Cannabinoid receptor function in the medial prefrontal cortex

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Chapter 1

General introduction
The endocannabinoid system

The term ‘endocannabinoid (eCB) system’ refers to a group of endogenous modulatory lipids called endocannabinoids (eCBs) that play a role in various physiological processes, both in the brain and in the periphery (Kano et al. 2009). In addition to the endogenous ligands, the eCB system contains at least two G protein-coupled receptors (GPCRs), cannabinoid type-1 receptors (CB1Rs) and cannabinoid type-2 receptors (CB2Rs), enzymes responsible for the synthesis and degradation of eCBs and eCB transporter proteins. The eCB system is present throughout the central nervous system (CNS) and the periphery and is involved in many functions of the CNS, including decision-making, working memory and other executive functions (Pattij et al. 2008).

Compounds interacting with the eCB system are found in nature. The most widely known cannabinoids are found in the plant *Cannabis sativa*. Cannabis, obtained from this plant, is one of the most widely used drugs of abuse worldwide. The major psychoactive component of cannabis, Δ⁹-tetrahydrocannabinol (Δ⁹-THC), was discovered in 1964 (Gaoni and Mechoulam 1964). Δ⁹-THC increases dopamine release in the striatum in animals and humans (Tanda and Goldberg 2003; Gardner 2005; Bossong et al. 2009). This characteristic of Δ⁹-THC is associated with the abuse liability of cannabis (Maldonado and Rodríguez de Fonseca 2002; Maldonado et al. 2011).

Since the discovery of Δ⁹-THC, other cannabinoids have been isolated from *Cannabis sativa* and synthetic cannabinoids have been discovered (Mechoulam and Hanus 2000; Pertwee et al. 2010). Although cannabis is classified as an illegal substance in most countries, cannabis is now officially prescribed to relieve symptoms of several diseases. For example, cannabis and Δ⁹-THC are used as antiemetic in patients with cancer and acquired immune deficiency syndrome (AIDS) and for the treatment of chronic pain in patients with cancer and spasticity in multiple sclerosis. Cannabis use in adolescents is associated with an increased risk of schizophrenia and chronic cannabis use with a reduction in cognitive control and neuropsychological decline (Arseneault et al. 2002; Harding et al. 2012; Meier et al. 2012). Medicinal use of cannabis is increasing, while our current understanding of the eCB system in the CNS is far from complete. eCB research aims to discover the role of the eCB system in the regulation of brain functions that are involved in psychopathological syndromes, such as addiction and cognitive disorders.

The work in this thesis was performed as a part of a TIPharma research consortium with the title ‘The neurophysiological role of the eCB system in support of smoking cessation, fighting addiction and treating cognitive decline’. The goal, as defined by this research consortium, is to elucidate the role of the eCB system in the development of psychological disorders. The consortium consists of several research groups, each with their own research techniques and specific research questions. The experimental work presented here relies on the use of electrophysiological and biochemical techniques and aims to investigate the functioning of the eCB system at the cellular and local network level. The targeted brain area is the medial prefrontal cortex (mPFC), a brain region involved in higher cognitive functions, such as working memory, planning complex behaviour, decision making and impulse control.
The mPFC has been implicated in various psychopathological and cognitive disorders and has traditionally received attention from researchers in the addiction field. Furthermore, although it is known that the eCB system is present in the mPFC, a detailed description of the functioning of this system in the mPFC is lacking. Here, our main research questions concern the role of CB receptors in neuronal communication, at the level of the local cortical network and individual neurons.

The eCB system and neuropathologies
Recent years have seen an increase in research into the involvement of the eCB system in a growing number of neuropathologies, such as multiple sclerosis, Huntington’s, Parkinson’s and Alzheimer’s diseases, schizophrenia, autism and epilepsy. Some research has focused on the potential role of cannabinoid ligands as therapeutics, whereas others have investigated harmful effects of cannabinoids (Arseneault et al. 2002; Scotter et al. 2010; Harding et al. 2012; Meier et al. 2012). A recent report shows that persistent cannabis use, particularly among adolescent-onset cannabis users, was associated with neuropsychological decline across several domains of functioning. Among adolescent-onset users, cessation of cannabis use did not fully restore performance on a broad range of neuropsychological tests (Meier et al. 2012). The mechanisms underlying the effects of persistent cannabis use in adolescents are unclear, but could be explained by effects of cannabinoid compounds on the still developing brain (Jager and Ramsey 2008; Rubino et al. 2009).

Cannabis use has been associated with the development of psychoses and schizophrenia by a multitude of studies performed in the last 50 years (Andréasson et al. 1987; Fergusson et al. 2006; Malone et al. 2010). However, whether cannabis use causes psychotic symptoms, precipitates them (in ‘schizophrenically vulnerable’ individuals) or can be seen as a form of self-medication by individuals prone to psychosis is still under debate. There is no consensus over the mechanisms that could be responsible for these negative effects of cannabis use, that may occur especially around adolescence. It has been suggested that the involvement of the eCB system in moderating neurodevelopmental processes could play a role (Malone et al. 2010).

The eCB system and CNS development
The development of the CNS involves dynamically regulated cellular processes such as cell generation, differentiation, migration, maturation and proper wiring. Developmental neurobiological studies are increasingly able to identify the molecular mechanisms underlying the specification and differentiation of particular neuronal cells. Several studies have shown that prenatal exposure to cannabinoids affects behavioural aspects regarding the control of emotions and cognitive responses and these results have further increased the interest for cannabinoid-mediated effects on CNS development (D’Souza et al. 2009; Jutras-Aswad et al. 2009; Diaz-Alonso et al. 2012). Early in development, eCBs act as signalling cues in ‘neurogenic niches’, microenvironments where neurons and glia are generated. Activation of the CB$_1$R plays an important role in neuronal fate decisions, such as cell cycle progression and
proliferation, neural cell specification and cell migration, morphogenesis and apoptosis. Importantly, dysfunction of the eCB system during CNS development may be involved in the onset of epileptogenesis, via an imbalance in excitatory and inhibitory neurotransmission (Katona and Freund 2008; Lutz and Monory 2008). During later, postnatal stages of development, exposure to cannabinoids or altered eCB signalling disrupts neuronal maturation and interferes with neuronal connectivity of developing networks, which could have consequences for neuronal functioning in the adult (Jutras-Aswad et al. 2009). To summarize, the eCB system is present in the CNS from an early stage and plays a key regulatory role in CNS development. Affecting the eCB system during development can have significant consequences for the adult brain, such as the tuning of the appropriate balance between excitatory and inhibitory input and the potential onset of neuropsychiatric disorders (Diaz-Alonso et al. 2012).

Cannabinoid receptors
CB1R expression
Following the discovery of Δ9-THC, the CB1R was cloned and characterized in 1990 (Matsuda et al. 1990). CB1Rs are one of the most abundantly expressed GPCRs in the CNS and are predominantly located on presynaptic terminals (Herkenham et al. 1991). CB1Rs are also expressed at much lower, yet functionally relevant, levels in various peripheral tissues (Hohmann and Herkenham 1999; Navarro et al. 2002; Bensaïd et al. 2003; Osei-Hyiaman et al. 2005). In the CNS, particularly high expression levels for CB1Rs are detected in the cerebellum and telencephalon, which is compatible with the effects of Δ9-THC on motor and cognitive functions. The CB1R mediates most psychoactive effects of cannabis (Ledent et al. 1999; Huestis et al. 2001). CB1Rs are expressed at low levels in lower brain stem areas that are responsible for the regulation of cardiovascular and respiratory functions. The relatively low expression of CB1Rs in brain areas involved in vital functions has been linked to the fact that even high doses of cannabinoids are not lethal (Herkenham et al. 1991). Similarly, analgesic, anti-anorexic and antiemetic effects of cannabinoids can be explained by CB1R expression in the spinal dorsal horn, the ventromedial hypothalamic nucleus and the caudal solitary nucleus (Kano et al. 2009).

CB1R signalling
Since their discovery, the expression and functionality of the CB1R in the CNS has been thoroughly investigated (Devane et al. 1988; Kano et al. 2009; Castillo et al. 2012; Katona and Freund 2012). CB1Rs are coupled to Gi/o proteins and activation of the receptor inhibits adenylyl cyclase and cAMP production in a variety of cells, including neurons expressing CB1Rs. However, under certain conditions, CB1R-coupling to Gs proteins has also been reported, thereby increasing cAMP production (Demuth and Molleman 2006). Furthermore, CB1Rs have been shown to link positively to mitogen-activated protein (MAP) kinase, a key signalling pathway that regulates many cellular functions, such as cell growth and apoptosis (Bouaboula et al. 1995; Demuth and Molleman 2006). MAP kinases can be stimulated through G protein-mediated mechanisms and activate transcription factors, which in turn modulate gene
expression. Importantly, CB₁R activation also modulates certain ion channels. Activation of CB₁Rs induces the opening of A-type K⁺ channels and G protein-coupled inwardly rectifying K⁺ (GIRK) channels and it inhibits N- and P/Q type voltage-dependent Ca²⁺ channels (VDCCs) as well as D- and M-type K⁺ channels (Hampson et al. 1995; Mackie et al. 1995; Twitchell et al. 1997; Mu et al. 1999; Schweitzer 2000; Kano et al. 2009). The inhibition of VDCCs and activation of GIRK channels upon presynaptic CB₁R activation is linked to the most commonly described effect of CB₁R activation, the suppression of neurotransmitter release, which will be described below.

**Cellular response to CB₁R activation**

The principal mechanism by which the eCB system is reported to play a functional role via CB₁Rs in neurons, is retrograde signalling (Kreitzer and Regehr 2001; Wilson and Nicoll 2001; Kano et al. 2009). Postsynaptic activity, consisting of Ca²⁺ influx and/or activation of GPCRs such as group 1 metabotropic glutamate receptors, leads to the synthesis of eCBs. eCBs travel backwards across the synapse and bind to a transmembrane binding site on the presynaptic CB₁R. There, activation of presynaptic CB₁Rs suppresses neurotransmitter release mainly by inhibition of Ca²⁺ influx through VDCCs and possibly the activation of GIRK channels (Fig. 1) (Daniel and Crepel 2001; Kreitzer and Regehr 2001; Wilson et al. 2001; Brown et al. 2003; Guo and Ikeda 2004). Neurotransmitters of which the release is modulated by CB₁R activation include the main excitatory and inhibitory neurotransmitters glutamate and GABA. CB₁R activation also inhibits the release of other neurotransmitters such as glycine, acetylcholine, dopamine, 5-HT and norepinephrine (Kano et al. 2009). CB₁R activation is involved in several forms of synaptic plasticity, both short-term and long-term, which will be discussed in more detail later on.

**CB₂R expression**

The second cannabinoid receptor, the CB₂R, was discovered in macrophages in 1993 (Munro et al. 1993). The human CB₂R shares 44% amino acid sequence identity with the human CB₁R and has a distinct pharmacological profile and expression pattern. The presence of CB₂Rs in the brain has been a matter of debate. Initially, CB₂Rs were not detected in the CNS. They were found to be predominantly expressed in immune cells and tissues outside the CNS and therefore called ‘peripheral’ cannabinoid receptors (Galiègue et al. 1995). Many conflicting reports on CB₂R expression in the brain have been published, varying from reports of widespread expression to complete absence in the CNS. For instance, the presence of CB₂R mRNA in the brain and CB₂Rs in neurons of the brainstem, cerebellum and hippocampus has been reported by various groups (Van Sickle et al. 2005; Gong et al. 2006; Brusco et al. 2008a, 2008b; Onaivi et al. 2008, 2012; García-Gutiérrez et al. 2010). The abundant presence of CB₂Rs was questioned by others, claiming that problems with visualization of CB₂Rs by immunohistochemical
stainings compromise reliable detection of CB2R brain expression (Atwood and Mackie 2010; Ashton 2012). Nevertheless, the presence of CB2Rs in microglia cells and other brain immune cells is now generally accepted (Stella 2010). CB2Rs were detected in perivascular microglia in the non-inflamed human brain; the current view supports the expression of functional CB2Rs in neurons upon brain stress or damage (Núñez et al. 2004; Viscomi et al. 2009).

CB2R signalling
CB2Rs couple to G\textsubscript{i/o} proteins and thereby inhibit adenylyl cyclase and cAMP production and stimulate the MAP kinase pathway (Bouaboula et al. 1996; Kobayashi et al. 2001). However, modulation of [Ca\textsuperscript{2+}], following CB2R activation has also been reported (Beltramo 2009). In calf pulmonary endothelial cells, activation of CB2Rs led to an increase in [Ca\textsuperscript{2+}], via the phospholipase C- (PLC) dependent production of inositol trisphosphate (IP\textsubscript{3}) (Zoratti et al. 2003). In contrast, in cardiac cells and adult dorsal root ganglion neurons, CB2R activation is negatively coupled to IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores (Sagar et al. 2005; Currie et al. 2008). Until recently, CB2Rs were assumed to poorly modulate Ca\textsuperscript{2+} channels, GIRK channels and other ion channels, unlike CB1Rs (Atwood and Mackie 2010). However, recent papers have demonstrated CB2R-mediated modulation of VDCCs (Atwood, Straiker, et al. 2012).

Cellular response to CB2R activation
Since CB2Rs were presumed absent from the CNS for many years, much less research has been performed to investigate the role of these cannabinoid receptors in the brain. However, several recent publications have demonstrated the functional presence of CB2Rs in the CNS by applying or administrating CB2R ligands, although the mechanism(s)
of action was/were unclear (Elmes et al. 2004; Jhaveri et al. 2008; Liu et al. 2009; Morgan et al. 2009; Xi et al. 2011; Atwood, Straiker, et al. 2012). These studies show varying roles for CB2Rs, for instance in the modulation of pain responses in the thalamus and dorsal horn neurons of rats in pain models (Elmes et al. 2004; Jhaveri et al. 2008). Others show that CB2Rs behave similarly to CB1Rs in the modulation of neurotransmitter release (Morgan et al. 2009; Atwood, Straiker, et al. 2012). Finally, other researchers found that brain CB2Rs modulate the rewarding and locomotor-stimulating effects of cocaine, likely occurring through a dopamine-dependent mechanism (Xi et al. 2011). These recent studies indicate that CB2Rs are functionally present in the CNS, but, so far, lack the description of the CB2R-mediated signalling pathways.

Other receptors activated by eCBs
In addition to CB1R and CB2R, other receptors that are sensitive to eCBs exist. Recently, an orphan GPCR called the GPR55 receptor has been shown to be activated by eCBs and phytocannabinoids, although this is cell- and tissue type-dependent (Sharir and Abood 2010). This has led to the hypothesis that GPR55 could be a third cannabinoid receptor. Relatively low expression levels of GPR55 have been detected in the brain. GPR55’s pharmacology remains controversial, although it is certainly distinct from that of CB1R and CB2R in terms of sensitivities for cannabinoid ligands (Sharir and Abood 2010; Zhao and Abood 2013). Future research on the GPR55 should determine whether GPR55 can be classified as a genuine cannabinoid receptor. It is important to note that it is not considered a CB receptor currently.

Another receptor that can be activated by eCBs is a Ca\(^{2+}\)-permeable, non-selective cation channel, which belongs to the transient receptor potential family, subfamily V member 1 (TRPV1). This receptor is peripherally present and is involved in pain signalling. It can be activated by heat greater than 43 °C, capsaicin (found in hot chili peppers) and allyl isothiocyanate (found in mustard and wasabi). Surprisingly, TRPV1 receptors are also expressed in the CNS, where their activation by heat and these chemicals are unlikely. Endogenous ligands that activate TRPV1 receptors are called endovanilloids and include some, but not all eCBs, as discussed below (Kano et al. 2009). In the CNS, these receptors are involved in synaptic plasticity (Gibson et al. 2008). TRPV1 receptors are not considered as components of the eCB system, because they cannot be activated by 2-AG and several synthetic cannabinoid ligands. However, shared ligands and similar tissue distribution, as well as opposing actions on the same intracellular signals imply a cross-talk between the eCB system and the endovanilloid system (Starowicz et al. 2007; Kano et al. 2009).

Finally, another class of receptors that can be activated by eCBs are peroxisome proliferator-activated receptors (PPAR). These nuclear receptors function as transcription factors regulating gene expression and are involved in cellular differentiation, lipid metabolism and inflammation (Burstein 2005; O’Sullivan 2007).
2-AG and anandamide

The two most important eCBs are N-arachidonoylenolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). The synthesis of both AEA and 2-AG, as briefly mentioned earlier, depends on cellular activity. The increase in $[\text{Ca}^{2+}]_i$, following neuronal depolarization and/or stimulation of GPCRs that are coupled to $G_{q/11}$ proteins, induce synthesis of both AEA and 2-AG, as discussed in more detail below.

The first identified eCB, AEA, was isolated from the porcine brain and named ‘anandamide’ after the Sanskrit word ‘ananda’ that means bliss (Devane et al. 1992). AEA is an agonist for CB$_1$Rs and CB$_2$Rs and is also an endogenous ligand for TRPV1 receptors and PPARs. The main pathway for AEA synthesis involves the activity of the enzyme N-acyltransferase (NAT). Activity of (most) NAT enzymes is stimulated by Ca$_2^+$ and this is considered the rate-limiting step in AEA synthesis. This enzyme converts arachidonic acid-containing phospholipids in the plasma membrane into N-acyl phosphatidylethanolamine (NAPE). NAPE is then cleaved by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), resulting in the formation of AEA (Kano et al. 2009; Luchicchi and Pistis 2012). Alternative pathways for AEA synthesis have also been investigated and described, driven by the observation that NAPE-PLD knock-out (KO) mice showed unchanged levels of AEA (Simon and Cravatt 2010). AEA is broken down predominantly by fatty acid amide hydrolase (FAAH), but - like for the synthesis of AEA - alternative pathways exist. For instance, N-acylphosphatidylethanolaminehydrolyzing acide amidase (NAAA), cyclooxygenase-2 (COX-2), lipoxigenase isoenzymes (LOX) and the P-450 cytochrome can break down AEA (Bornheim et al. 1993; Ueda et al. 1995, 2010; Yu et al. 1997) (Fig. 2.). The extent to which these alternative degradation pathways are physiologically relevant, remains to be investigated.

The second important eCB, 2-AG, was identified a few years after the discovery of AEA (Mechoulam et al. 1995; Sugiura et al. 1995). Diacylglycerols (DAGs), the major source of 2-AG, are produced via PLC-dependent pathways (Fig. 2) (Bisogno et al. 1997). 2-AG is synthesized predominantly by diacylglycerol lipase $\alpha$ (DAGL$\alpha$) and $\beta$ (DAGL$\beta$) from DAGs. Both DAGL$\alpha$ and DAGL$\beta$ are sensitive to Ca$_2^+$ and dependent on physiological levels of glutathione. Although Ca$_2^+$ influx alone can lead to 2-AG synthesis, the simultaneous increase in PLC activity following stimulation of GPCRs that are coupled to $G_{q/11}$ proteins and Ca$_2^+$ influx is thought to lead to maximal 2-AG synthesis (Min et al. 2010). Alternative, presumably less relevant, routes for 2-AG synthesis have also been described (Fig. 2) (Luchicchi and Pistis 2012). 2-AG is principally hydrolyzed by monoacylglycerol lipase (MGL), with a more recently discovered role for a series of serine hydrolase $\alpha$-$\beta$-hydrolase domain 6 or 12 (ABHD6, ABHD12) (Mechoulam et al. 1995; Sugiura et al. 1995; Dinh et al. 2002, 2004; Marrs et al. 2010; Savinainen et al. 2012).

2-AG and AEA, which are by far the most studied eCBs, were initially regarded as very similar eCBs, in terms of their pharmacological properties. 2-AG and AEA are both found in the CNS as well as the periphery. Despite difficulties with the precise quantification of brain levels for these eCBs, there is consensus with respect to 2-AG
levels exceeding those for AEA (Stella et al. 1997; Buczynski and Parsons 2010). Since the affinities of AEA and 2-AG for CB receptors are comparable (Table 1), this indicates that 2-AG is, generally speaking, the most relevant eCB. Thanks to the development of mouse lines lacking genes for FAAH or MAGL and pharmacological tools that selectively inhibit eCB-degrading or -synthesizing enzymes, researchers have increasingly disentangled the roles for 2-AG and AEA (Luchicchi and Pistis 2012).
Since eCBs are lipids, it is generally assumed they cannot be stored in vesicles like classical neurotransmitters. They are released from the postsynaptic cell after which they cross the synaptic cleft, presumably with the help of chaperone proteins such as the FAAH-like AEA transporter (Fu et al. 2012). As described, eCB levels increase when neurons are stimulated. This has led to the concept of ‘on demand’ synthesis. It has recently been suggested that eCBs, aside from the ‘on demand’ synthesis, are present in a ‘basal pool’, being constantly produced and released (Alger and Kim 2011; Di Marzo 2011; Alger 2012). In particular for 2-AG, these pools could be related to signalling, and basal, non-signalling levels. It is now obvious that the synthesis, release and possibly storage of eCBs are organized in a complex manner.

Other eCBs
Other (putative) eCBs have also been discovered, such as dihomo-γ-linolenoyl ethanolamide, docosatetra enoyl ethanolamide, 2-arachidonyl glycerol ether (noladin ether), O-arachidonoylethanolamine (virodhamine), N-arachidonoyldopamine (NADA), palmitoylethanolamide (PEA) and oleamide. These other eCBs have varying affinities and specificities for both cannabinoid receptors. For instance, members of the N-acylethanolamide family (which also contains AEA) like dihomo-γ-linolenoyl ethanolamide and docosatetra enoyl ethanolamide, bind to CB1Rs, but have lower affinities for CB2Rs. The specific functions of these putative eCBs are still to be determined. Noladin ether also has a lower affinity for CB2Rs than for CB1Rs and its presence in the CNS is disputed (Oka et al. 2003; Richardson et al. 2007). However, a recent study demonstrated that noladin ether increased food intake in rats, possibly by increasing the incentive value of food (Jones and Kirkham 2012). Virodhamine, which was isolated from rat brain, acts as a full agonist at CB2Rs, as an antagonist or partial agonist at CB1Rs and as a partial agonist at putative cannabinoid receptor GPR55 (Sharir et al. 2012). It produces hypothermia in mice, similar to AEA (Porter et al. 2002). The eCB NADA binds to both cannabinoid receptors as well as TRPV1 receptors and has been shown to induce TRPV1-dependent cell death (Davies et al. 2010). Although the affinity of the fatty acid amide PEA for CB1R and CB2R is still under scrutiny, PEA is considered by some as a bone fide eCB (Mackie and Stella 2006). PEA plays a role in protection against inflammation and pain (Re et al. 2007). For oleamide, in vitro efficacy at the CB1R has been shown, although this is controversial (Fowler 2004; Leggett et al. 2004). Oleamide induces sleep in rats, independent of its effects on body temperature, blood pressure and heart rate (Huitrón-Reséndiz et al. 2001). A complication in the investigation of the role of oleamide as an eCB is the leaching of this compound from disposable laboratory plasticware (McDonald et al. 2008). For some of these potential eCBs, a debate regarding their qualification as eCBs is still ongoing. Additional research is needed to elucidate the pharmacological profiles and functions of these putative eCBs. Here, we will focus on the roles of the eCBs 2-AG and AEA. For binding affinities, expressed as $K_I$ values, of commonly used cannabinoid agonists and antagonists, see Table 1 and Table 2.
eCB-mediated modulation of synaptic transmission

Experiences and memories are presumed to be represented in the brain by a vast amount of interconnected networks of synapses. Changes in the strengths of these synapses, synaptic plasticity, are thought to underlie processes like learning and memory. The activation of CB1Rs is associated with several forms of synaptic plasticity, both long-term plasticity and short-term plasticity. Short-term plasticity, mediated by activation of CB1Rs lasting a few seconds, depends on the retrograde neurotransmission process of eCBs and has been described for many brain areas, e.g. hippocampus, cerebellum, basal ganglia and cortex. Two short-term plasticity phenomena called depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) are CB1R-dependent (Kreitzer and Regehr 2001; Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001).

DSI and DSE, typically lasting less than a minute, rely on postsynaptic activity triggering Ca^{2+} influx through VDCCs. In addition, other Ca^{2+} sources, such as activated NMDARs and release of Ca^{2+} from internal stores can play a role as well.

Table 1. Ki values for various, commonly used cannabinoid agonists. Ratios of Ki values indicate the selectivity for either cannabinoid receptor. Large variations are due to variable experimental conditions, e.g. different mammalian species, tissue origin, cell culture background etc. For eCBs, an additional complicating factor is the rapid metabolism of these compounds. Their potencies depend on whether their metabolic pathways are inhibited. Adapted from Fowler (2008), Pertwee et al. (2010) and Pertwee (2008).

<table>
<thead>
<tr>
<th>agonist</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (nM)</th>
<th>CB&lt;sub&gt;1&lt;/sub&gt;R</th>
<th>CB&lt;sub&gt;2&lt;/sub&gt;R</th>
<th>CB&lt;sub&gt;1&lt;/sub&gt;R/CB&lt;sub&gt;2&lt;/sub&gt;R</th>
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<tbody>
<tr>
<td>AEA</td>
<td>61-543</td>
<td>279-1940</td>
<td>0.2-0.3</td>
<td></td>
</tr>
<tr>
<td>2-AG</td>
<td>58-472</td>
<td>145-1400</td>
<td>0.3-0.4</td>
<td></td>
</tr>
<tr>
<td>Δ&lt;sup&gt;a&lt;/sup&gt;-THC</td>
<td>5-80</td>
<td>3-75</td>
<td>1-2</td>
<td></td>
</tr>
<tr>
<td>WIN-552512</td>
<td>2-123</td>
<td>0.3-16.2</td>
<td>1-2</td>
<td></td>
</tr>
<tr>
<td>CP-55940</td>
<td>0.5-5</td>
<td>0.7-2.8</td>
<td>0.7-1.8</td>
<td></td>
</tr>
<tr>
<td>JWH-133</td>
<td>677</td>
<td>3.4</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>HU-308</td>
<td>10000</td>
<td>23</td>
<td>441</td>
<td></td>
</tr>
<tr>
<td>ACEA</td>
<td>1-5</td>
<td>195-2000</td>
<td>0.01</td>
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Table 2. Ki values for various, commonly used cannabinoid antagonists. Ratios of Ki values indicate the selectivity for either cannabinoid receptor. Adapted from Lunn et al. (2008).

<table>
<thead>
<tr>
<th>antagonist</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (nM)</th>
<th>CB&lt;sub&gt;1&lt;/sub&gt;R</th>
<th>CB&lt;sub&gt;2&lt;/sub&gt;R</th>
<th>CB&lt;sub&gt;1&lt;/sub&gt;R/CB&lt;sub&gt;2&lt;/sub&gt;R</th>
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<tbody>
<tr>
<td>rimonabant</td>
<td>2-12</td>
<td>514-13200</td>
<td>0.001-0.0035</td>
<td></td>
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<tr>
<td>Sch.356036</td>
<td>4387</td>
<td>1</td>
<td>3375</td>
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<td>AM630</td>
<td>5152</td>
<td>31</td>
<td>165</td>
<td></td>
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(Castillo et al. 2012). The influx of Ca\(^{2+}\) into the cell results in the production of eCBs. Alternatively, or in addition to Ca\(^{2+}\) influx-dependent eCB production, eCB synthesis can be induced by the activation of postsynaptic G\(_{q/11}\)-coupled receptors. G\(_{q/11}\)-coupled receptors induce eCB-mediated short-term plasticity include group I metabotropic glutamate receptors (mGluR 1/5), M\(_1\)/M\(_3\) muscarinic receptors, glucocorticoid receptors, oxytocin receptors and orexin receptors (Kano et al. 2009). Activation of G\(_{q/11}\)-coupled receptors stimulates PLC\(\beta\) production. PLC\(\beta\), component of the pathway for 2-AG synthesis, is thought to act as a coincidence detector for postsynaptic activity and Ca\(^{2+}\) influx (Castillo et al. 2012).

eCB-mediated long-term synaptic plasticity has been described in several brain areas, such as the nucleus accumbens, cortex, hippocampus and cerebellum (Kano et al. 2009). The induction of eCB-mediated plasticity starts with increased glutamate release from afferents which induces the postsynaptic synthesis of eCBs, which then

![Figure 3. Molecular mechanisms that underlie eCB-mediated short- and long-term plasticity. (A) Postsynaptic activity causes Ca\(^{2+}\) influx through VDCCs (VGCCs). Increased Ca\(^{2+}\) induces eCB synthesis via activation of DAGL\(\alpha\) (DGL\(\alpha\)). Presynaptic activity can also induce eCB synthesis via activation of G\(_{q/11}\)-coupled GPRCs, such as group I mGluRs, which stimulates PLC\(\beta\) production. PLC\(\beta\) can act as a coincidence detector that integrates pre- and postsynaptic activity. DAGL\(\alpha\) promotes 2-AG synthesis, which retrogradely activates CB\(_1\)Rs to reduced neurotransmitter release. (B) Glutamate released from presynaptic neurons induces postsynaptic production of eCBs, which retrogradely activate presynaptic CB\(_1\)Rs on the original afferent (homosynaptic eCB-LTD) or other nearby afferents (heterosynaptic eCB-LTD). A G\(_{\alpha_{i/o}}\)-dependent decrease in adenylyl cyclase (AC) and PKA activity reduces neurotransmitter release. At inhibitory synapses, reduced PKA activity together with activation of Ca\(^{2+}\)-sensitive phosphatase calcineurin (CaN) alters the phosphorylation status of an unidentified presynaptic target (T) necessary for iLTD. The presynaptic active zone protein RIM1\(\alpha\) and Rab3B are necessary for iLTD. Adapted from Castillo et al. 2012.](image-url)
Chapter 1

Cannabinoid receptors in glia and astrocytes

Both cannabinoid receptors are expressed by astrocytes and glial cells and their activation regulates differentiation, function and viability of these cells (Stella 2010). Resting microglia in the healthy brain do not express (many) cannabinoid receptors, but activated microglia can express both types of receptor (Stella 2010). Activation of these receptors in tissues under cell-culture conditions (when microglia are activated) regulates some of the immune-related functions that these cells are responsible for. In astrocytes, CB1R activation controls metabolic functions and reduces the ability of astrocytes to produce inflammatory mediators (Stella 2010).

Interestingly, astrocytic CB1Rs couple to PLC via G_{q/11}, thereby increasing intracellular Ca^{2+} and inducing glutamate release from astrocytes (Navarrete and Araque 2008). These results demonstrate eCB-mediated neuron-astrocyte signalling and imply that astrocytes are involved in cannabinoid-mediated non-synaptic interneuronal communication. In the hippocampus, eCBs released by pyramidal neurons increased the probability of transmitter release at CA3-CA1 synapses. This occurs via CB1R-dependent glutamate release from astrocytes that activates presynaptic glutamate receptors. In short, eCBs can both suppress neurotransmitter release from neurons, over a short distance (< 20 µm), and enhance neurotransmitter release CB1Rs on the original afferent (homosynaptic eCB-LTD) or other nearby afferents (heterosynaptic eCB-LTD) (Castillo et al. 2012). In eCB-LTD, presynaptic CB1Rs are activated for several minutes. Their activation is only required during the induction of eCB-LTD, not for the remaining duration of eCB-LTD. The predominant mechanism underlying eCB-mediated long-term plasticity involves the α_i/o effector limb of the CB1R G protein signalling cascade, which inhibits adenylyl cyclase and the cAMP/protein kinase A (PKA) pathway (Heifets and Castillo 2009) (Fig. 3). Other forms or LTD and LTD are also known to depend on the modulation of the cAMP/PKA pathway, which can change the transmission release machinery. For eCB-LTD, a presynaptic active zone protein called RIM1α was identified as a key protein (Chevaleyre et al. 2007). RIM proteins are multidomain molecules that form an important part of the cytomatrix at the presynaptic active zone. In the nucleus accumbens, prolonged inhibition of the cAMP/PKA pathway and presynaptic P/Q-type VDCCs are necessary for eCB-LTD to occur (Mato et al. 2008). eCB-LTD has been heavily investigated in recent years and is now considered one of the best examples of presynaptic forms of long-term plasticity (Castillo et al. 2012). Most results suggest that 2-AG is the main eCB responsible for the regulation of synaptic plasticity, with a minor role for AEA. However, the ability of AEA to activate other receptors, such as TRPV1 receptors, means that AEA might modulate synaptic plasticity via different routes. Interestingly, a recent study showed that both 2-AG and AEA can induce eCB-mediated long-term depression (LTD) via CB1R activation in the striatum. Different stimulation protocols, 100 Hz and 20 Hz stimulation, induced the synthesis of AEA and 2-AG, respectively (Lerner and Kreitzer 2012).
release, via astrocytes over a larger distance (Fig. 4) (Navarrete and Araque 2010). Both of these forms of short-term plasticity are CB₁R-dependent. In addition, astrocytic CB₁Rs were found to be necessary and sufficient for spike timing-dependent LTD (Min and Nevian 2012). eCBs released from neocortical pyramidal neurons activated CB₁Rs on astrocytes, evoking the release of glutamate which activates presynaptic NMDARs. These findings demonstrate that the eCB system can influence brain functioning through activation of cannabinoid receptors on both neuronal and non-neuronal cells.

CB₂Rs are mostly known for their immunosuppressive effects in the periphery. However, CB₂R expression in astrocytes and glial cells indicate that this receptors plays a role in the CNS immune response as well. Several recent studies demonstrate a key role for the CB₂R in the regulation of macrophage/microglia functions, both under physiological and pathological conditions (Benito et al. 2008; Zurolo et al. 2010). The fact that their expression levels are increased by inflammatory stimuli indicates their involvement in pathogenesis and/or the endogenous response to injury (Maresz et al. 2005; Benito et al. 2008). Data from *in vitro* experiments and animal models demonstrate that CB₂Rs decrease glial reactivity and are part of the general neuroprotective action of the eCB system. In the human CNS, CB₂R expression was also found in immune cells. Here too, upregulation of the CB₂R serves as a response against various types of chronic CNS injury (Benito et al. 2008). A recent study confirmed induced CB₂R expression in macrophages/microglia in human epileptogenic lesions (Zurolo et al. 2010). The anti-inflammatory effects following CB₂R activation suggest that this receptor could be a target for the development of novel anti-inflammatory therapies.
The prefrontal cortex
A specific region of the neocortex called the prefrontal cortex (PFC) consists of 5 layers since layer IV is lacking in most species, although not in humans and primates. In this brain region, the anterior part of the frontal lobe, many thalamocortical inputs come from the mediiodorsal nucleus in the thalamus and terminate onto dendritic spines of layer III and layers V/VI neurons. From layer III, neurons can project locally to layers II, III and V, but also further away, to layer III neurons in the contralateral cortical area. From layers V/VI, neurons project back to the thalamus (Fig. 5) (Kuroda et al. 1998). More generally, input into layer I of the neocortex is considered to be cortical, but thalamic input also converges here (Douglas and Martin 2007; Rubio-Garrido et al. 2009).
The PFC consists of a number of interconnected neocortical areas and sends and receives projections from almost all cortical sensory systems, motor systems and many subcortical structures (Miller and Cohen 2001). In humans, the PFC can be subdivided in three major areas: orbital, medial and lateral. The orbital and medial regions in humans are involved in emotional behaviour and the lateral region plays a role in cognitive processes (Groenewegen and Uylings 2000; Fuster 2001). More specifically, dorsolateral regions of the PFC are involved in the integration of sensory and mnemonic information, the regulation of cognitive functions and actions, working memory, planning, problem solving and predicting forthcoming events (Seamans et al. 2008). The dorsolateral PFC is connected to another brain area that plays an important role in the monitoring of actions and outcomes to guide decisions, the anterior cingulate cortex (ACC, the frontal part of the cingulate cortex). In rodents, the PFC can be subdivided into regions with different connectivities, a medial, a lateral and a ventral, orbital region (Uylings et al. 2003). In terms of anatomy and

![Image](image_url)

Figure 5. Simplified example of the cortical circuit of the rodent mPFC. Excitatory terminals (open triangle) from the mediodorsal thalamic nucleus (MD) predominantly end onto dendritic spines of layer III and layers V/VI pyramidal neurons. Non-pyramidal cells in layer III also receive input from the MD. From layer III, pyramidal neurons project to other layer III pyramidal neurons. In layers V/VI, some pyramidal neurons project back to the MD. Adapted from Kuroda et al. 1998.
electrophysiological properties, the rat medial PFC (mPFC) is related to both the primate and human anterior cingulate cortex (ACC), and the dorsolateral PFC. Overall, it has been suggested that the rat mPFC combines elements from human and primate ACC and the dorsolateral PFC at a more rudimentary level (Uylings et al. 2003; Seamans et al. 2008).

The PFC is much more elaborately developed in primates than in other mammals. Phylogenetically, it is one of the most recent cortices to develop. This neocortical area is associated with many cognitive functions, such as working memory, attention, decision making and inhibitory control of actions (Miller and Cohen 2001; Koechlin et al. 2003). The PFC is thought to underlie the orchestration of thoughts and actions and it is believed that this brain area provides the foundation for complex behaviour in primates (Damasio 1995; Miller et al. 2002). Since addiction is a brain disorder that is associated with impairment of many of these PFC functions, it is easily understood why researchers have focused on the PFC in understanding addiction (Wistanley 2007; Perry et al. 2011).

The eCB system is involved in many of the cognitive functions that are linked to the PFC (Pattij et al. 2008). Although eCB-mediated signalling in the PFC is largely unexplored, the presence of components of the eCB system has been reported by several studies. For instance, the presence of functional CB1Rs, FAAH, MGL and DAGL in the PFC has been demonstrated (Herkenham et al. 1991; Eggan and Lewis 2007; Hansson et al. 2007; Lafourcade et al. 2007; Chiu et al. 2010; Volk et al. 2010; Yoshino et al. 2011). Intake of cannabinoid ligands, such as cannabis, affects PFC regional blood flow, metabolism and immediate early gene expression, which indicates that cannabinoid compounds can affect neural activity (Egerton et al. 2006). In both humans and rats, studies have shown that interference with the eCB system impairs several forms of working memory, attentional function and reversal learning (Jentsch et al. 1998; Lichtman et al. 2002; Arguello and Jentsch 2004; Egerton et al. 2005, 2006). The mechanisms underlying the effects of cannabinoids on such cognitive processes are thought to involve the altered release of neurotransmitters and synaptic plasticity (Egerton et al. 2006; Puighermanal et al. 2012). More research is needed to understand the role of eCB system in the PFC and, eventually, the effects of disruption of this system on cognitive processes.

Aim and approach
As mentioned earlier, the research presented in this thesis was performed within a research line of a TIPharma consortium, which focused on the role of the eCB system in the development of psychological disorders, in particular addiction. Several research groups contributed to this broad goal by trying to answer various specific research questions. Since impaired functioning of the PFC is associated with the brain disorder addiction, amongst other disorders, the PFC constitutes an interesting target brain area. Furthermore, it is known that the eCB system is present here, although many questions concerning effects of cannabinoid ligands on PFC functioning remain. The main aim of this research is to elucidate the role of these receptors in neuronal communication in a subregion of the rodent PFC, the mPFC.
Here, we used *in vitro* electrophysiological and biochemical techniques to investigate the role of CB receptors at the cellular and local network level in the mPFC. Firstly, using *in vitro* electrophysiological tools allowed for the recording of the activity of a neuronal population in this cortical area. Secondly, recordings were made of various electrophysiological properties of cells as well as input onto these cells. Cannabinoid ligands could be applied to brain slices in order to investigate the effects of CB receptor activation/blockade on these parameters. In addition, biochemical tools such as Western blotting, immunohistochemistry and radioactive binding assays enabled us to detect CB receptor proteins and their binding sites.

**Outline of this thesis**

This thesis represents a culmination of experimental research over a period of four years. Aside from this general introduction, the thesis contains four experimental chapters and a general discussion.

In **chapter two**, we examined the effects of CB₁R- and CB₂R-activation on general network excitability in the mPFC. We first characterized local field potentials recorded in layer II/III. We then used a mixed CB₁R/CB₂R agonist to evaluate the concentration-dependent effects on field potential amplitudes. In addition, specific cannabinoid ligands were used to investigate whether the observed effects were mediated by CB₁Rs and/or CB₂Rs.

Following the results of the second chapter, we continued our investigation by examining the functional presence of CB₂Rs in the mPFC at the cellular level, in **chapter three**. This study was specifically based on the previous observation that not all effects of a mixed CB₁R/CB₂R agonist on field potentials in the mPFC could be explained by the activation of CB₁Rs. We used a combination of electrophysiological techniques, Western blotting and a radioactive binding assay to assess the presence and functional role of CB₂Rs in this neocortical area. In addition, we performed experiments to determine effects of a CB₂R agonist on neuronal excitability.

In **chapter four**, we specifically investigated the activation of CB₂Rs in layer II/III of the mPFC by endogenous ligands. We hypothesized that stimulation protocols used to evoke eCB synthesis and subsequent CB₁R activation, could also result in CB₂R activation. First, we determined the effects of treating slices with 2-AG or a metabolically stable analog of AEA (methanandamide) and compared these effects. In addition, we performed experiments with a stimulation protocol that evokes action potential (AP) firing to induce eCB synthesis. Finally, we investigated CB₂R-mediated effects following repeated AP firing on neuronal excitability.

In **chapter five**, we investigated the role of the eCB system in neurotransmission and the maintenance of the E/I balance in the mPFC. We started our investigation by recording miniature synaptic currents, excitatory and inhibitory, to confirm the supposed presynaptic localization of CB₁Rs. We then visualized CB₁Rs using immunohistochemistry in healthy animals and animals that were repeatedly injected with a CB₁R/CB₂R agonist to corroborate their presynaptic localization and investigate the natural distribution of CB₁Rs and their internalization into cell bodies. Finally, we made use of a method to decompose synaptic input conductances onto layer II/III.
pyramidal cells into its excitatory and inhibitory component to assess the role of CB1Rs in the regulation of the E/I balance.

In chapter six, we summarize the main findings of these studies and discuss the results of the research.