Cannabinoid receptor function in the medial prefrontal cortex

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Chapter 5

Modulation of the balance between excitation and inhibition in the rat medial prefrontal cortex by type-1 cannabinoid receptors

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Abstract

Although type 1 cannabinoid receptors (CB₁Rs) are abundantly expressed in many brain areas and well characterized, less is known about their role in the medial prefrontal cortex (mPFC). Using electrophysiological and immunohistochemical techniques, we demonstrate that functional CB₁Rs are located on both excitatory and inhibitory inputs to layer II/III pyramidal neurons and that their activation results in a reduction of ~30% of the release of both GABA and glutamate. More importantly, by decomposing the evoked synaptic response into its excitatory and inhibitory components, we show that CB₁R activation modulates the balance between excitation and inhibition (E/I balance) by causing a shift of this balance towards excitation, from ~20/80% to 25/75%. Finally, when animals were injected with a cannabinoid receptor agonist, we observed a E/I balance of 30/70%. The modulation of the E/I balance by presynaptic CB₁Rs may be fundamental in the regulation of local mPFC network excitability and consequently in the modulation of higher order cognitive functions fulfilled by the mPFC.
Introduction

Cannabinoid type-1 receptors (CB₁Rs) are among the most abundantly expressed G protein-coupled receptors (GPCRs) in the central nervous system (CNS) (Herkenham et al. 1991). Together with at least one other GPCR, type-2 cannabinoid receptors (CB₂Rs), they are part of the endocannabinoid (eCB) system. This system also contains lipid endogenous cannabinoids (eCBs), eCB transporters and enzymes responsible for the synthesis and degradation of eCBs (Kano et al. 2009; Fu et al. 2012). The expression and functionality of CB₁Rs in the CNS have been heavily investigated (Devane et al. 1988; Kano et al. 2009; Castillo et al. 2012; Katona and Freund 2012). The principal mechanism by which the eCB system is reported to play a functional role is through retrograde signalling (Kano et al. 2009). Briefly, postsynaptic activity, consisting of Ca²⁺ influx and/or activation of GPCRs such as group 1 metabotropic glutamate receptors, leads to the synthesis of eCBs in the plasma membrane. eCBs can travel backwards across the synapse and bind to presynaptic CB₁Rs. There, activation of presynaptic CB₁Rs suppresses neurotransmitter release by inhibition of Ca²⁺ influx through voltage-dependent Ca²⁺ channels and activation of G protein coupled inwardly rectifying K⁺ (GIRK) channels (Kreitzer and Regehr 2001; Wilson et al. 2001; Brown et al. 2003; Guo and Ikeda 2004). CB₁Rs are expressed in the PFC where their activation suppresses spontaneous IPSCs (sIPSCs), evoked EPSCs (eEPSCs) and which mediates synaptic plasticity of inhibitory synapses (Auclair et al. 2000; Yoshino et al. 2011). However, the expression pattern of CB₁Rs in this cortical area remains relatively unknown.

The neuronal population in the cerebral cortex consists of ~20% GABAergic interneurons and ~80% non-GABAergic cells (Peters and Kara 1985; Somogyi et al. 1998; Markram et al. 2004). Despite the small number of interneurons, the relative contribution of excitatory and inhibitory input conductances (balance between excitation (E) and inhibition (I), E/I) is, across different layers, dynamically maintained at approximately 20% excitation and 80% inhibition (Le Roux et al. 2006, 2007, 2008; Zhang et al. 2011). The E/I balance is thought to result from the coordinated activities of direct and recurrent excitation together with feed-forward and feedback inhibition. It determines proper cortical network rhythms responsible for higher order cognitive functions (Shu et al. 2003; Haider et al. 2006a). Disturbances in the E/I balance are associated with a broad spectrum of neuropsychiatric and neurological diseases, such as autism, schizophrenia and epilepsy (Cobos et al. 2005; Lewis et al. 2005; Rubenstein 2010). In particular, in the mPFC, an elevated E/I balance elicits impairments in cellular information processing and social dysfunction (Yizhar et al. 2011). Since dysregulation of the eCB system is associated with various psychiatric disorders that could be linked to a disturbed E/I balance (Ishiguro et al. 2010; Parolaro et al. 2010; Roche and Finn 2010), we set out to investigate the effects of CB₁R activation on the E/I balance in the mPFC.

In the present study, we have determined the effects of CB₁R-activation in the mPFC on miniature synaptic currents in order to confirm the supposed presynaptic localization of these receptors. Furthermore, we made use of immunohistochemical stainings to determine the preferential distribution of CB₁Rs on GABAergic cells and non-GABAergic (principle) cells in the mPFC. Based on the expression of CB₁Rs, we
investigated the effects of CB₁R-activation by the superfusion of a cannabinoid agonist on the E/I balance in the mPFC. We made use of a method that enabled us to decompose the total synaptic conductance into excitatory and inhibitory components (Borg-Graham et al. 1998; Wehr and Zador 2003). These methods allow for the estimation of the E/I balance, without altering the functional interactions between glutamatergic and GABAergic cells, since the use of pharmacological tools is avoided (Monier et al. 2008). In addition, the total, excitatory and inhibitory conductances can be monitored dynamically over the course of the synaptic response. Finally, we used the same method to determine the E/I balance in slices obtained from animals that had been treated with a cannabinoid agonist.

Materials and Methods

Animals
Wistar rats (Harlan, the Netherlands) between the age of postnatal day 14 (P14) and postnatal day 20 (P20) were used for this study. All experiments were performed in accordance with the committee on animal bioethics of the University of Amsterdam.

Electrophysiology recordings
Coronal slices (300 µm) of the mPFC were obtained from male rats aged 17-20 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). mPFC 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 used for recordings of miniature synaptic currents (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). Whole-cell voltage clamp recordings were made at 32 °C from the soma of layer II/III pyramidal neurons. For the experiments determining the effects of cannabinoid ligands on the balance between excitatory and inhibitory input, a pipette solution with a physiologically relevant Cl⁻ concentration was used, containing (in mM): 131.25 KGluconate, 8.75 KCl, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, 0.5 Na-GTP. Recordings were made using an Axopatch 200b (Axon, USA) and in-house software running under Matlab (MathWorks, USA). Signals were filtered at 5 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.

Miniature synaptic currents analysis
The amplitude and instantaneous frequency were determined for every miniature synaptic event and analysed. Distributions of binned mEPSC/mIPSC instantaneous
frequencies and amplitudes (40-50 bins) were constructed for every cell and then averaged. For mEPSCs, at least 250 events were analysed per cell, for mIPSCs, at least 100 events were analysed per cell.

**Synaptic response analysis**

Data were analysed off-line with in-house software running under Matlab. The method that was used to decompose the excitatory and inhibitory synaptic input is based on the continuous measurement of conductance dynamics of evoked synaptic responses, first described *in vivo* in cat cortex (Borg-Graham et al. 1998; Monier et al. 2003). This method has since then been validated by several groups and in different cortices (Shu et al. 2003; Wehr and Zador 2003; Higley and Contreras 2006; Le Roux et al. 2006, 2007, 2008; Cruikshank et al. 2007). For synaptic stimulation, a stimulation electrode was placed in layer I, through which currents (100-600 μA, 0.1 ms) were injected. Stimulation amplitudes were adjusted to be large enough to evoke postsynaptic responses of half-maximal amplitude and which did not evoke action potentials (APs) in the current clamp configuration. Evoked synaptic responses were recorded in layer II/III and averaged (four traces) at different Vm levels (-90 to -50 mV, in 500 ms steps of 5 mV). This allowed for the construction of an average I/V relationship during rest (50 ms) and an I/V relationship for each delay (t) per 1 ms after synaptic stimulation. The evoked synaptic conductance ($g_{\text{syn}(t)}$) was calculated by subtracting the slope of the best linear fit (mean least square criterion) of the average I/V curve prior to stimulation ($g_{\text{rest}}$) from the slope of the best linear fit for the I/V curve for each delay (t) after synaptic stimulation (Fig. 4B). The voltage abscissa of the intersection point between the average rest I/V curve and a I/V curve at time (t) was taken as the reversal potential of the synaptic current ($E_{\text{syn}(t)}$). In order to decompose the evoked synaptic conductance ($g_{\text{syn}(t)}$) into its excitatory ($g_e(t)$) and its inhibitory ($g_i(t)$) component, we used the following simplifications, as described by others (Borg-Graham et al. 1998; Wehr and Zador 2003):

$$E_{\text{syn}(t)} = \frac{g_e(t) \times E_e + g_i(t) \times E_i}{g_e(t) + g_i(t)}$$

$$g_{\text{syn}(t)} = g_e(t) + g_i(t)$$
And solving for $g_e(t)$ and $g_i(t)$:

$$g_i(t) = \frac{g_{syn}(t) \times (E_e - E_{syn}(t))}{E_e - E_i}$$

$$g_e(t) = g_{syn}(t) - g_i(t)$$

where $g_e(t)$ and $g_i(t)$ are the excitatory and inhibitory conductances, respectively; $E_e$ and $E_i$ are the reversal potentials for excitatory and inhibitory conductances, respectively. These latter values were determined by the intracellular and extracellular solutions. The value for $E_e$ was set at 0 mV, the value for $E_i$ was set at -86 mV. The integral (int) of the conductances over a time window of 250 ms after synaptic stimulation was used to quantify changes in conductances. The contributions of the excitatory and inhibitory components were expressed by the ratio of their integrals (integral for excitatory conductances, int$_e$ and integral for inhibitory conductances, int$_i$) to the integral of the total conductance (int$_t$), as previously described (Borg-Graham et al. 1998; Wehr and Zador 2003). Since we performed somatic recordings, the conductance measurements are representative of the proximal excitatory and inhibitory inputs to the neuron and may underestimate the contribution of distal synaptic events. Nevertheless, they reflect the relative changes in excitatory and inhibitory conductances read out at the soma (Haider et al. 2006b; Le Roux et al. 2006).

**CB$_1$R internalization and immunohistochemistry**

To determine the expression of CB$_1$Rs in the mPFC of P14 male pups, some animals were injected with the mixed CB$_1$/CB$_2$ agonist CP-55940 (0.7 mg/kg, i.p., dissolved in DMSO and a physiological saline solution) 24, 12 and 2 hours prior to being sacrificed. Animals, treated and untreated (vehicle injections), were deeply anaesthetized with pentobarbital (80 mg/kg, i.p.) and ketamine (60 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde (PFA). Brains were dissected out and left in PFA overnight for postfixation. 60-μm slices of the mPFC were cut on a vibratome (Leica VT1000S) and washed with saline phosphate buffer (0.01 M, pH 7.4; PBS). Slices were permeabilized for 30 min in PBS-0.25% Triton-X (PBST), kept for one hour in PBST + 10% normal goat serum (NGS) and incubated overnight at 4 °C with primary antibody in PBST + 5% NGS. The next day, slices were washed with PBS and incubated for 2 hours at room temperature in secondary antibody in PBST + 5% NGS. Slices were washed again in PBS and mounted on slides using Vectashield Hard Set Mounting Medium (Vector Laboratories, Peterborough, UK). Primary antibody against the CB$_1$R was a rabbit polyclonal antibody raised against the C-terminal portion of the CB$_1$R (1:1000) that was produced by Eurogentec (Seraing, Belgium) and has been characterized and used previously (Letertier et al. 2004; Vitalis et al. 2008). Primary antibodies against the mouse glutamate decarboxylase (GAD) 65 (1:5000, ab26113, Abcam) and 67 (1:1000, MAB5406, Millipore) were coincubated with some slices. Secondary antibodies (goat
anti-mouse IgG Alexa fluor 488, 1:200 and goat anti-rabbit IgG Alexa fluor 594, 1:200) were obtained from Invitrogen (the Netherlands). Images from stained slices were made using a confocal microscope (LSM 510, Zeiss) with 488- and 543- nm lines of an Argon/Krypton laser.

**Cannabinoid agonist treatment**

For experiments that investigated the effects of *in vivo* cannabinoid receptor activation on the *in vitro* E/I balance, animals were injected with the mixed CB1R/CB2R agonist CP-55940 (0.7 mg/kg, i.p., dissolved in dimethyl sulfoxide (DMSO) and a physiological saline solution) 60 min prior to being sacrificed and their brains being sliced. Control animals were injected with a vehicle DMSO solution.

**Drugs**

CP-55940, DL-AP5 (AP5), CNQX disodium salt, bicuculline methochloride and DMSO were purchased from Tocris (the Netherlands). TTX was purchased from Latoxan (France). WIN55212-2, rimonabant (SR141716) and Sch.356036 were generous gifts from Abbott Healthcare Laboratories (the Netherlands).

**Statistical analysis**

Data were statistically tested with t-tests and the Kolmogorov-Smirnov test. In the figures the significance is indicated with asterisks (*P<0.05, ** P<0.01 and *** P<0.001). Results are given as mean ± standard error of the mean. The number of observations are given in the bar charts (Figs. 1 and 2) or in the figure legends (Figs. 5 - 7).

**Results**

**CB1R activation reduces mEPSC and mIPSC frequency**

With a pipette solution with [Cl\(^-\)]\(_i\) = 30 mM and our standard ACSF in the recording chamber (reversal potential for Cl\(^-\): -38.3 mV), both excitatory and inhibitory postsynaptic currents could be easily detected as inward currents when cells were clamped at -70 mV (Fig. 1A, 2A). We isolated miniature postsynaptic currents by superfusion of TTX (0.5 μM). CNQX (20 μM) and AP5 (10 μM) were used to isolate miniature inhibitory postsynaptic currents (mIPSCs), bicuculline (20 μM) was used to isolate miniature excitatory postsynaptic currents (mEPSCs). To determine the effects of cannabinoid ligands on mEPSCs and mIPSCs, control recordings (5 min) were made at least 5 min after going whole-cell. Prior to determining the effects on mEPSCs and mIPSCs (5 min), cannabinoids were washed in for 15 min. The application of the mixed CB1R/CB2R agonist WIN55212-2 (WIN) changed the cumulative probability distribution plot of mEPSC instantaneous frequencies (Fig. 1C), but not the cumulative distribution plot of the amplitudes (Fig. 1D). After preincubation (of at least 10 min) and in the continuous presence of the CB1R antagonist rimonabant (5 μM), we did not detect a change in the frequency or
Figure 1. CB₁R activation reduces the frequency, but not the amplitude of mEPSCs. (A) Representative current trace of mEPSCs (control). Inset shows an example current trace of a single mEPSC. (B) Representative current trace of recorded mEPSCs after the application of WIN (5 µM). (C) Cumulative distribution plot of mEPSC frequency showing that bath-applied WIN (5 µM) caused a left-ward shift of the distribution (Kolmogorov-Smirnov test, P < 0.01). (D) Cumulative distribution plots of mEPSC amplitude were not different before and after bath application of WIN (5 µM). (E) Cumulative distribution plot of mEPSC frequency demonstrating that the frequency of mEPSC recorded after preincubation with, and in the continuous presence of rimonabant (5 µM), is not different from the frequency of mEPSCs recorded after preincubation with and in the
continuous presence of rimonabant (5 μM) and WIN (5 μM). (F) Cumulative distribution plots of mEPSC amplitude in the presence of rimonabant (5 μM) and rimonabant (5 μM) plus WIN (5 μM) are not different. (G) Normalized mEPSC frequency is reduced by ~30% in the presence of WIN (5 μM) (P < 0.01). This reduction by WIN was prevented after preincubation with, and in the continuous presence of rimonabant (5 μM). (H) Normalized mEPSC amplitude is not changed after the application of WIN (5 μM). After preincubation with, and in the continuous presence of rimonabant (5 μM), normalized mEPSC amplitude was also not different after application of WIN (5 μM).

amplitude of mEPSCs following WIN application (Fig. 1E, F), indicating that this effect was mediated by CB1Rs. We show that the frequency, but not the amplitude, of mEPSCs was reduced in the presence of WIN by ~30% (Fig. 1G). We next investigated the effects of WIN on mIPSCs. WIN changed the cumulative probability distribution plot of mIPSC instantaneous frequencies (Fig. 2C), but not the cumulative distribution plot of the amplitudes (Fig. 2D). Preincubation (of at least 10 min) and continuous presence of rimonabant (5 μM) could prevent this effect on mIPSC frequency, which indicates that the reduction was mediated by CB1Rs (Fig. 2E, F). The frequency, but not the amplitude, of mIPSCs was reduced in the presence of WIN by ~30% (Fig. 2G). These data demonstrate that CB1R activation leads to a reduction in frequency of both excitatory and inhibitory miniature synaptic events. The frequency of mEPSCs and mIPSCs was affected, but not the amplitude, which indicates that WIN acted on CB1Rs at presynaptic terminals.

**Immunohistochemical detection of CB1Rs in the mPFC**

To confirm the presynaptic localization of CB1Rs in the rat mPFC, we performed immunohistochemical stainings. We stained mPFC slices of P14 pups to demonstrate the presence of CB1Rs. CB1R immunostaining was detected throughout the mPFC, but higher concentrations were observed in layer II/III and in the deeper layer V (Fig. 3A). High immunoreactivity was found on axonal fibers surrounding the unstained cortical cell bodies (Fig. 3B). In order to determine whether both GABAergic and non-GABAergic cells express CB1Rs, we performed immunohistochemistry on slices obtained from animals that were treated with three injections (24, 12 and 2 hours prior to sacrifice) of a mixed CB1R/CB2R agonist (CP-55940, 0.7 mg/kg). Internalization of CB1Rs induced by treatment with CP-55940 combined with immunohistochemistry for markers of GABAergic cells, glutamate decarboxylase (GAD) 65 and 67, allowed for the detection of CB1Rs on inhibitory GABAergic cells (Fig. 3C). We found that a small number of CB1R-expressing cells also expressed GAD65/67, whereas most CB1R-expressing cells did not (Fig. 3C, Table 1). Although it is likely that a portion of GABAergic cells failed to display a detectable amount of GAD65/67, we assume that a majority of CB1R-positive cells that were GAD65/67 negative are non-GABAergic cells. Together, this data shows that CB1Rs are mainly expressed on axonal fibers and that they are expressed by both GABAergic and non-GABAergic cells, which is in line with the effects of CB1R activation on miniature synaptic events (Fig. 1 and 2).
Figure 2. CB1R activation reduces the frequency, but not the amplitude of mlPSCs. (A) Representative current trace of mlPSCs (control). Inset shows an example current trace of a single mlPSC. (B) Representative current trace of recorded mlPSCs after the application of WIN (5 µM). (C) Cumulative distribution plot of mlPSC frequency showing that the application of WIN (5 µM) resulted in a left-ward shift of the distribution (Kolmogorov-Smirnov test, P < 0.05). (D) Cumulative distribution plots of mlPSC amplitude are not different before and after bath application of WIN (5 µM). (E) Cumulative distribution plot of mlPSC frequency which demonstrates that the frequency of mlPSCs recorded after preincubation with, and in the continuous
presence of rimonabant (5 µM), is not different from the frequency of mIPSCs recorded after preincubation with, and in the continuous presence of rimonabant (5 µM) and in the presence of WIN (5 µM). (F) Cumulative distribution plots of mIPSC amplitude in the presence of rimonabant (5 µM) and rimonabant (5 µM) plus WIN (5 µM) are not different. (G) Normalized mIPSC frequency is reduced by ~30% in the presence of WIN (5 µM) (P < 0.001). The reduction by WIN was prevented after preincubation with, and in the continuous presence of rimonabant (5 µM). (H) Normalized mIPSC amplitude is not changed after the application of WIN (5 µM). Normalized mIPSC amplitude was not different following application of WIN (5 µM), if slices were preincubated with, and in the continuous presence of rimonabant (5 µM).

Characterization of the E/I balance in layer II/III pyramidal neurons of the mPFC in response to synaptic stimulation in layer I

We investigated the E/I balance of synaptic input from layer I to pyramidal neurons in layers II/III, using previously described methods. We used a pipette solution with a physiologically relevant [Cl-], (8.75 mM), in combination with our standard ACSF. Electrical stimulation of layer I evoked complex current responses in layer II/III cells (Fig. 4A1). We used CNQX (20 µM), bicuculline (20 µM) and AP5 (10 µM) to block AMPA/kainate, GABA_A and NMDA receptor-mediated currents, respectively, showing that the current responses were mediated by glutamate and GABA (Fig. 4A2). Figure 4B shows, as example, the average rest I/V curve and the I/V curve at 8 ms after electrical stimulation, which is around the peak of the synaptic response. We analysed traces over a total duration of 250 ms as to cover the whole range of the synaptic response. Figure 4C shows the decomposition of the total synaptic conductance (g total) into inhibitory (g inhibition) and excitatory (g excitation) components. The contributions of the excitatory and inhibitory components were expressed by the ratio of their integrals to the integral of the total conductance. The E/I balance typically consisted of ~80% inhibition and ~20% excitation (inset Fig. 4C). This is consistent with previous findings in the neocortex and it shows that excitability in layer II/III of the mPFC is mainly controlled by inhibition (Le Roux et al. 2006, 2007, 2008). We next used cannabinoid ligands to investigate whether CB1R activation affected conductance amplitudes and the ratio between excitation and inhibition.

CB1R receptor activation changes the E/I balance of synaptic input

We investigated the effects of CB1R activation on the ratio between excitation and inhibition of synaptic input onto layer II/III mPFC pyramidal neurons (Fig. 5A-D). Control recordings, performed at least 5 min after going whole-cell, show that the balance between excitation and inhibition under control conditions is maintained at approximately 20-80% (Fig. 5C, E). Separate control recordings were performed in which only vehicle was applied for 15 min and in which no changes in E/I balance or conductances were observed (data not shown). To investigate CB1R-mediated effects on the E/I balance, the cannabinoid receptor agonist WIN was washed in for 15 min after which (still in the presence of the ligand) recordings were performed to determine WIN effects (Fig. 5B, D, E, F). Bath application of WIN (5 µM) resulted in a reduction of the inhibitory component relative to the total synaptic conductance and a corresponding relative increase in the excitatory component, shifting the balance
Modulation of the E/I balance in the rat medial prefrontal cortex by CB1Rs

Figure 3. CB1R immunoreactivity was found on axonal fibers in layers II/III and layer V, belonging to GABAergic and non-GABAergic cells. (A) Overview of CB1R expression in the various layers of the mPFC in an untreated brain. CB1R-positive immunoreactivity was predominantly found in layers II, III and the deeper layer V. Five slices from three animals showed similar staining patterns. (B) CB1R-positive immunoreactivity in more detail in layer III, particularly high immunoreactivity was found in axonal fibers surrounding the unstained cortical cell bodies. (C) When animals were repeatedly treated with the cannabinoid agonist CP-55940, CB1R internalization could be induced. This internalization, combined with staining for markers of GABAergic GAD65/67, allowed for the detection of CB1R-positive interneurons. Only a small number of CB1R-expressing cells also expressed GAD65/67, see also Table 1.

Table 1. Distribution of CB1R in GABAergic and non-GABAergic neurons of the mPFC. The detection of CB1R-positive interneurons showed that in both superficial (I-III) and deeper (V-VI) layers only a small percentage of CB1R-positive cells also expressed GAD65/67. A total of 4 slices obtained from 3 animals were used to determine co-localisation of CB1R- and GAD65/67-positive immunostaining. The values between brackets indicate the % of neurons that co-localise CB1R and GAD65/67 immunoreactivity.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Superficial layers</th>
<th>Deep layers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB1R</td>
<td>318 (6.6 %)</td>
<td>70 (2.9 %)</td>
</tr>
<tr>
<td>GAD65/67</td>
<td>470 (4.5 %)</td>
<td>171 (1.2 %)</td>
</tr>
<tr>
<td>Co-localized</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>Analyzed area (mm²)</td>
<td>7.22</td>
<td>5.31</td>
</tr>
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</table>
Figure 4. Electrical stimulation of layer I of mPFC brain slices resulted in synaptic response currents in layer II/III pyramidal neurons that could be decomposed into excitatory and inhibitory components. (A) Representative current traces of synaptic responses to electrical stimulation (arrow) in layer I at various holding potentials (-90 to -50 mV) under control conditions (A1) and in the presence of CNQX (20 µM), bicuculline (20 µM) and AP5 (10 µM) (A2). The black and grey vertical bars indicate two 1-ms time points at which current amplitudes were measured for plotting I/V relationships (see panel B) at rest and at the peak of the synaptic response, respectively. (B) Average rest I/V relationship (50 ms) and I/V relationship at time \( t \) (1 ms), taken at the peak of the synaptic response. The voltage intersection point of the average rest I/V curve is taken as the resting membrane potential. The slopes of the linear fits of the average rest I/V curve and the I/V curve at time \( t \) are the rest conductance and synaptic conductance, respectively. The voltage abscissa of the intersection point between the average rest I/V curve and the I/V curve at time \( t \) represents the reversal potential of the synaptic current. (C) Decomposition of the total conductance (black) into excitatory (light grey) and inhibitory (dark grey) components. Inset: the percentage of the integrals of inhibitory and excitatory conductance compared to the integral of total conductance, typically existing of ~80% inhibition and ~20% excitation.

towards excitation (Fig. 5E). When total, excitatory and inhibitory conductances were normalized to control (before WIN application), we observed a decrease in all conductances following the application of WIN (Fig. 5F). However, the reduction in inhibitory conductance exceeded the reduction in excitatory conductance, which explains the shift of the balance towards excitation. To confirm the involvement of CB\(_1\)Rs and to exclude CB\(_2\)R-mediated effects, we repeated the experiments in the presence of the selective CB\(_1\)R antagonist rimonabant (5 µM) or the selective CB\(_2\)R
Figure 5. Effects of CB₁R activation on balanced synaptic input. (A) Representative current traces of synaptic responses recorded in a layer II/III mPFC pyramidal neuron to electrical stimulation in layer I at various holding potentials (-90 - -50 mV) under control conditions. (B) Representative current traces of synaptic responses in the same cell as in A, 15 min after application of WIN (5 µM). (C) Decomposition of total (black), excitatory (light grey) and inhibitory (dark grey) conductances of the current traces displayed in A. (D) Decomposition of the conductances of the current traces displayed in B after application of WIN (5 µM). (E) Average integral values of inhibitory and excitatory conductances (as percentage of the integral value of the total conductance) were changed after application of WIN (5 µM), control: 82.1±0.8% and 17.9±1.5%, respectively; WIN: 75.3±1.5% and 24.7±1.5%, respectively (P < 0.001, n = 16). (F) WIN reduced total (black), inhibitory (dark grey) and excitatory (light grey) normalized conductances by 36.2±9.1%, 38.6±9.4% and 19.5±7.9%, respectively (P < 0.01, P< 0.001 and P < 0.05, respectively, n = 16).
Figure 6. Effects of CB₁R activation on balanced synaptic input after preincubation (of at least 10 min) with, and in the continuous presence of selective CB₁R and CB₂R antagonists. (A) Integral values of inhibitory and excitatory conductances (as percentage of the integral value of the total conductance) were not changed after application of WIN (5 µM) following preincubation with, and in the continuous presence of 5 µM rimonabant (rim); control: 83.0±1.7% and 17.0±1.7%, respectively; WIN: 80.6±1.2% and 19.4±1.2%, respectively (n = 7). (B) WIN did not change total (black), inhibitory (dark grey) and excitatory (light grey) normalized conductances in the presence of rimonabant (5 µM), -4.1±11.5%, -6.1±12.3% and +9.7±11.4%, respectively (n = 7). (C) Integral values of inhibitory and excitatory conductances (as percentage of the integral value of the total conductance) were changed following application of WIN (5 µM) after preincubation with and in the continuous presence of 5 µM Sch.356036 (Sch); control: 79.8±1.8% and 20.2±1.8%, respectively; WIN: 73.5±2.4% and 26.5±1.2%, respectively (n = 8). (D) In the presence of Sch.356036 (5 µM), WIN (5 µM) reduced total (black), inhibitory (dark grey) and excitatory (light grey) normalized conductances by 38.25±7.9%, 43.7±8.6% and 26.9±5.4%, respectively (P < 0.01, n = 8).

antagonist Sch.356036 (5 µM) (Fig. 6). After preincubation with and in the continuous presence of rimonabant, the application of WIN did not affect the E/I balance (Fig. 6A) or the normalized conductances (Fig. 6B). In contrast, after preincubation with and in the continuous presence of Sch.356036, the application of WIN changed the E/I balance (Fig. 6C). This effect on the E/I balance was comparable to the effects of WIN, when it was applied alone (Fig. 5E). In addition, when CB₂Rs were blocked, the normalized total, excitatory and inhibitory conductances were decreased after the application of WIN.
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(Fig. 6D). These data show that the effects observed after WIN application are mediated by CB₁Rs.

In vivo cannabinoid receptor activation

We next investigated whether slices obtained from animals acutely treated with a cannabinoid receptor agonist showed a change in the E/I balance compared to vehicle-injected animals. Rats were injected with the mixed CB₁R/CB₂R agonist CP-55940 (0.7 mg/kg, i.p.) or vehicle 60 minutes prior to being sacrificed. mPFC slices obtained from these animals were tested and the decomposition method revealed that the E/I balance was different compared to control slices, in favour of excitation, in CP-55940-treated animals (Fig. 7). We did not observe such a shift in vehicle-treated animals (Fig. 7), which showed ratios comparable to non-injected animals (Fig. 5).

Discussion

In this study, we have investigated the effects of the activation of CB₁Rs on miniature synaptic currents and the E/I balance in the mPFC. We show that CB₁R activation reduced the frequency, but not the amplitude, of both mEPSCs and mIPSCs. This indicates a presynaptic localization of CB₁Rs on excitatory and inhibitory terminals in the rodent mPFC, which is in line with earlier studies which have reported the presynaptic presence of CB₁Rs on pyramidal neurons and various classes of inhibitory interneurons (Katona et al. 1999, 2006; Tsou et al. 1999; Bodor et al. 2005; Hill et al. 2007). In addition, we performed immunohistochemical stainings for CB₁Rs that confirmed their predominant presence on axonal fibers. Previously, treatment of pregnant females with CP-55940 was shown to result, in their embryos, in the internalization of CB₁Rs to cell bodies (Vitalis et al. 2008). Another study showed the internalization of CB₁Rs by repeated injections of CP-55940 in adult rats (Thibault et al. 2012). In the present study, when CB₁Rs were internalized following several injections with CP-55940, we observed that CB₁Rs were present on both GABAergic and non-GABAergic neurons. This is in agreement with our electrophysiological findings (see above), showing that CB₁R activation affects spontaneous release of excitatory and inhibitory neurotransmitters.

To better understand the repercussions of the activation of presynaptic CB₁Rs, we investigated the effects of CB₁R activation on the E/I balance in the mPFC. We first characterized the E/I balance of synaptic input onto layer II/III pyramidal neurons after stimulation of layer I. Pyramidal neurons in layer II/III are thought to be key players in the cortical network, receiving cortical and subcortical input and projecting to other cortical areas. Input into layer I is thought to be crucial for feedback processes that are involved in cognitive functions (Gilbert and Sigman 2007). The excitatory input into this layer is considered to be cortical, but thalamic input also converges here (Douglas and Martin 2007; Rubio-Garrido et al. 2009). Although glutamatergic cells, like layer II/III pyramidal neurons, constitute more than 80% of the neuronal population in the neocortex, GABAergic interneurons are highly connected to glutamatergic neurons, allowing inhibitory conductances to dominate (Peters and Kara 1985). Inhibitory interneurons, which vary greatly in their morphology and physiology
Figure 7. *In vivo* cannabinoid receptor activation changed the *in vitro* E/I balance. (A) Representative current traces of synaptic responses recorded in a layer II/III mPFC pyramidal neuron of a vehicle-treated rat to electrical stimulation in layer I at various holding potentials (-90 - -50 mV). (B) Decomposition of total (black), excitatory (light grey) and inhibitory (dark grey) conductances of the current traces displayed in A. (C) Representative current traces of similarly recorded synaptic responses in a layer II/III mPFC pyramidal neuron of a rat treated with CP-55940. (D) Decomposition of total (black), excitatory (light grey) and inhibitory (dark grey) conductances of the current traces displayed in C. (E) Average integral values of inhibitory and excitatory conductances (as percentage of the integral value of the total conductance) were different for cells from two vehicle- and two CP-55940-treated animals; vehicle: 80.7±1.4% and 19.3±1.4% (n = 15), respectively; CP-55940 (CP): 70.0±3.2% and 30.0±3.2% (n = 18), respectively (P < 0.01).
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(Markram et al. 2004), regulate local cortical circuit activity. It has been suggested that it is the great diversity of interneurons that enables them to regulate inhibition to dynamically match and balance complex excitation (Markram et al. 2004; Isaacson and Scanziani 2011). The E/I balance in the neocortex is maintained at around 20% excitation and 80% inhibition over different layers and a variety of stimuli and deviations from this ratio are associated with a range of neuropsychiatric disorders (Cobos et al. 2005; Lewis et al. 2005; Rubenstein 2010). Here, we show that the balance between excitation and inhibition following stimulation in layer I is similar to previously reported values, at ~20% - ~80%, respectively (Le Roux et al. 2006, 2007, 2008). When CB₃Rs are activated by the bath application of WIN, the E/I balance is shifted towards excitation. The decomposition method we applied in this study allowed us to compare the conductance amplitudes before and after CB₃R activation for total, excitatory and inhibitory conductances, separately. We report a decrease in all conductance amplitudes, which is in line with our results that demonstrate the modulation of both excitatory and inhibitory neurotransmitter release following the application of the mixed CB₁R/CB₂R agonist WIN. The most prominent reduction in conductance amplitude was found for inhibition, which explains the shift in favour of excitation in the E/I balance. WIN induced a similar range of effects in the presence of a selective CB₂R antagonist, but not in the presence of a selective CB₁R antagonist. This indicates that these effects are mediated by CB₁R activation and were not dependent on CB₂Rs, which – as postsynaptic receptors – are involved in regulating layer II/III pyramidal cell excitability (den Boon et al. 2012). Our immunohistochemical data and miniature synaptic current recordings demonstrate that functional CB₁Rs are located presynaptically. Therefore, we conclude that the WIN-mediated reduction of the conductances, following synaptic stimulation, is mediated by the presynaptic modulation of neurotransmitter release by CB₁R activation. Altogether, this points to a general role of the eCB system in the modulation of neurotransmission via presynaptic CB₁Rs.

In order to investigate the effects of *in vivo* cannabinoid receptor activation on the E/I balance we used slices obtained from animals that were treated with the cannabinoid receptor agonist CP-55490. Slices from these animals showed a shift in E/I balance in favour of excitation, compared to slices from control animals. This shift was comparable to the shift induced by bath application of WIN. These data show that when cannabinoid receptors are acutely activated *in vivo* by exogenous cannabinoids, changes in the E/I balance are robust and long-lasting enough to be determined with the decomposition method used on *in vitro* data, collected during a period of recording lasting several hours. To compare, it is known that effects of cannabis administration may last up to 24 hours, which is relatively long compared to other drugs of abuse (Leirer et al. 1991; Grotenhermen 2003). The duration of effects mediated by endogenous ligands, such as eCB-mediated short-term synaptic plasticity, last much shorter (< 1 min) (Kreitzer and Regehr 2001; Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001). Our results could suggest that (part of) the effects of cannabinoid ligands on mPFC functioning may be mediated by a change in the E/I balance. Changes in the
E/I balance can have profound consequences not only for network excitability, but also for the tuning of neurons to specific stimuli and in shaping of their activity in time (Kavalali et al. 2011). Several studies showed that in sensory cortical areas, the E/I balance also depends on the property of the sensory stimulus (Wu et al. 2008; Poo and Isaacson 2009; Liu et al. 2011; Tan et al. 2011). The modulation of the E/I balance by presynaptic CB1Rs may be a key determinant in the regulation of higher order cognitive functions associated with the mPFC. The shift in the E/I balance we observed, is associated with a relative increased excitatory input onto cells. A recent paper reported that Ca\(^{2+}\) transients evoked by backpropagating APs were potentiated by the activation of CB1Rs, which was mediated by suppression of GABAergic transmission (Hsieh and Levine 2012). This could reinforce coincidence detection of feedback and feedforward processes.

Interestingly, an increase in E/I ratio in the mPFC, obtained using optogenetic tools, results in impairments in cellular information processing and social dysfunction (Yizhar et al. 2011). In that study, evidence that supports a causal relationship between an increased E/I balance and behavioural deficits was uncovered. Future studies should also determine whether intake of cannabinoids, e.g. the smoking of marijuana, can induce its behavioural effects through a changed E/I balance and whether long-term effects of cannabis use during adolescence are mediated by the same mechanism.

Taken together, the data reported in the present study indicate that presynaptic CB1Rs are involved in the modulation of both evoked and spontaneous neurotransmission. Furthermore, in vitro CB1R-activation and in vivo cannabinoid receptor activation changed the E/I balance in favour of excitation. Future studies could shed more light on the regulation of neuronal communication by the eCB system, both under control conditions and during excessive stimulation of the eCB system, e.g. following cannabis use.

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