to 60 mV. Sustained current amplitudes were measured as the average current during the last 30 ms of the current. To allow the detection of CB₂R-mediated currents, currents were evoked in the voltage clamp configuration every 15 s by series of rectangular voltage steps (200 ms-duration) from a holding potential of -80 mV to voltage potentials ranging from -90 or -105 mV to -20 or -55 mV in 10 mV increments (Fig. 1A). To estimate CB₂R-mediated currents, currents evoked by rectangular voltage steps before and after bath application of cannabinoids or a stimulation protocol (in the current clamp configuration) that evoked APs (Fig. 2A), were compared. Due to difficulties with washing out bath-applied cannabinoid ligands, every cell was treated with one concentration. Concentration-response curves for cannabinoid ligands were fitted with the following logistic equation:

\[
\text{Current density} = \frac{\text{Maximal current density}}{1 + \frac{\text{EC}_{50}}{[\text{Ligand}]}}
\]  

(eq.1)

Under current clamp conditions, a stimulation protocol was used to evoke APs with 60 suprathreshold current injections (1 nA, 5 ms duration, 45 ms interval) into the soma via the patch pipette (Fig 2A). For experiments with synaptically evoked APs and/or EPSPs, currents ranging from 300-600 μA (5 ms) were injected (at 20 Hz) via a separate stimulation electrode that was placed in layer I (Fig. 2D inset).

Experiments determining eCB-mediated effects on cell excitability were done in the whole-cell current clamp configuration. We used a slow feedback system that guaranteed that current clamp recordings started at a defined membrane voltage of -80 mV (Fig. 3) or -70 mV (Fig. 4). Frozen filtered Gaussian noise (time constant = 10 ms) was injected via the patch pipette with a variance adjusted for each neuron to result in a mean spike frequency of ~0.95 Hz. Firing frequency was calculated using 1-min bins. Prior to the stimulation protocol, 5-min recordings were used as control. Maximal effects of 20-Hz AP firing were observed within 2-6 min after the stimulation protocol was applied. Recordings were made using an Axopatch 200b (Axon, USA) and in-house software running under Matlab (MathWorks, USA). Signals were filtered at 2.9 kHz and sampled at 10 kHz. Series resistance ranged from 5–15 MΩ and was compensated to ~65%. Signals were corrected for liquid junction potential. Current densities were calculated using cell capacitance and expressed in pA/pF.

**Data analysis**

Data were compared with t-tests. When data was not normally distributed a Mann-Whitney test was used. In the figures the significance is indicated with asterisks.
Endocannabinoids produced upon action potential firing evoke a Cl⁻ current via CB₂Rs

(*P<0.05, ** P<0.01 and *** P<0.001) and the number of observations are given between brackets (Figs. 1 and 2) or in the bar charts (Figs. 3 and 4). Results are given as mean ± standard error of the mean.

Drugs
mAEA, 2-AG, RHC-80267 and ω-conotoxin were purchased from Tocris (the Netherlands). Sch.356036 and JWH-133 were generous gifts from Abbott Healthcare Laboratories (the Netherlands). 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS) and nefidipine were purchased from Sigma-Aldrich (the Netherlands), TTX was purchased from Latoxan (France). URB602 and URB597 were purchased from Cayman Chemical (USA). Cannabinoid receptor ligands were dissolved in DMSO or ethanol to 50 mM and diluted in ACSF with a final concentration of DMSO or ethanol that was always lower than 0.1%. Some cells were filled with biocytin (Sigma, 1 mg/ml dissolved in pipette solution) during whole-cell patch recordings. Biocytin was visualised using previously described methods (Chameau et al. 2009).

Results

2-AG and mAEA induce a Cl⁻ current
To investigate whether eCBs can induce the previously described Ca²⁺-activated Cl⁻ current (Den Boon et al. 2012), we first performed whole-cell voltage clamp recordings of layer II/III pyramidal neurons. These experiments were done in the presence of K⁺, Ca²⁺ and Na⁺ channel blockers (mACSF) and with a pipette solution containing a high Cl⁻ concentration and no K⁺ (modified pipette solution with 30 mM Cl⁻). Using a series of step potentials before and during the bath application of 2-AG (25 out of 35 cells responded) or mAEA (24 out of 33 cells responded) allowed the construction of a current-voltage relationship that reversed (E_{rev}) close to the calculated reversal potential for Cl⁻ (E_{Cl⁻}) (Fig. 1B). Both 2-AG (0.1-50 µM) and mAEA (1-30 µM) concentration-dependently evoked a Cl⁻ current. The current density values determined at -90 mV were plotted as a function of ligand concentration and fitted with a logistic equation (eq. 1), yielding EC₅₀ values of 1.2±1.0 µM and 4.9±1.4 µM for mAEA and 2-AG, respectively (Fig. 1C). The maximal current density recorded at -90 mV for 2-AG exceeded that for mAEA (Fig. 1B,C), which demonstrates that 2-AG is a more efficacious agonist than mAEA. However, the estimated EC₅₀ values indicate that mAEA is a slightly more potent agonist than 2-AG (Fig. 1C). When 2-AG (30 µM) and mAEA (10 µM) were applied simultaneously, the maximal current density was not different from that evoked by mAEA (10 µM) alone, which suggests that both ligands compete for the same binding site (Fig. 1B). The selective CB₂R agonist JWH-133 (1 µM) induced a Cl⁻ current that was comparable to that elicited by 2-AG, suggesting that the current
Figure 1. 2-AG and mAEA induce a Cl⁻ current in layer II/III mPFC pyramidal neurons. (A) Representative traces of voltage steps (A1), evoking currents during baseline (A2), and currents including Cl⁻ currents during cannabinoid-ligand application (A3); the isolated Cl⁻ currents (A4) are obtained by subtracting the traces of A2 from the traces of A3. (B) I/V relationships of 2-AG- and mAEA-induced Cl⁻ currents at their maximal effective concentrations (30 μM and 10 μM, respectively). E<sub>rev</sub> for 2-AG (-38.7±1 mV) and mAEA (-42.2±2.4 mV) is close to E<sub>Cl⁻</sub> (-38.9 mV). When 2-AG (30 μM) and mAEA (10 μM) were applied simultaneously, current densities were not different than for mAEA (10 μM) alone (E<sub>rev</sub> = -40.4±1.2 mV). Application of the CB<sub>2</sub>R-selective agonist JWH-133 (1 μM) induced a current (E<sub>rev</sub> = -41.1±2.1 mV) that was comparable to the current induced by 2-AG (30 μM). (C) Concentration-response curves for 2-AG and mAEA, constructed from the current densities recorded at -90 mV (4-10 cells per concentration). The data points were fitted with eq. 1. The estimated maximal current density at -90 mV was larger for 2-AG (3.1±0.3 pA/pF, 30 μM) than for mAEA (1.3±0.3 pA/pF, 10 μM, p < 0.01). The estimated EC<sub>50</sub> value for mAEA (1.2±1 μM) was smaller than that for 2-AG (4.9±1.4 μM, p < 0.01).
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Together, these experiments show that 2-AG and mAEA can induce a Cl\(^-\) current, presumably via the activation of CB\(_2\)Rs, and that 2-AG is a more efficacious, yet slightly less potent agonist for the CB\(_2\)R.

**AP firing (20 Hz) results in a CB\(_2\)R-mediated Cl\(^-\) current**

We hypothesized that a stimulation protocol that induces eCB synthesis and CB\(_1\)R activation, may also lead to CB\(_2\)R activation. We tested whether 60 APs fired at 20 Hz (Fig. 2A) could induce the release of sufficient eCBs to activate CB\(_2\)Rs. In the voltage clamp configuration, we recorded currents evoked by 60 APs firing at 20 Hz. We found that this protocol evoked a current in 10 out of 14 tested neurons and that this reversed close to E\(_{Cl^-}\) (Fig. 2B). The stimulation protocol did not evoke a current when neurons were preincubated with the CB\(_2\)R antagonist Sch.356036, which indicates that the current is mediated by CB\(_2\)Rs (Fig. 2B). To exclude the involvement of CB\(_1\)Rs, we repeated the experiment in the presence of the selective CB\(_1\)R antagonist rimonabant (5 µM) and detected a current that was comparable to the one recorded in the absence of rimonabant (Fig. 2B). Decreasing [Cl\(^-\)]\(_i\) to 8.75 mM shifted the reversal potential of the current (E\(_{rev}\)) toward the newly established E\(_{Cl^-}\) (-70 mV, data not shown). This experiment confirmed the identity of the CB\(_2\)R-mediated current as a Cl\(^-\) current.

Since the synthesis of eCBs is sensitive to the influx of Ca\(^{2+}\) (Di Marzo 2011), we next examined the possible involvement of N-type and L-type voltage-dependent Ca\(^{2+}\) channels (VDCCs). VDCC-mediated currents were recorded in the absence and presence of \(\omega\)-conotoxin (0.25 µM) or nifedipine (10 µM) to block N-type or L-type VDCCs, respectively. Ca\(^{2+}\) current densities were clearly reduced in the presence of either blocker, which shows the presence of these two channels in layer II/III pyramidal neurons (Fig. 1C inset). In the presence of the N-type VDCC blocker \(\omega\)-conotoxin (0.25 µM), but not the L-type VDCC blocker nifedipine (10 µM), 60 current injections at 20 Hz resulted in a markedly reduced Cl\(^-\) current (Fig. 2C). This indicates that Ca\(^{2+}\) influx through N-type VDCCs following AP spiking at 20 Hz contributes to the synthesis of eCBs that mediate CB\(_2\)R activation. To investigate whether the CB\(_2\)R-mediated current could be evoked under more physiological conditions, we next applied synaptic stimulation to evoke APs, instead of current injections into the soma. These experiments were performed with normal ACSF and with a pipette solution containing a physiologically relevant Cl\(^-\) concentration ([Cl\(^-\)]\(_i\) = 8.75 mM). With electrical synaptic stimulation in layer I, excitatory postsynaptic potentials (EPSPs) large enough to induce APs could be generated in layer II/III neurons. Following 60 synaptically evoked APs at 20 Hz, we recorded a current that reversed close to E\(_{Cl^-}\) (Fig. 2D). This current could not be induced by the synaptic stimulation protocol if only EPSPs that did not generate APs were evoked (Fig. 2D). If APs were evoked, but recordings were performed after preincubation (of at least 10 minutes) with and in the continuous presence of the CB\(_2\)R antagonist Sch.356036 (5 µM), we did not detect the current (Fig. 2D). These results show that the synthesis of eCBs resulting in CB\(_2\)R activation (to open CaCCs) depends on
Figure 2. 60 APs at 20 Hz result in a CB2R-mediated Cl⁻ current in 71% of layer II/III mPFC neurons. (A) Cropped traces of 60 evoked APs (A1) and injected currents (A2) at 20 Hz. (B) I/V relationships showing that AP firing evoked with 60 current injections at 20 Hz induced a CB2R-mediated Cl⁻ current, \( E_{\text{rev}} = -42.4 \pm 2.3 \text{ mV} \) (\( E_{\text{Cl}^-} = -38.3 \text{ mV} \)). Such current injections could not evoke a current after preincubation with and in the continuous presence of the CB2R antagonist Sch. 356036 (5 µM). After preincubation with and in the continuous presence of the CB1R antagonist rimonabant (5 µM), a similar current, \( E_{\text{rev}} = -38.7 \pm 4.9 \text{ mV} \), could be detected following 60 current injections at 20 Hz. (C) The N-type VDCC blocker \( \omega \)-conotoxin, but not the L-type blocker nifedipine, markedly reduced the amplitude of CB2R-mediated Cl⁻ currents evoked with 60 current injections at 20 Hz. \( E_{\text{rev}} \) for currents recorded with \( \omega \)-conotoxin (-41.4±2.3 mV) and with nifedipine (-42.9±1.4 mV) are close to \( E_{\text{Cl}^-} \) (-38.3 mV). The current in the absence of VDCC blockers is the same as the control current depicted in Fig. 2B (closed circles). Inset shows Ca²⁺ currents recorded in the absence and presence of \( \omega \)-conotoxin (0.25 µM) and nifedipine (10 µM). VDCC-mediated current densities were clearly reduced in the presence of either blocker, showing the presence of both N- and L-type VDCCs in these neurons. (D) I/V relationship of CB2R-mediated current following 60 synaptically evoked APs at 20 Hz reversed at -71.7±1.2 mV, which is close to \( E_{\text{Cl}^-} \) (-70 mV). The Cl⁻ current could not be observed if no APs were evoked or after the preincubation with and in the continuous presence of Sch. 356036 (5 µM). Inset shows a biocytin-filled layer II/III neuron and the schematic representation of a stimulation electrode in layer I and a recording electrode for registering APs or EPSPs in layer II/III neurons.
AP firing and that the subsequent Ca\(^{2+}\) influx through N-type VDCCs is a major contributor to the response.

**2-AG is the main eCB involved in CB\(_2\)R-mediated effects following 60 APs at 20 Hz**

We next performed experiments in the current clamp configuration to elucidate whether 2-AG and/or AEA are involved in the activation of CB\(_2\)Rs following the firing of 60 APs at 20 Hz. In the current clamp configuration, at an experimental membrane potential of -80 mV and with E\(_{Cl^-}\) = -38.3 mV, opening of CaCCs resulted in a transient depolarization of the neurons (Fig. 3A). These experimental conditions allow the determination of the delay, duration and amplitude of the depolarization of CB\(_2\)R-mediated responses, similar to what we observed with a synthetic CB\(_2\)R ligand (Den Boon et al. 2012). The transient depolarization of the membrane, evoked by the 20-Hz current injection protocol, was absent in neurons that were preincubated (for at least 10 min) with and in the continuous presence of the selective CB\(_2\)R antagonist Sch.356036 (5 µM, Fig. 3B). To corroborate the involvement of Cl\(^-\) channels, we performed a similar experiment in the presence of the Cl\(^-\) channel blocker DIDS (0.2 mM) and observed a reduced depolarization of the membrane potential (Fig. 3B).

Finally, we confirmed the pharmacological evidence that the depolarization is mediated via CB\(_2\)R activation by using brain slices from CB\(_2\)R knock-out (KO) and wild type (Wt) mice. Following 60 suprathreshold current injections at 20 Hz we observed a depolarization of Wt layer II/III mPFC neurons, but not of KO neurons (Fig. 3B).

In order to differentiate between 2-AG and AEA, we blocked eCB hydrolysis by their most important catabolic enzymes, MGL and FAAH, respectively. We used URB602 to inhibit MGL activity and URB597 to inhibit FAAH activity (Fegley et al. 2005; Hohmann et al. 2005; King et al. 2007; Chávez et al. 2010). In the presence of either the MGL inhibitor URB602 (100 µM) or the FAAH inhibitor URB597 (1 µM), 60 APs at 20 Hz resulted in depolarizations that were not different in amplitude from control recordings (Fig. 3C). However, when the MGL inhibitor URB602 was present, the duration of the depolarization was larger than in control conditions, whereas the FAAH inhibitor URB597 did not affect the duration of the response (Fig. 3D). This suggests that 2-AG, but not AEA, mediates CB\(_2\)R activation. In control conditions, the depolarization occurred with a delay of ~150 seconds after 60 current injections at 20 Hz. Delays were not different when neurons were tested in the presence of either URB602 or URB597 (Fig. 3E). We further implicated 2-AG as the main eCB involved in the CB\(_2\)R-mediated depolarization by showing a decreased depolarization in the presence of the diacylglycerol lipase inhibitor RHC-80267 (100 µM), which is known to disrupt 2-AG synthesis (Puente et al. 2011) (Fig. 3C). Taken together, these data indicate that 2-AG is the main eCB involved in CB\(_2\)R activation following 60 APs at 20 Hz.
Figure 3. Stimulation with 60 AP-evoking current injections at 20 Hz induces eCB synthesis and subsequent CB2R activation in layer II/III mPFC pyramidal neurons. (A) Representative trace recorded from a neuron that depolarizes following 60 current injections at 20 Hz (arrow); the depolarization generated AP firing. (B) In current clamp recordings with ECl = -38.3 mV, 60 current injections at 20 Hz resulted in a depolarization (24.3±2.2 mV) which was reduced after preincubation with and in the continuous presence of the CB2R antagonist Sch. 356036 (5 µM) (2.2±0.8 mV, P < 0.001) and in the presence of the Cl⁻ channel blocker DIDS (10.8±1.3 mV, P < 0.001). The same stimulation protocol depolarized layer II/III mPFC neurons of Wt mice, but not of CB2R KO mice (16.8±2.4 mV and 1±1.1 mV, respectively, P < 0.001). (C) The magnitude of the depolarization following 60 current injections at 20 Hz was not different in the presence of the MGL inhibitor URB602 (17.7±2.4 mV) or the FAAH inhibitor URB597 (24.6±2.6 mV). Incubation with the DGL inhibitor
CB$_2$R activation following 60 APs at 20 Hz decreases neuronal excitability

We have shown that activation of CB$_2$Rs with the synthetic agonist JWH-133 reduces neuronal excitability under physiologically relevant intracellular Cl$^-$ conditions ($E_{Cl} = -70$ mV) (Den Boon et al. 2012), most likely through the opening of CaCCs. We next tested whether endogenous activation of CB$_2$Rs has a similar effect in a series of current clamp experiments. Neuronal firing was evoked by Gaussian current injection, which led to fluctuations around resting membrane potential, resulting in the occasional firing of APs. For each neuron, the variance of the injected current was adjusted to cause a stable firing rate of ~0.95 Hz. Two to six minutes after stimulation with 60 APs at 20 Hz the normalized firing rate was transiently reduced by ~30%. This reduction could be prevented by preincubation (of at least 10 min) with and in the continuous presence of the CB$_2$R antagonist Sch. 356036 (5 µM) (Fig. 4). These results indicate that, when AP firing is evoked with an input that could resemble spontaneous background synaptic activity, CB$_2$R activation following 60 APs at 20 Hz modulates the regular firing rate of mPFC neurons, presumably through the opening of CaCCs.

Discussion

We have previously demonstrated that the activation of intracellular CB$_2$Rs by synthetic cannabinoid ligands in layer II/III pyramidal neurons of the mPFC leads to the IP$_3$R-dependent opening of CaCCs (Den Boon et al. 2012). Here, we show that bath application of 2-AG and mAEA (the stable analog of AEA) can, concentration-dependently, induce a similar Cl$^-$ current. Both compounds evoked a comparable Cl$^-$ current as was evoked by the selective CB$_2$R agonist JWH-133, suggesting that 2-AG and mAEA activate CB$_2$Rs. The estimated EC$_{50}$ values indicate that 2-AG, although more efficacious, has a slightly lower affinity for CB$_2$Rs than mAEA. Future studies on the binding of eCBs at the CB$_2$R in the rodent mPFC could show how 2-AG and AEA behave at their binding sites. When both agonists are bath applied simultaneously at their respective maximal effective concentrations, the resulting current density does not differ from experiments in which mAEA was applied alone. These data suggest that 2-AG and mAEA compete for the same CB$_2$R binding site and that they are able to activate a common downstream signalling cascade. This is in line with earlier findings that AEA can attenuate the agonistic activity of 2-AG (Gonsiorek et al. 2000; Sugiura 2009). 2-AG and AEA are considered as the two major eCBs and they have been shown to be involved in several neuronal processes via abundantly expressed CB$_2$Rs.
Figure 4. Endogenous CB2R activation decreases firing activity of rat mPFC layer II/III pyramidal neurons. (A) Stimulation with 60 current injections at 20 Hz led to a reduction of the neuronal firing rate. Firing was induced by a Gaussian current input into the soma. Mean baseline firing frequency of 0.95±0.09 Hz was normalized over slices to 100±3% and reduced to 72±6% 2-6 minutes after 60 current injections at 20 Hz (P < 0.01). After preincubation with and in the continuous presence of the CB2R antagonist Sch. 356036 (5 µM) (baseline firing frequency 0.93±0.08 Hz, normalized to 100±2%), 60 current injections at 20 Hz could not induce a response (99±9%). (B) Representative traces of current clamp recordings showing AP firing of layer II/III neurons before and after 60 current injections at 20 Hz, in the absence and presence of Sch. 356036 (5 µM).

(Terranova et al. 1995; Stella et al. 1997; Wilson and Nicoll 2001; Lafourcade et al. 2007; Kano et al. 2009). Despite difficulties with the precise quantification of brain eCB levels, it is clear that 2-AG levels exceed those for AEA (Stella et al. 1997; Buczynski and Parsons 2010). Since the discovery of 2-AG and AEA and the development of selective pharmacological tools and transgenic mice strains, the roles of 2-AG and AEA have been increasingly disentangled (Luchicchi and Pistis 2012). So far, collected data suggests that 2-AG is the principal eCB that mediates synaptic plasticity, with a smaller role for AEA (Kano et al. 2009; Kim and Alger 2010). In the PFC specifically, 2-AG, but not AEA, was reported to mediate long term depression (LTD) and depolarization-induced suppression of inhibition (DSI) (Lafourcade et al. 2007; Yoshino et al. 2011). However, AEA has also been shown to be involved in some forms of synaptic plasticity in the rodent PFC (Lourenço et al. 2011). In this study, we confirm earlier findings that 2-AG is a full agonist at the CB2R, whereas mAEA behaves as a partial agonist (Gonsiorek et al. ...)
Endocannabinoids produced upon action potential firing evoke a Cl⁻ current via CB₂Rs

2000; Sugiura 2009). This means that CB₂R-mediated effects depend on the local concentrations of both 2-AG and AEA.

The synthesis and release of eCBs that lead to CB₁R-mediated effects are described to follow a wide range of stimulation protocols, such as voltage steps and AP firing (Llano et al. 1991; Brown et al. 2003; Lafourcade et al. 2007). In particular, eCBs can be synthesized upon Ca²⁺-entry through VDCCs after repetitive firing of the postsynaptic cell and then travel to presynaptic CB₁Rs. Alternatively, activation of GPCRs coupled to Gq/11, such as group I mGluRs and M₁/M₃ muscarinic receptors, can enhance or by itself lead to eCB production (Kano et al. 2009). So far, research has focused on CB₁R-mediated effects following eCB synthesis. We hypothesized that similar protocols that lead to CB₂R activation may also result in CB₂R activation. We tested this by administering 60 AP-evoking current injections at 20 Hz that were described to lead to CB₁R-mediated effects in the mouse PFC (Yoshino et al. 2011). Using this stimulation protocol, we demonstrate that CB₂Rs are activated and that this results in the opening of CaCCs. To demonstrate that CB₂Rs mediated this effect, we used the selective CB₂R antagonist Sch.356036 to block the Cl⁻ current following 60 APs at 20 Hz (Shankar et al. 2005; Lunn et al. 2008). Importantly, we excluded the involvement of CB₁Rs by performing similar experiments in which we observed the Cl⁻ current in the presence of the selective CB₁R antagonist rimonabant. Both the Erev of the CB₂R-mediated current and the reduction in the membrane depolarization in the presence of the Cl⁻ channel blocker DIDS (Akasu et al. 1990; Hogg et al. 1994) identified this current as a Cl⁻ current. In the present study, we did not find evidence for the modulation of other ion channels following CB₂R activation. This is in contrast with a recent study which reported a CB₂R-mediated reduction of neurotransmission in cultured autaptic hippocampal neurons of CB₁R null mice (Atwood, Straiker, et al. 2012). The authors determined that the CB₂R exerted its function from a presynaptic localization, presumably through the inhibition of VDCCs. It is possible that this discrepancy can be explained by differences in preparation, brain region and species.

Here, we show that blocking N-type VDCCs reduced the amplitude of the Cl⁻ current elicited with our stimulation protocol (repetitive neuronal firing), whereas blocking L-type VDCCs did not. This indicates that Ca²⁺ influx through N-type VDCCs following 20-Hz AP firing is sufficient to induce the synthesis of eCBs. Blocking 2-AG synthesis reduced the amplitude of the CB₂R-mediated effect and inhibiting its degradation prolonged it. This finding indicates that due to a reduction in the degradation of 2-AG, local 2-AG levels are elevated for a longer period of time, which results in a longer lasting depolarization of the membrane potential. In addition, this points to a CB₂R-evoked effect that is most likely mediated by 2-AG. Interfering with AEA degradation did not affect the response. However, a minor role for AEA cannot be excluded under these experimental conditions. Although the main route of AEA synthesis involves the conversion of NAPE into AEA, alternative pathways have been
described (Leung et al. 2008; Katona and Freund 2012; Luchicchi and Pistis 2012). A lack of specific pharmacological tools to inhibit AEA synthesis complicates the exclusion of AEA from playing a role in CB2Rs-mediated effects. In the striatum, both 2-AG and AEA were reported to induce eCB-mediated LTD through CB1Rs (Lerner and Kreitzer 2012). Interestingly, different stimulation protocols, high- and low-frequency stimulation, induced the synthesis of AEA and 2-AG, respectively. This is in line with the notion that 2-AG and AEA play specialized roles in the modulation of synaptic plasticity (Luchicchi and Pistis 2012).

In this study we demonstrate that CB2R activation following eCB release - under physiologically relevant intracellular Cl⁻ conditions - results in a decrease of neuronal firing rate, presumably via the opening of CaCCs. If E_cl is at or close to the resting membrane potential of neurons, the opening of CaCCs following activation of CB2Rs will stabilize or even clamp the membrane potential around that value. We observed a similar reduction in neuronal excitability when CB2Rs were activated with a selective, synthetic cannabinoid (Den Boon et al. 2012). Here we demonstrate that 20-Hz AP firing of layer II/III pyramidal neurons can, with a delay of 2-6 minutes, reduce neuronal excitability via CB2Rs. This suggests that in the mPFC, the eCB system plays a slow modulatory role via the activation of CB2Rs, possibly preventing excessive firing via a feedback mechanism after an initial brief period of high activity. We have previously shown that the application of the CB2R antagonist Sch. 356036 increased the regular firing rate in a similar experiment, which points to a basal pool of eCBs and/or constitutive activity of CB2Rs (Alger and Kim 2011; Di Marzo 2011; den Boon et al. 2012). The activity-induced self-inhibition via CB2Rs we demonstrate here is likely to depend on the on-demand pool. The timescale of the onset of this feedback mechanism (several minutes) is different from well described and generally much faster modulatory eCB-mediated processes via presynaptic CB1Rs (seconds), such as depolarization-induced suppression of excitation (DSE) and DSI. Both cannabinoid receptors may thus modulate neuronal activity, albeit through different mechanisms and on different time-scales. Future studies on the role of CB1Rs and CB2Rs in the cortex could shed more light on the precise role of each receptor and their interplay in cortical network functioning.

**Funding**
This work was supported by Dutch Top Institute Pharma (grant number T5-107-1). MM was partly supported by Fondazione Italiana Sclerosi Multipla (grant 2010), and by Ministero dell’Istruzione, dell’Università e della Ricerca (grant PRIN 2011-2012).