Chapter 6

General discussion
Scope of this thesis
This thesis focusses on the role of the endocannabinoid (eCB) system with respect to interneuronal communication and neuronal excitability in the neocortex. The studies described here were performed within a research project of a TIPharma consortium (grant T5-107-1). This research project investigated the role of the eCB system in the regulation of brain functions involved in psychopathological syndromes with high medical need, e.g. addiction and cognitive disorders.

Summary
The eCB system is a powerful modulator of communication between cells. Since the discovery of its constituents in the 90s, much work has been done to unravel the workings of this neurotransmitter system in various physiological processes. One of the key features of the eCB system is its involvement in the modulation of neurotransmitter release. Many different studies have shown that the activation of type-1 cannabinoid receptors (CB1Rs), which are abundantly present in the central nervous system (CNS), results in the attenuated release of GABA, glutamate, glycine, acetylcholine, dopamine, 5-HT and norepinephrine (Kreitzer and Regehr 2001; Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001; Kano et al. 2009). Furthermore, CB1Rs have been shown to be involved in several forms of synaptic plasticity (Heifets and Castillo 2009; Castillo et al. 2012). In contrast, the role of type-2 cannabinoid receptors (CB2R) in the CNS has been relatively unclear, although more and more research groups are now focusing their efforts on this component of the eCB system in order to determine its role in the brain.

In chapter two, we examined the effects of CB1R- and CB2R-activation on local evoked field potentials in the medial prefrontal cortex (mPFC). We demonstrated that activation of these receptors resulted in a concentration-dependent reduction of the amplitude of the fEPSP component of the evoked mPFC field potential amplitude. This shows that the eCB system is present in this brain area and that activation of CBRs results in the modulation of neuronal activity, as detected by the recording of field potentials.

In chapter three, we focused on investigating the presence of CB2Rs in layer II/III pyramidal cells of the mPFC. We were the first to demonstrate that CB2Rs are expressed intracellularly in layer II/III pyramidal neurons. We showed that CB2R-activation, by application of synthetic, selective agonists, resulted in the opening of Ca\(^{2+}\)-activated Cl\(^-\) channels (CaCCs). The opening of CaCCs was mediated by the release of Ca\(^{2+}\) from intracellular IP\(_3\)-sensitive Ca\(^{2+}\) stores, since this could be prevented by blocking IP\(_3\)Rs, which could be located e.g. on the endoplasmic reticulum. Furthermore, to investigate the functional role of CB2R activation, we induced action potential (AP) firing with the injection of noisy current and observed a CB2R-mediated reduction of neuronal firing rate.

In chapter four, we hypothesized that stimulation protocols suitable to evoke eCB synthesis and subsequent CB1R activation, could also result in CB2R activation. We first determined that the application of the eCB 2-arachidonoylglycerol (2-AG) and methanandamide (mAEA, a stable analog of anandamide, AEA) could result in a similar
Cl⁻ current as was evoked with synthetic cannabinoid ligands. We found that although both 2-AG and mAEA can evoke a Cl⁻ current, 2-AG is a more efficacious agonist for the CB₂R. In addition, we performed experiments to show that a stimulation protocol that evokes 20 Hz AP firing can induce eCB synthesis and result in CB₂R-activation. We performed additional experiments that demonstrate the involvement of Ca²⁺ influx through N-type VDCCs in this process. Further experiments demonstrated that 2-AG is the main eCB involved in this process. Finally, we showed that under physiologically relevant Cl⁻ conditions, AP firing could lead to a CB₂R-dependent reduction of neuronal excitability.

In chapter five, we investigated the role of the eCB system in neurotransmission and the maintenance of the excitation/inhibition (E/I) balance in the mPFC. We showed that CB₁R activation reduced both inhibitory and excitatory neurotransmission, indicative of a presynaptic localization of these receptors. With immunohistochemical stainings for CB₂Rs we confirmed the presynaptic localization and found the CB₂Rs to be expressed by both excitatory and inhibitory cells. We furthermore described that the application of a commonly used, mixed CB₁R/CB₂R agonist, could alter the E/I balance in the mPFC, in favour of excitation. We showed that this shift in E/I balance is mediated by CB₁Rs. Importantly, we showed that a similar shift in the E/I balance due to in vivo treatment with a cannabinoid agonist could be detected using the decomposition method to analyse in vitro data.

In summary, the data presented in this thesis show the functional presence of both cannabinoid receptors, CB₁Rs and CB₂Rs, in the mPFC. The consequences and physiological relevance of these findings will be discussed next.
Discussion

Cannabinoid receptors in the cortical network

In chapter two, we have described effects of cannabinoid receptor activation on excitability of the local mPFC cortical network. Recordings in layers II/III of evoked field potentials in mPFC brain slices are indicative of a highly complex cortical network with components sensitive to glutamate receptor blockers. In experiments with two stimuli separated by varying interpulse intervals (IPIs), we also show that these field potentials are modulated by inhibitory network components through GABA_A and GABA_B receptors. The technique of field potential recordings has been proven especially successful in recordings in the hippocampus. The organized structure of the hippocampus has traditionally made this technique a suitable method of hippocampal research into a broad range of topics (Leung 1979; Foy et al. 1987; Kerr et al. 1989). In this thesis, we present results from field potential recordings in the cortex, a brain area relatively little investigated with field potential methods.

The data reported in chapter two demonstrates that cannabinoid receptor activation leads to the reduction of fEPSP amplitudes. This reduction was particularly strong when the mixed CB_1R/CB_2R agonist WIN55212-2 (WIN) was applied to mPFC brain slices, but could also be observed following the application of selective CB_1R and CB_2R agonists. Interestingly, the application of WIN resulted in the switch from paired-pulse depression to paired-pulse facilitation, when two stimuli were given with an IPI of 25 ms. This reversal in short-term plasticity was not induced by separate CB_1R and CB_2R activation with selective CB_1R and CB_2R agonists, respectively. Furthermore, additional experiments with the simultaneous application of the selective CB_1R and CB_2R ligands could not replicate the effects induced by WIN application (data not shown). These results indicate the complexity of cannabinoid receptor-mediated effects at the level of the cortical network. Furthermore, additional knowledge about the connectivities in this cortical network would be very useful for the interpretation of the field potential data. In recent years, the use of multielectrode arrays enabled researchers to answer ever more detailed questions in other brain areas. The use of such multielectrode arrays could shed more light on the functioning of the local mPFC network.

As discussed in the introduction of this dissertation, the PFC is associated with several executive functions such as working memory, reversal learning and attentional function. Studies have shown that these higher cognitive functions are impaired when the eCB system is modulated (Jentsch et al. 1998; Lichtman et al. 2002; Arguello and Jentsch 2004; Egerton et al. 2005, 2006). The mechanisms that could underlie these effects include the altered release of neurotransmitters and synaptic plasticity (Egerton et al. 2006; Puighermanal et al. 2012). Following the data presented in this thesis, future studies should determine the role of both CB_1Rs and CB_2Rs in the modulation of network activity and the eventual disturbance of executive functions performed by the PFC by cannabinoid ligands.
The presence of CB2Rs in the CNS
Since the discovery of CB2Rs in macrophages in the marginal zone of the spleen (Munro et al. 1993), CB2Rs were assumed to be exclusively present in the periphery. Their expression was detected in cells of the immune system, such as in the spleen and thymus, as well as in several circulating immune cell populations (Galiègue et al. 1995; Klein et al. 2003). A few years after the discovery of CB2Rs, a study reported the presence of CB2R mRNA in cerebellar granule cells (Skaper et al. 1996). Later, CB2Rs were discovered on perivascular microglial cells and in cultured cerebrovascular endothelium (Golech et al. 2004; Núñez et al. 2004). More than a decade after their discovery, an important paper was published in which the presence of CB2Rs in the brainstem, cerebellum and cortex of several rodent species was reported (Van Sickle et al. 2005). In that study, the authors also demonstrated a role for CB2Rs in the reduction of emesis in ferrets, showing that these receptors are functional. More recently and in addition to this evidence, several other articles were published that report a variety of functional effects mediated by CB2Rs in the brain (Gong et al. 2006; Brusco et al. 2008a, 2008b; Jhaveri et al. 2008; Onaivi et al. 2008; Morgan et al. 2009; Xi et al. 2011; Atwood, Straiker, et al. 2012). It is now generally accepted that the CB2R is expressed in neurons upon brain stress and damage (Viscomi et al. 2009).

Despite the studies claiming the (functional) presence of CB2Rs in the healthy brain, the existence of such central CB2Rs has remained controversial. One of the main objections raised against a large portion of the evidence for CB2Rs in the brain concerns immunohistochemical stainings of these receptors. It is well known that the visualization of many G protein-coupled receptors (GPCRs) is problematic due to the lack of specific antibodies (Ashton 2012). While the staining of CB1Rs with specific antibodies has been validated and matches the pattern of CB1R expression detected with autoradiography methods (Grimsey et al. 2008), antibodies against the CB2R are presumed to lack specificity (Atwood and Mackie 2010; Ashton 2012). Other problems could arise from slight differences in staining protocols, species-specific isoform expression patterns and complications with negative controls such as CB2R-KO mice (Liu et al. 2009; Atwood and Mackie 2010; Ashton 2012; Onaivi et al. 2012). In conclusion, this highlights the importance of investigations into CB2R expression in the brain with tools that do not (solely) rely on antibodies.

In chapter three, we have avoided the controversial use of antibodies against CB2Rs in order to visualize their expression with immunohistochemistry. Nevertheless, we did use a different biochemical technique relying on antibodies, Western blotting, to show the presence of these receptors in the mPFC. A lack of specificities of antibodies in one assay (e.g. immunohistochemistry) does not mean that the same antibody will not be specific in another assay (e.g. Western blotting). Fixatives used in immunohistochemistry may mask the epitope of interest so that the antibody no longer correctly binds to the protein (Ramos-Vara 2005). In our Western blotting experiments, we detected a band for mPFC tissue of the correct molecular weight (~45 kD). Subsequently, when mPFC samples were fractionated in a plasma membrane fraction and an intracellular fraction, we were only able to detect a similar band in the intracellular fraction. These unexpected results were confirmed by additional
experiments performed with a radioactive binding assay on similarly fractionated samples, and corroborated our hypothesis based on electrophysiological data that functional CB2Rs are intracellularly present in the mPFC. In addition to these results, with fluorescence imaging experiments on a neuronal cell line (human neuroblastoma cells transiently transfected with GFP-tagged CB2Rs), we found that CB2Rs were almost exclusively localized in intracellular membranous structures and that they were not present in the plasma membrane. The intracellular presence of several GPCRs was described earlier for receptors such as the mGluR1, mGluR5, apelin receptor, angiotensin AT1 receptor and the bradykinin B2 receptor (Lee et al. 2004b; Jong et al. 2005b, 2007). Functional CB2Rs were also found to be present intracellularly in guinea pig heart cells (Currie et al. 2008). Other studies reported that the CB2R protein was found to be associated with the rough endoplasmic reticulum and Golgi apparatus in hippocampal pyramidal neurons, although the functional role of these intracellular CB2Rs was not elucidated (Brusco et al. 2008a, 2008b; Onaivi et al. 2012).

Some papers have reported CB2R-mediated effects on synaptic currents, that indicate a presynaptic locus for these receptors (Morgan et al. 2009; Atwood, Straiker, et al. 2012). The reduction of spontaneous excitatory postsynaptic currents (EPSCs) described in cultured hippocampal autaptic neurons from CB1R-KO mice could be explained, according to the authors, by a CB2R-mediated inhibition of voltage-dependent Ca2+ channels (VDCCs) (Atwood, Straiker, et al. 2012). In short, these studies suggest that the CB2R has a similar role as the CB1R, i.e. mediating the presynaptic reduction of neurotransmitter release. In our preparation of the mPFC, we were not able to detect CB2R-mediated changes in the frequency or amplitude of miniature excitatory postsynaptic currents (mEPSCs) or miniature inhibitory postsynaptic currents (mIPSCs), (data not shown). One of the mentioned studies that reported presynaptic CB2R-mediated effects was performed on cultured neurons (Atwood, Straiker, et al. 2012). This could explain differences in CB2R functioning, since CB2R expression is thought to be highly inducible by immune response-triggering conditions, such as injury and culturing (Wotherspoon et al. 2005; Viscomi et al. 2009). The other study was performed in acute slices from the entorhinal cortex (Morgan et al. 2009). In this study, no CB2R-mediated effects on mIPSCs were found and changes in sIPSC frequency were inconsistent. Despite these results, the authors conclude that CB2Rs mediate their AP-dependent effects from a presynaptic locus, since they observed a small change in sIPSC decay time. Taken together, the results from various studies into CB2R-mediated effects imply that the CB2R may come to expression at various locations in neurons, presynaptically and postsynaptically, and that their activation can couple to different downstream signalling pathways.

Localisation of CB2R
Our findings point to a postsynaptic, intracellular localisation for CB2Rs in layer II/III pyramidal neurons of the mPFC. Since we have not been able to use antibodies for immunohistochemical visualization of the receptors, one of the main questions that remain to be answered concerns the exact localisation of CB2Rs (Fig 1). As described in chapter three, CB2R activation involves a IP3-mediated rise in [Ca2+]. Most GPCRs that
mediate a rise in [Ca\(^{2+}\)]\(_i\) can be found on the plasma membrane. The binding of an agonist to such a receptor results in the production of IP\(_3\), which diffuses into the cytosol and binds to IP\(_3\)Rs. IP\(_3\)Rs are located within the membrane of intracellular Ca\(^{2+}\)-stores such as the endoplasmic reticulum, which is a continuum with the perinuclear space (Gerasimenko et al. 1995). Consequently, a rapid rise in [Ca\(^{2+}\)]\(_i\) can be detected. The ability of IP\(_3\) in reaching its receptor in a different part of the cell means that the GPCR and IP\(_3\)R do not necessarily need to be localized close together.

Many other GPCRs that are found intracellularly are specifically localized at the cell nucleus (Gobeil et al. 2006a). Stimulation of such GPCRs can take place intracellularly. Particularly interesting examples of such GPCRs are the receptors activated by bioactive lipids such as prostaglandin, platelette-activating factor and lysophosphatidic acid (Zhu et al. 2006). These ligands are formed from membranes, including the nuclear membrane, in close proximity to their receptors. Alternatively, ligands may be transported from the extracellular space into cells to reach their receptors. This has been described for the polar compounds glutamate and quisqualate and their binding to nuclear mGluR5 in neurons, which is followed by a rise in [Ca\(^{2+}\)] (O’Malley et al. 2003a; Jong et al. 2005b). Other nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs), can be activated by AEA and other lipids that reach their target by, so far, unknown means (O’Sullivan 2007). The possible involvement in this process of chaperone proteins is currently a topic of intense investigation (Fu et al. 2012).

In addition to GPCRs, many components of downstream signal transduction machinery are present at the nuclear membrane, such as G proteins (G\(_s\), G\(_i/o\)), enzyme effectors and ion channels (Gobeil et al. 2006b). An interesting point, brought up in the article describing functional, nuclear mGluR5 (O’Malley et al. 2003b), is the orientation of these receptors in the nuclear membrane. Since the luminal side of the endoplasmic reticulum corresponds to the extracellular side of the plasma membrane, the authors predict that ligand binding domains of mGluR5 are on the luminal side. This means that ligands need to pass the nuclear envelope to reach their binding site, which should be possible for lipid eCBs. In turn, this means that the G protein binding domains are on the cytosolic side, where interaction with downstream signalling machinery is possible. In this model, mGluR5 are ideally located to activate nuclear PLC, IP\(_3\) and Ca\(^{2+}\) cascades. Possibly, CB\(_2\)Rs are present at a similar location and in a similar orientation to enable interaction with the G protein signalling machinery. Both the exact localization of CB\(_2\)Rs and their orientation in the intracellular membrane are interesting topics for future investigations.

**CB\(_2\)R activation**

eCB synthesis depends on the influx of Ca\(^{2+}\) through VDCCs and/or activation of GPCRs such as metabotropic glutamate receptors or M1/M3 muscarinic acetylcholine receptors (Kano et al. 2009). In chapter four, we have shown that a stimulation protocol (inducing 20 Hz AP firing), used to evoke eCB-mediated CB\(_1\)R activation, can result also in CB\(_2\)R activation. We performed additional experiments that demonstrate the involvement of N-type VDCCs and that 2-AG is the main eCB in this process.
influx through VDCCs is thought to be a major contributor to eCB synthesis (Fig. 1), likely through the activation of diacylglycerol lipase (DAGL), the enzyme that synthesizes 2-AG from diacylglycerol (DAG). Alternatively, it has been suggested that Ca\(^{2+}\) influx could release presynthesized eCBs from intracellular pools so that eCBs are available to then bind to intracellular CB\(_2\)Rs (Min et al. 2010; Alger and Kim 2011; Alger 2012). With CB\(_2\)R-activation involving Ca\(^{2+}\) influx-induced eCB synthesis in the same cell, the process of the activation of this receptor and the consequent opening of CaCCs both require an increase in [Ca\(^{2+}\)]. If Ca\(^{2+}\) flows into the cell through VDCCs upon repeated depolarizations, is this Ca\(^{2+}\) itself not sufficient to activate CaCCs? It is possible that compartmentalization in the cell requires the complex machinery of a CB\(_2\)R-mediated rise in [Ca\(^{2+}\)]. This will provide the cell with a more precise control over the final opening of CaCCs.

An interesting parameter in the described process is the relatively long delay following our stimulation protocol until the depolarization response and the all or nothing nature of this response, which has a Cl\(^{-}\) current as underlying conductance. Presumably, the stimulation protocol results in the immediate synthesis of eCBs, mainly 2-AG. The G protein signal transduction pathway, including IP\(_3\) synthesis, the activation of IP\(_3\)Rs and the rise in [Ca\(^{2+}\)], normally occur within seconds (Berridge 2009). Release of Ca\(^{2+}\) from intracellular stores following activation of IP\(_3\)Rs or ryanodine receptors (RyRs) is described to be a process that occurs abruptly (Berridge 1998, 2009). In fact, like RyRs, IP\(_3\)Rs are sensitive to intracellular Ca\(^{2+}\) levels and this gives rise to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), which is a nonlinear cooperative process (Ross 2012). CICR, depending on activation of IP\(_3\)Rs or RyRs, is assumed to underlie the sudden upstroke of [Ca\(^{2+}\)], typical for Ca\(^{2+}\) release from intracellular stores (Berridge 1998, 2009). The abrupt increase in [Ca\(^{2+}\)], following Ca\(^{2+}\) release from intracellular stores could underlie the all or nothing character of the CB\(_2\)R-mediated depolarization. However, it is important to mention that experiments should still be performed to investigate the mechanism behind the opening of CaCCs, which is prevented when intracellular Ca\(^{2+}\) is chelated (chapter 3).

In complement with these fast processes, changes in [Ca\(^{2+}\)] can instantaneously induce Cl\(^{-}\} currents through CaCCs (Osipchuk et al. 1990). The long delay (several minutes) we reported in chapter four could be due to the time it takes for eCBs to travel to their binding sites on intracellular CB\(_2\)Rs. This delay could be enhanced by the fact that the whole-cell patch clamp method is associated with the dialysis of the cytosol with pipette solution, effectively reducing the concentration of the intracellular contents. In chapter three, we have shown that the delay following application of synthetic cannabinoids until the CaCC-mediated depolarization of the cell could be reduced by the intracellular application of the ligand. This is indicative of the slow penetration of cannabinoid ligands into the cell. The lipophilic nature of both synthetic cannabinoids and eCBs enable these compounds to enter lipid membranes.

Researchers have investigated whether eCBs exiting membranes is a passive or active process (Ligresti et al. 2004; Chicca et al. 2012; Fowler 2012; Fu et al. 2012). Despite 2-AG being the predominant eCB in the CNS, 2-AG transport has received
relatively little attention. In contrast, AEA transport has been the topic of intense investigation. Initially, the intracellular degradation of AEA by FAAH was thought to drive AEA uptake, but several studies reported that specific transporters mediate bidirectional transport of this eCB and that a carrier protein can shuttle AEA to intracellular targets (Fowler 2012; Fu et al. 2012). Recently, it was demonstrated that a common eCB membrane transporter controls the cellular AEA and 2-AG trafficking in both directions (i.e. release and uptake) and metabolism (Chicca et al. 2012). The authors indicate that this eCB membrane transporter could play an important role in various aspects of eCB signalling, since eCBs bind to several intracellular targets such as PPARs and intracellular cannabinoid receptors, as well as to extracellular targets. Despite the fact that to date no intracellular carrier protein has been described for 2-AG, the existence of such a protein is an intriguing possibility which could help explain our findings regarding the delay of the CB2R-mediated response.

Opening and function of CaCCs
Interest in Cl- channels in general has been driven by the finding that multiple human diseases are Cl- channelopathies, like cystic fibrosis (Duran et al. 2010). However, neuronal Cl- channels have received relatively little attention from researchers, compared to cation channels. In immature neurons with high [Cl-]i, GABA produces depolarizing postsynaptic potentials via Cl- conducting GABA_A receptors, which could play a role in stabilizing developing synapses (Ben-Ari et al. 2007). In mature neurons, [Cl-]i is much lower, so that Cl- ions are in electrochemical equilibrium across membranes. In these mature neurons, the reversal potential of IPSCs mediated by GABA_A receptors, is very close to the resting membrane potential. Maintaining appropriate [Cl-], following Cl- influx when GABA_A receptors are activated is typically attributed to several cation Cl- cotransporters, most notably the K+-Cl- cotransporter (Blaesse et al. 2009). The dramatic changes in [Cl-], can occur slowly during the development from immature to mature neurons. More acute changes in [Cl-], may occur in response to (sustained) synaptic activity due to the accumulation of intracellular Cl- and, following that, the local collapse of the Cl- gradient (Kuner and Augustine 2000; Isomura et al. 2003; Berglund et al. 2006).

In chapter three, we have described the opening of CaCCs following the activation of CB2Rs with synthetic cannabinoids. We reported that the opening of these channels is dependent on a rise in [Ca2+]i, mediated by IP3Rs (Fig. 1). It is known that CaCCs can be activated by Ca2+ influx through VDCCs, ligand-operated Ca2+ channels, as well as Ca2+ release from intracellular stores (Frings et al. 2000). Alternatively, some CaCCs may not possess a Ca2+ binding site, but instead are activated by Ca2+/calmodulin-dependent protein kinase II (CaMK II) (Hartzell et al. 2005). In some neurons, CaCCs can be localized in the dendritic membrane, so that their contribution to the processing of synaptic input depends on local Cl- concentrations (Frings et al. 2000). Their activation can amplify or attenuate the cellular response to such input. Recent years have seen important developments in the field of CaCCs (Flores et al. 2009; Hartzell et al. 2009) with the almost simultaneous publications of three papers that reported the description of the transmembrane protein Anoctamin 1 (also known
as TMEM16A) as a CaCC (Caputo et al. 2008; Schroeder et al. 2008; Yang et al. 2008). At least one other member of the Anoctamin family, Anoctamin 2, generates Ca\(^{2+}\)-activated Cl\(^{-}\) currents. Aside from Anoctamins, other, more controversial transmembrane proteins could also function as CaCCs (Sun et al. 2002). Reports on the molecular identity of CaCC proteins could help us to understand details of the functioning of these channels. Some important questions concern the exact localization of these channels as well as their sensitivity to [Ca\(^{2+}\)]_i and CaMK II. In particular, future knowledge on the molecular identity of CaCCs should help with the development of specific antibodies, which in turn could provide us with information on the subcellular localization of these channels.

As described in chapters 3 and 4, CB\(_2\)R activation under conditions with high [Cl\(^{-}\)]\(_i\), resulted in transient, but relatively long-lasting (several min) depolarizations. In experiments in chapters 3 and 4 we examined CB\(_2\)R-mediated modulation of neuronal excitability with a physiologically relevant [Cl\(^{-}\)]\(_i\), where the reversal potential for Cl\(^{-}\) was close to the resting membrane potential (~70 mV). Under these experimental conditions, which were designed to more closely mimic the physiological situation for mature neurons, the opening of CaCCs did not (or hardly) mediate a Cl\(^{-}\) current. Under these conditions, the opening of CaCCs reduced the input resistance of the cell in a process which is reminiscent of GABA\(_A\) receptor-mediated shunting inhibition. This could be the process through which CB\(_2\)R-activation affects neuronal excitability. If CB\(_2\)Rs are functionally expressed by immature neurons, the activation of these receptors should result in the depolarization of the cell. It would be interesting to investigate whether such CB\(_2\)R-mediated responses can be detected in immature neurons.

Figure 1. Schematic overview of the proposed eCB signalling pathway. Ca\(^{2+}\)-entry through VDCCs induces eCB synthesis. eCBs then bind to intracellular G\(_{i/o}\)-coupled CB\(_2\)Rs which results, via the activation of PLC, into IP\(_3\) production. IP\(_3\) activates IP\(_3\)Rs and this induces release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores, which in turn leads to the opening of CaCCs.
CB₁R-mediated modulation of neurotransmission

There is broad consensus regarding the notion that CB₁Rs mediate the vast majority of their modulatory effects in the CNS from a presynaptic locus (Chapter 1, Fig. 1). The initial discovery of eCB-mediated retrograde signalling via the activation of CB₁Rs was followed by many descriptions of this mechanism in different brain areas (Kano et al. 2009; Castillo et al. 2012). However, non-retrograde, autocrine eCB signalling via CB₁Rs has also been reported (Bacci et al. 2004). In cortical interneurons and layer II/III pyramidal neurons, repetitive AP firing can trigger a CB₁R-dependent postsynaptic hyperpolarization via the opening of GIRK channels (Bacci et al. 2004; Marinelli et al. 2008, 2009). This process was termed somatodendritic slow self-inhibition (SSI) and provides an alternative route via which CB₁Rs can influence neuronal communication.

In chapter five, we demonstrated the presynaptic localization of CB₁Rs by means of electrophysiology and immunohistochemistry. We reported a reduction of the frequency of both mEPSCs and mIPSCs following CB₁R activation. These findings are consistent with a presynaptic localization for CB₁Rs and were confirmed by the immunohistochemical visualization of CB₁Rs on axonal fibers. Importantly, both the observed CB₁R-mediated reduction in the frequency of mEPSCs and mIPSCs and our immunohistochemical data are in line with results from earlier studies performed in the PFC, which reported modulation of inhibitory and excitatory input (Lafourcade et al. 2007; Yoshino et al. 2011).

In chapter five, we performed CB₁R internalization experiments and with immunohistochemistry we showed that most of the CB₁R-positive neurons did not express the interneuron marker GAD65/67. Although it is likely that some GABAergic neurons did not display a detectable amount of GAD65/67, we assume that most of the CB₁R-positive neurons that were GAD65/67 negative were non-GABAergic, principal neuron. Most investigations into the localization of CB₁Rs report their predominant presence on inhibitory interneurons, in particular the subtypes of GABAergic interneurons that contain the neuropeptide cholecystokinin (CCK) or the Ca²⁺-binding protein calbindin (CaBP) (Katona et al. 1999; Bodor et al. 2005; Eggan et al. 2010; Thibault et al. 2012). However, CB₁Rs have also been detected in the rodent brain at excitatory synapses where they were shown to modulate the release of glutamate (Auclair et al. 2000; Katona et al. 2006; Kawamura et al. 2006). It has been reported that the relative CB₁R expression in interneurons and principal neurons may depend on the developmental stage of the CNS (Vitalis et al. 2008). In a recent paper, authors describe that, in a mouse model of Huntington’s disease, the functionality of CB₁Rs in GABAergic and glutamatergic neurons is differently affected (Chiodi et al. 2012). As a result, the net effect of CB₁R activation is profoundly altered in these animals, compared to healthy wild type mice. This could indicate an association between appropriate functioning of CB₁Rs in both neuron types and proper CNS functioning.
CB₁R activation modulates the E/I balance
Although inhibitory interneurons constitute only ~20% of the neocortical neuronal population, they are heavily connected and are able to match and balance excitatory input conductance derived from the remaining ~80% excitatory neurons (Peters and Kara 1985; Somogyi et al. 1998; Markram et al. 2004). In fact, the relative contribution of excitatory and inhibitory input conductance is dynamically maintained at ~20% excitation and ~80% inhibition (Le Roux et al. 2006, 2007, 2008; Zhang et al. 2011). As discussed in chapter five, we found that CB₁R activation in the mPFC reduced both inhibitory and excitatory spontaneous neurotransmission onto layer II/III pyramidal cells by ~30%. This means that, despite our predominant detection of CB₁Rs on non-GABAergic cells, CB₁R activation affects spontaneous inhibitory and excitatory neurotransmission similarly. This could be explained by the multitude of inhibitory synaptic contacts made by only a small amount of inhibitory interneurons.

When we investigated the E/I balance in layer II/III pyramidal neurons of the mPFC we found that this balance was very close to previously reported values at ~20% excitation and ~80% inhibition (Le Roux et al. 2006, 2007, 2008). The activation of CB₁Rs with a synthetic agonist resulted in the reduction of both inhibitory and excitatory conductances. However, the relatively larger reduction in inhibitory conductance meant a shift in the E/I balance towards excitation. If the E/I balance is shifted, one would expect that, overall, neurons are exposed to more excitatory input than normally, which increases the chance of firing APs. In this way, a changed E/I balance could have profound physiological consequences. Deviations from the ‘normal’ E/I balance (~20~/~80%) are associated with a range of neuropsychiatric disorders, such as epilepsy, schizophrenia and autism spectrum disorders (Cobos et al. 2005; Lewis et al. 2005; Rubenstein 2010). A causal relationship between an increased E/I balance in the mPFC and behavioural deficits and social dysfunction was uncovered in a recent study (Yizhar et al. 2011). These findings and our data described in chapter five suggest the possibility that CB₁R activation following cannabis intake could alter the E/I balance which in turn could result in behavioural deficits. The effects of cannabis intake on executive functioning are thought to be mediated predominantly by CB₁Rs and the involvement of this receptor in the modulation of the E/I balance forms an interesting potential mechanism to explain neuropsychological deficits associated with cannabis use (Ledent et al. 1999; Huestis et al. 2001; Grant et al. 2012). In addition to overactivation of CB₁Rs by exogenous cannabinoids, physiological activation of CB₁Rs (either by induced eCB release or the basal eCB tone) could play a role in the maintenance of the proper E/I balance.

CB₂R and CB₁R interplay
The findings represented in chapters three and four show the presence and functionality of intracellular CB₂Rs in pyramidal neurons. CB₂R activation leads to the
opening of CaCCs and the reduction of neuronal excitability in the mPFC. The findings in chapter five demonstrate that CB1R activation modulates both excitatory and inhibitory neurotransmission and changes the E/I balance in favour of excitation in the same brain region. Taken together, these studies show the diverse mechanisms by which the eCB system can play a role in the modulation of neuronal communication. The intracellular presence of CB2Rs and the CB2R-mediated effects described in chapters three and four have not been reported earlier and discredit the general assumption to exclude these receptors from investigations into the CNS eCB system.

Since similar stimulation protocols can induce CB1R and CB2R activation, it is a distinct possibility that both cannabinoid receptors may be activated following certain patterns of neuronal activity, although perhaps at a different time scale. The activation of presynaptic CB1Rs is reported to immediately follow the induced synthesis of eCBs in short-lasting (~1 min) phenomena such as DSI and DSE (Kreitzer and Regehr 2001; Wilson et al. 2001; Fortin et al. 2004; Yoshino et al. 2011). In chapter four, we have demonstrated that, under our experimental conditions, a CB2R-mediated Cl\(^{-}\) current could be recorded only after a (highly variable) delay of ~2.5 min. As previously discussed, the delay we reported could be an overestimation of the delay in a more physiological situation, due to our experimental conditions. Nonetheless, there seems to be a marked difference between the timescale at which CB1Rs and CB2Rs are activated following induced eCB synthesis. This means that the CB1R-mediated reduction of the release of glutamate at an excitatory synapse will precede the transiently reduced excitability of the postsynaptic principal neuron through CB2Rs, in the case of eCB synthesis in this postsynaptic neuron. This hypothetical principal neuron would initially experience a reduced activation of its glutamate receptors, followed by a reduction in excitability due to the opening of CaCCs. In the case of longer lasting CB1R-mediated effects, such as eCB-LTD, CB1R-mediated effects and CB2R-mediated effects may exist simultaneously. This would mean that the neuron might receive reduced synaptic input and that it is less sensitive to synaptic input at the same time. In the mPFC, pharmacological activation of CB1Rs leads to a decrease in both excitatory and inhibitory input conductance, summing up to the net effect of a relative decrease in inhibitory input conductance. If CB2Rs are activated simultaneously, the relative increase in excitatory input conductance could be balanced by the CB2R-mediated opening of CaCCs, which leads to a reduction of neuronal excitability. Taking into account the CB1R and CB2R, the eCB system is endowed with powerful means to influence both synaptic input (on a short and long time-scale) and the neuronal sensitivity to synaptic input (on a long time-scale).

Complicating the situation further, a recent article reports that CB1Rs and CB2Rs can form plasma membrane heteromers in transfected neuronal cells and tissue from subcortical brain regions (Callén et al. 2012). The authors show that heteromer formation led to bidirectional cross antagonism (i.e. the ability of a CB1R antagonist to
block the effect of a CB$_2$R agonist and the ability of a CB$_2$R antagonist to block the effect of a CB$_1$R agonist). The authors suggest that these heteromers could be the basis for several contradicting and controversial reports regarding the expression levels of CB$_1$Rs and CB$_2$Rs and their activation by different levels of ligands. More research is required to elucidate the role of plasma membrane CB$_1$R-CB$_2$R heteromers in the eCB system in the CNS.

Taken together, results from the data presented in this thesis show that CB$_1$Rs and CB$_2$Rs are in a powerful position to modulate neurotransmission, the balance between inhibitory and excitatory input conductance and neuronal excitability. CB$_1$Rs are abundantly expressed in the CNS and most of our knowledge on the eCB system in the brain derives from investigations into CB$_1$R-functioning. Recent developments have highlighted the importance of investigations into CB$_2$R-functioning (Elmes et al. 2004; Jhaveri et al. 2008; Morgan et al. 2009; Xi et al. 2011). The results described in chapters three and four of this thesis are relevant additions to this work and strengthen the importance of exploring the role CB$_2$R in the CNS.

Concluding remarks and future directions
In this dissertation I have investigated how the eCB system can exert its effects through the two GPCRs currently classified as cannabinoid receptors, in layer II/III pyramidal neurons of the mPFC. The work presented in chapters three and four represent the first descriptions of neuronal CB$_2$Rs as intracellular receptors that, upon activation, can induce a Cl$^{-}$ current. The surprising localization of these receptors brings to mind various questions, some of which have been discussed earlier. One of the most obvious questions concerns the exact localization of intracellular CB$_2$Rs. Since reliable antibodies against CB$_2$Rs for immunohistochemistry are currently unavailable, experiments with fluorescent CB$_2$R ligands could be performed in order to answer this question. In fact, such a fluorescent ligand exists and it has been used to evaluate CB$_2$R binding in high throughput screening in cultured cell lines expressing CB$_2$Rs (Sexton et al. 2011). However, ligands that may be used in slice experiments are still to be discovered.

Another question regarding the localization and downstream signalling pathway of CB$_2$Rs concerns the expression of these receptors by inhibitory interneurons. Since cortical interneurons can synthesize eCBs (Bacci et al. 2004), it would be very useful to investigate whether interneurons show a delayed reduction in excitability due to the opening of Cl$^{-}$ channels, mediated by CB$_2$Rs. Answering the question whether CB$_2$Rs are exclusively expressed by pyramidal neurons or also by inhibitory interneurons would provide valuable information for understanding the role of CB$_2$Rs at the network level. The question of CNS-wide generalizability of the described CB$_2$R-mediated effects is also an important issue. It would be very interesting to perform additional studies to
determine the presence, as well as the function of CB₂Rs in principal neurons in other layers of the cortex and other brain areas.

We have reported, in **chapter five**, that CB₁R activation resulted in the shift of the E/I balance in favour of excitation. These findings raise the question whether CB₂R activation by Δ⁹-THC in cannabis can cause a similar shift in this balance. Future studies into the change of the E/I balance by modulation of the eCB system could increase our understanding of the effects of cannabis use on the brain.

It is known that both cannabinoid receptors play a role during neurodevelopment. In light of the findings presented in **chapter five**, it would be very interesting to investigate the involvement of the eCB system in the proper wiring of the mPFC network. Such experiments could be of particular importance for evaluating of the risk of cannabis use in pregnant mothers.

The eCB system represents possible therapeutic targets for a wide range of pathologies including neurodegenerative disorders (such as multiple sclerosis and Parkinson’s disease), neuropsychiatric disorders (such as depression and schizophrenia), epilepsy, ischemia, neuropathic and inflammatory pain, autoimmune and cardiovascular and gastrointestinal diseases (Vinod and Hungund 2006; Bisogno and Di Marzo 2007; Di Marzo 2009; Fernández-Ruiz et al. 2010; Pacher and Mechoulam 2011; Skaper and Di Marzo 2012). Within the eCB system, the CB₂R could be of particular interest as therapeutic target, since the psychoactive effects of cannabis are believed to be mediated exclusively by CB₂Rs. However, it must be noted that, since the eCB system is abundantly present in the CNS and the periphery, the risk of side effects of cannabinoid compounds is large. This is illustrated by the development of the selective CB₁R antagonist/inverse agonist rimonabant, which was used as an anti-obesity drug. Rimonabant was redrawn from the market in 2009 due to reports of increased risk of psychiatric events such as depressed mood disorders, anxiety and an increased risk of suicide (Christensen et al. 2007). Clearly, knowledge of the basic properties of the eCB system is essential for the development of new eCB system-modulating therapeutic drugs which display less side effects.