Revised models of endocannabinoid signaling at inhibitory synapses in the brain
The research presented in this thesis was carried out at the Department of Integrative Neurophysiology of the Center for Neurogenomics and Cognitive Research at the Vrije Universiteit Amsterdam, the Netherlands.

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Revised models of endocannabinoid signaling at inhibitory synapses in the brain

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Rogier Min

geboren te Hoorn
promotoren: prof.dr. A.B. Brussaard
              prof.dr. N. Burnashev
‘Tell me one last thing,’ said Harry. ‘Is this real? Or has this been happening inside my head?’

Dumbledore beamed at him, and his voice sounded loud and strong in Harry’s ears even though the bright mist was descending again, obscuring his figure.

‘Of course it is happening inside your head, Harry, but why on earth should that mean that it is not real?’

J.K. Rowling, “Harry Potter and the Deathly Hallows”
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The principle task of the brain is to translate the multitude of sensory signals that it receives from the external and internal environment into appropriate behavioral output. In order to achieve this goal the billions of neurons in the brain assemble into complex neuronal networks capable of intricate computations (Figure 1.1A). These neurons can be divided into different subtypes. Cortical neuronal networks consist mainly (~80-90%) of excitatory principal neurons, which release the excitatory neurotransmitter glutamate at their synaptic terminals. The remaining 10-20% are interneurons, most of which are inhibitory and release the neurotransmitter \( \gamma \)-aminobutyric acid (GABA). Excitatory neurons form a relatively homogeneous population (DeFelipe and Farinas, 1992). They form local intracortical connections, but are also the main output neurons of cortical circuits. Interneurons on the other hand show a wide variety of morphological and physiological characteristics (Markram et al., 2004; Somogyi and Klausberger, 2005) and are, as their name implies, mainly involved in local circuit processing (Figure 1.1B,C).

**The neuronal microcircuit as a functional unit in the brain**

To understand the way in which neurons in the brain work together in complex networks, one can assume that such networks are built consisting of repeated copies of smaller building blocks. However, single neurons do not qualify as these building blocks, since their input/output relation is not sufficiently capable of complex computations. Instead, we can think of minimal circuits of interconnected neurons capable of collectively producing functional output. Evidence for the existence of such “canonical microcircuits” is accumulating (for example see Douglas and Martin, 1991). Such a microcircuit should consist of balanced interactions between excitatory and inhibitory neurons, to prevent overexcitation on the one hand, and complete silence on the other.

Interactions between excitatory and inhibitory neurons can have fundamentally different features, depending on the ways in which these neurons are connected. Three functionally different examples of such connectivity are shown in figure 1.2A. First, interneurons can provide stabilizing feedback inhibition to the principal neuron, if both neurons are interconnected. Second, feed-forward inhibition can dampen afferent input to principal neurons (Buzsaki, 1984), thereby increasing temporal fidelity of spike-firing (Pouille and Scanziani, 2001). Third, lateral inhibition can segregate neuronal populations, by specific inhibition of neighbouring neurons (see also Buzsaki, 2006).
However, the anatomical hardwiring of the microcircuit is not the only determinant of its functioning. Other important features are the strength and dynamic properties of its synapses. Even apparently minor changes in the strength and/or timing of excitatory drive to interneurons are crucial for microcircuit remodeling, as are differences in inhibitory synaptic strength or timing (Figure 1.2B). Indeed, both inhibitory and excitatory synapses are highly plastic, meaning that they may undergo use-dependent changes in synaptic strength on scales ranging from milliseconds to hours, days or even years. Such synaptic plasticity changes the ‘assembly’ of the microcircuit, thereby dramatically altering the way in which it processes incoming information. Therefore, understanding synaptic plasticity is pivotal to understanding microcircuit functioning.

**Different modes of synaptic plasticity**

As mentioned before, changes in synaptic strength are critical for information processing in neuronal networks. One can distinguish three different forms of such synaptic plasticity. First, short-term plasticity signifies transient changes in synaptic efficacy typically occurring on the timescale of milliseconds to minutes, is thought to underlie critical computational functions performed by neuronal networks (Abbott and Regehr, 2004). Second, long-term plasticity, a change in synaptic efficacy typically lasting for hours to days/months, is thought to be the cellular correlate of learning and memory (Martin et al., 2000). Third, homeostatic plasticity denotes processes by which circuits maintain...
a certain balance between excitation and inhibition despite changes in the strength of individual synapses (Turrigiano and Nelson, 2004). Research described in this thesis will focus on established and novel mechanisms underlying short-term synaptic plasticity.

Synaptic transmission is, principally, a unidirectional process: action potentials in the presynaptic neuron lead to neurotransmitter release at its axon terminals, subsequently activating postsynaptic receptors. Likewise, many forms of synaptic plasticity run in the same direction: they are induced by presynaptic activity, and are expressed either pre- or postsynaptically (left arrows in Figure 1.3). However, there are also forms of synaptic plasticity, which are induced by activity in the postsynaptic neuron (right arrows in Figure 1.3). For example, activity in the postsynaptic neuron can change the properties of postsynaptic neurotransmitter receptors, or change the presynaptic release probability. In the latter case the postsynaptic neuron will have to send a message back to the presynaptic neuron. This process is termed ‘retrograde signaling’, and the

Figure 1.2 Different modes of inhibitory transmission in microcircuits
(A) Negative (inhibitory) feedback provides stability (left). Feedforward inhibition dampens (“filters”) the effect of afferent excitation (middle). Lateral inhibition provides autonomy (segregation) of neurons by suppressing the similarly activated neighboring neurons (“winner take all”; right). (B) Inhibition is essential for cell assembly selection. Slight differences in synaptic strengths between the afferent input and neurons in assembly 1 and 2 can completely silence the competing assembly. In case of equal input strengths, the earlier input selects the assembly and silences the competing assembly by feedforward and lateral inhibition. Figures adapted from (Buzsaki, 2006).
substance mediating the message from the post- to the presynapse is called a ‘retrograde messenger’.

Several substances have been suggested to act as retrograde messengers. For example, dendrites of postsynaptic neurons release vesicles filled with messenger molecules in an activity dependent way (de Kock et al., 2003; Zilberter, 2000; Zilberter et al., 1999). These messengers subsequently bind to presynaptic receptors, which in turn modulate presynaptic release probability. Next to vesicular release, other possible mechanisms of retrograde signaling have been proposed. Gaseous molecules like nitric oxide and carbon monoxide have been suggested to act as retrograde messengers in hippocampal synaptic plasticity (O’Dell et al., 1991; Zhuo et al., 1993). These molecules are able to freely cross synaptic membranes, allowing them to travel to the presynaptic terminal without an additional release step.

More recently, the endocannabinoid system has been shown to be involved in several retrograde signaling pathways. Endocannabinoids are molecules which are synthesized ‘on-demand’ from membrane lipid precursors, and which can bind to the cannabinoid CB₁ receptor (CB₁R), the most abundant G-protein coupled receptor in the brain. Activation of the CB₁R leads to a powerful reduction in presynaptic release probability in CB₁R containing terminals. However, much is unknown about the mechanisms involved in endocannabinoid signaling, and it is not clear whether the CB₁R is the only brain target for endocannabinoids. The research described in this thesis will focus on the role of the endocannabinoid system in synaptic plasticity. In the rest of the introduction I will introduce the endocannabinoid system, and the processes in which it is thought to play a role.

Cannabinoïd signaling in the brain
The term cannabinoid was introduced with the isolation and structural characterization of a specific component from the marihuana plant (Cannabis sativa), with psychoactive properties (Gaoni and Mechoulam, 1964). This compound, Δ⁹-tetrahydrocannabinol (Δ⁹-THC; Figure 1.4A), has unique chemical properties when compared to other drugs. It is highly hydrophobic, which led to the early theory that cannabinoids might affect the nervous system by influencing membrane fluidity, rather than by interaction with a specific receptor. However, at the end of the 20th century two cannabinoid receptors were identified: the CB₁R (Matsuda et al., 1990) and the CB₂ receptor (CB₂R; Munro et
These cannabinoid receptors are metabotropic receptors and signal transduction after receptor stimulation is mediated by a G-protein-coupled second-messenger signaling cascade (Howlett et al., 1986; Pertwee, 1997). The two cannabinoid receptors show a different distribution in the body. The CB$_1$R shows high expression levels throughout the central nervous system (CNS), with especially high densities present in the cerebellum, the hippocampus, the striatum and, more diffusely, in the neocortex (Galiegue et al., 1995; Herkenham et al., 1991). It is, in fact, the most abundant G-protein coupled receptor in the brain, with levels of expression similar to those of ligand-gated GABA and glutamate receptors (Howlett et al., 2004). In contrast, the CB$_2$R is abundantly localized in the immune system (e.g. tonsils and spleen) and, less abundant, in other organs (Galiegue et al., 1995). A low amount of CB$_2$R expression has been found in the brain (Van Sickle et al., 2005). This distribution is complementary to the role that the two cannabinoid receptors are thought to play in the body: the CB$_1$R is thought to account for most of the behavioral effects of cannabinoids, while the CB$_2$R is mainly involved in immune functions.

The fact that these highly specialized cannabinoid receptors are present at high levels in the body suggests the existence of endogenous ligands. Indeed, apart from being activated by plant-derived cannabinoids, the body itself produces endogenous cannabinoids (endocannabinoids). The two most studied and best understood endocannabinoids are anandamide (AEA; Devane et al., 1992) and 2-arachidonoylglycerol (2-AG; Mechoulam et al., 1995; Sugiura et al., 1995; Figure 1.4D). However, several other endogenously occurring ligands for cannabinoids have been identified,
although their role in cannabinoid signaling is not yet fully understood (for review see De Petrocellis et al., 2004). Like Δ⁹-THC, endocannabinoids are fatty substances with hydrophobic properties.

**Biosynthesis and degradation of endocannabinoids**

Endocannabinoids differ from classical neurotransmitters in that they are not pre-synthesized and then stored until released, but they are thought to be synthesized ‘on demand’ from lipid precursors. For both AEA and 2-AG the biochemical pathways leading to synthesis are starting to become clear (Figure 1.5).

AEA synthesis is achieved by a two step process (for review see Okamoto et al., 2007; Schmid et al., 2002). First, it involves the calcium dependent formation of N-acyl phosphatidylethanolamines (NAPEs) by acyl transfer from phospholipids to the N-position of phosphatidylethanolamine. This process, which is catalyzed by a calcium dependent N-acyl transferase, is thought to be the rate limiting step in AEA formation. Next, these NAPEs are enzymatically hydrolyzed into AEA. The enzyme which catalyzes this hydrolysis is a phospholipase D (PLD) with high selectivity for NAPEs (termed NAPE-PLD; Okamoto et al., 2004; Figure 1.5). Studies on the anatomical localization of NAPE-PLD show that it is present mainly in axon terminals of hippocampal mossy fiber synapses, associated with intracellular calcium stores (Nyilas et al., 2008). However,
these terminals do not express the CB$_1$R, suggesting that AEA is involved in CB$_1$R independent signaling at these terminals. Additionally, studies on NAPE-PLD knockout mice have shown that AEA can be formed by parallel pathways, independent of NAPE-PLD activity (Leung et al., 2006; Liu et al., 2006).

The deactivation of AEA, and thereby the termination of its signaling, may involve two mechanisms. Firstly, it has been proposed that AEA can be taken up by a carrier system that is present in both neurons and glial cells. This process is described as a form of facilitated diffusion, meaning that AEA is internalized by energy-independent carriers (Beltramo et al., 1997; Hillard et al., 1997). However, there is controversy as to the existence of these carriers, since to date they have not been molecularly characterized. Proof of their existence mainly comes from selective inhibition of AEA internalization by 'transport inhibitors', like the substance AM404 (Beltramo et al., 1997; Moore et al., 2005). Others argue that AEA transport can be explained by pure passive diffusion (Kaczocha et al., 2006). The second step in termination of AEA signaling is its breakdown. This process is mediated by the intracellular membrane bound enzyme fatty acid amide hydrolase (FAAH; Cravatt et al., 1996), which breaks down AEA into arachidonic acid (AA) and ethanolamine (Bracey et al., 2002; Figure 1.5). The distribution of FAAH in the brain suggests that it is primarily present in postsynaptic compartments, namely the somata and dendrites of neurons (Gulyas et al., 2004). A FAAH knockout mouse has been generated, which shows increased sensitivity to AEA, together with increased endocannabinoid signaling (Cravatt et al., 2001).

2-AG, the second well-characterized endocannabinoid, is usually present in tissues at a much higher level than AEA (Sugiura et al., 1995). This does not necessarily imply that all this 2-AG is uniquely used to activate cannabinoid receptors, since 2-AG is at the crossroads of several metabolic pathways involving other important signaling lipids like AA and diacylglycerol (DAG). The biosynthetic pathway for 2-AG to act as an endocannabinoid messenger is thought to be mediated by the hydrolysis of 2-arachidonate containing DAGs, catalyzed by an $sn$-1-selective DAG lipase ($sn$-1 DAGL). The DAGs that serve as 2-AG precursors can be produced from the hydrolysis of 2-arachidonate-phosphohydrolase, or of phosphoinositides, catalyzed by phosphoinositide selective phospholipase C (PI-PLC; Figure 1.5). To date, two $sn$-1 DAGLs have been characterized (Bisogno et al., 2003). The distribution of these $sn$-1 DAGLs supports their roles as mediators of endocannabinoid signaling in the brain: during development, when endocannabinoids are thought to direct axon outgrowth, they are localized to axonal tracts, while they are mostly expressed in dendritic fields in the adult, consistent with a role in regulation of synaptic transmission at this stage (Bisogno et al., 2003). However, pharmacological inhibition of DAGL activity does not eradicate all 2-AG signaling in the brain, suggesting that alternative pathways for the biosynthesis of 2-AG exist (Chevaleyre and Castillo, 2003; Edwards et al., 2006; Szabo et al., 2006; chapter 2 of this thesis). An alternative pathway for 2-AG synthesis could begin with the formation of a 2-arachidonoyl-lysophospholipid by phospholipase A1 (PLA1), which could be hydrolyzed to 2-AG by lyso-PLC activity. There is no direct evidence for the involvement of this pathway in 2-AG synthesis, but the high expression levels of the
involved enzymes in the brain makes it an attractive hypothesis for further investigation (Piomelli, 2003).

As with AEA, there is controversy about the uptake of 2-AG by neurons and glial cells. It has been suggested that, like AEA, 2-AG is taken up by cells through facilitated diffusion (Beltramo and Piomelli, 2000; Bisogno et al., 2001), although this hypothesis is controversial. 2-AG transport seems to show a comparable sensitivity to AM404 as AEA transport, but whether this means that the same enzyme is responsible is unknown. Inactivation of 2-AG occurs through several separate pathways. First, 2-AG can be hydrolyzed by the enzyme monoacylglycerol lipase (MGL; Dinh et al., 2002; Figure 1.5). This enzyme shows a broad expression in the central nervous system. Its expression partially overlaps with that of FAAH, but it seems to be predominantly present in presynaptic structures, whereas FAAH is found mainly postsynaptic (Gulyas et al., 2004). Second, FAAH also seems to be able to hydrolyze 2-AG (Di Marzo et al., 1998). A third pathway leading to 2-AG breakdown is its oxidative metabolism by the enzyme cyclooxygenase-2 (COX-2; Kozak et al., 2000). Electrophysiological studies support a role for both MGL metabolism (Hashimotodani et al., 2007a; Makara et al., 2005) and COX-2 oxidative metabolism (Kim and Alger, 2004), but not of FAAH (Makara et al., 2005) in the termination of synaptic 2-AG signaling (Figure 1.6).

Both AEA and 2-AG are formed in the brain in an activity dependent way (Di Marzo et al., 1994; Stella et al., 1997). This activity dependence is attributed to the fact that several of the enzymes governing the synthesis of endocannabinoids are calcium dependent. Furthermore, activation of G-protein coupled receptors, including metabotropic glutamate receptors (Maejima et al., 2001), muscarinic acetylcholine receptors (Kim et al., 2002) and dopamine receptors (Giuffrida et al., 1999) also boosts endocannabinoid production.

**CB₁R signaling in the nervous system**

CB₁Rs appear to be mainly expressed on presynaptic axon terminals. Activation of this receptor type causes G-protein mediated inhibition of N- and P/Q-type calcium channels (Mackie and Hille, 1992; Twitchell et al., 1997). Subsequently, calcium influx into the presynaptic terminal is reduced, which causes a decrease in the probability of neurotransmitter release (Hoffman and Lupica, 2000; Wilson et al., 2001). Furthermore, it has been suggested that CB₁R activation can induce opening of presynaptic potassium channels, which subsequently inhibit presynaptic action potential efficacy in releasing neurotransmitter (Diana and Marty, 2003). Third, CB₁R signaling might directly modulate the presynaptic release machinery, thereby causing a reduction in release probability (Takahashi and Linden, 2000).

CB₁R signaling is involved in several forms of short- and long-term synaptic plasticity in several brain areas. The first process in which CB₁R signaling was shown to be involved was depolarization induced suppression of inhibition (DSI) in the hippocampus and cerebellum (Llano et al., 1991; Pitler and Alger, 1992; Figure 1.6A). In this process, a postsynaptic depolarization initiates a large calcium influx into the postsynaptic neuron. This calcium influx subsequently activates a retrograde signaling
cascade which causes a presynaptic reduction in release probability. At the same time both hippocampal and cerebellar DSI were shown to depend on presynaptic, endocannabinoid mediated CB₁R activation (Kreitzer and Regehr, 2001a; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). A similar process appears to occur at excitatory synapses in hippocampus and cerebellum (termed depolarization induced suppression of excitation; DSE; Kreitzer and Regehr, 2001b; Ohno-Shosaku et al., 2002). DSI and DSE have later been described in various other brain regions (for review see Chevaleyre et al., 2006). Furthermore, synaptic endocannabinoid signaling cannot only be initiated by a postsynaptic calcium influx, but also by activation of metabotropic receptors, or by a combination of the two (for review see Hashimotodani et al., 2007b). Finally, endocannabinoid mediated CB₁R signaling is involved in multiple forms of long-term depression of synaptic transmission in several brain regions (for review see Chevaleyre et al., 2006).

A key question is which endocannabinoid (AEA, 2-AG or a novel, unidentified CB₁R ligand) mediates endocannabinoid signaling in the brain. To answer this question, several studies have tried interfering with either the synthesis or breakdown of 2-AG and AEA using pharmacological tools. In this way, it was shown that inhibition of FAAH, the major enzyme mediating AEA degradation, does not affect hippocampal DSI (Figure 1.6B), while inhibiting the 2-AG degrading enzyme MAGL lengthens DSI (Figure 1.6C; Hashimotodani et al., 2007a; Kim and Alger, 2004; Makara et al., 2005). This suggested that 2-AG is responsible for endocannabinoid signaling during hippocampal DSI. However, studies where inhibitors of 2-AG synthesis have been used have yielded controversial results. In some studies inhibition of DAGL blocks hippocampal DSI (Hashimotodani et al., 2007a; Hashimotodani et al., 2008), while in others it is ineffective (Chevaleyre and Castillo, 2003; Edwards et al., 2006; Szabo et al., 2006; chapter 2 of this thesis). The reason for this controversy is currently unclear, but this will be the topic of chapter 2 of this thesis.

Endocannabinoid signaling in other brain regions has been attributed to either 2-AG or AEA. For example, endocannabinoid mediated long-term depression (LTD) of cerebellar parallel synapses onto Purkinje cells depends on postsynaptic 2-AG synthesis, because it can be blocked by DAGL inhibitors (Safo and Regehr, 2005). Similarly, short-term endocannabinoid signaling at this synapse as well as at synapses onto cerebellar stellate cells evoked by repetitive parallel fiber stimulation is also sensitive to DAGL inhibition (Beierlein and Regehr, 2006). However, depolarization induced suppression of excitation (DSE) at exactly the same synapses has both been reported to be insensitive to DAGL inhibition (Safo and Regehr, 2005) and to be blocked by DAGL inhibition (Hashimotodani et al., 2008; Szabo et al., 2006), suggesting that mechanisms controlling the formation of endocannabinoids can differ within the same cell type, and even at the same synapse. There are also reports of AEA involvement in endocannabinoid signaling in the brain. For example, AEA signaling seems to be responsible for long-term plasticity in the striatum (Ade and Lovinger, 2007).
Irrespective of which endocannabinoid is involved, the results described above have led to the generally accepted hypothesis that endocannabinoids act as retrograde messengers in the central nervous system. According to this hypothesis, postsynaptic calcium influx, activation of postsynaptic metabotropic receptors, or a combination of the two, would trigger the postsynaptic synthesis of endocannabinoids, which subsequently travel through the postsynaptic membrane to reach the presynaptically located CB$_1$R.

Direct ion channel modulation by cannabinoids

Almost all studies on cannabinoid modulation of the nervous system in the last decennium have focused on activation of the G-protein coupled CB$_1$R, and its downstream effects on synaptic transmission and neuronal excitability. However, cannabinoids can still affect animal behavior both in the presence of CB$_1$R antagonists as well as in CB$_1$R knockout (CB$_1$R$^{-/-}$) mice (Adams et al., 1998; Baskfield et al., 2004; Di Marzo et al., 2000; Wise et al., 2007), which suggests the existence of additional brain targets for cannabinoids. Indeed, several studies have suggested the existence of novel G-protein coupled cannabinoid receptors (Breivogel et al., 2001; Hoffman et al., 2005). Additionally, (endo)cannabinoids have been shown to interact with vanilloid receptors (for review see Szallasi et al., 2007).

But it is also important to note that at the same time when AEA was characterized to be an endogenous ligand for the CB$_1$R, independent work showed that AEA may directly
bind to L-type voltage-gated calcium channels (VGCCs; Johnson et al., 1993). Recent studies have shown that not only AEA, but also other endo- and exocannabinoids can modulate the functional properties of a number of ligand-gated and voltage-gated ion channels. The wide targets of direct cannabinoid modulation include several subtypes of VGCCs (Chemin et al., 2001; Fisyunov et al., 2006; Oz et al., 2004a; for review see Lozovaya et al., 2009), voltage-gated potassium channels (Oliver et al., 2004; Poling et al., 1996), and ligand-gated ion channels. The latter group includes the NMDA receptor (Hampson et al., 1998), the serotonin 5HT3 receptor (Barann et al., 2002; Oz et al., 2002), the nicotinic acetylcholine receptor (nACHR; Oz et al., 2004b) and the glycine receptor (Hejazi et al., 2006; Lozovaya et al., 2005). In this thesis we describe a direct modulation of the GABAA receptor, the most abundant inhibitory neurotransmitter receptor in the brain (Chapters 3 and 4).

Concerning the mechanism by which cannabinoids modulate ion channels, there are two possibilities. Either cannabinoids bind directly to a site on the ion channel protein, thereby affecting its gating properties, or cannabinoids modulate ion channels by an interaction with the plasma membrane lipid bilayer in which these channels are embedded. Which of the two mechanisms give rise to direct ion channel modulation is unclear. Furthermore, the physiological roles of this direct receptor modulation in neuronal network functioning to date has hardly been studied (but see Chapter 4).

**Aims of this study**
As described above, the endocannabinoid system in the brain may play an important role in the regulation of synaptic transmission. Endocannabinoids are thought to act as retrograde messengers at both excitatory and inhibitory synapses in numerous brain regions, although there are several open questions as to how they exactly function. Furthermore, (endo)cannabinoids also appear to directly modulate a large group of voltage-gated and ligand-gated ion channels. The aim of this study has been to expand our knowledge about endocannabinoid signaling both at inhibitory synapses containing the CB1R and at CB1R lacking inhibitory synapses. The following research questions have been addressed:

1. **Where and how are endocannabinoids synthesized in hippocampal DSI?**
Hippocampal DSI remains the best studied endocannabinoid mediated mechanism in the brain. However, there are several controversies about the molecular machinery involved in the synthesis of the endocannabinoids that mediate DSI. In chapter 2 we addressed these controversies by studying CB1R expressing inhibitory synaptic contacts (CB1R containing interneuron to pyramidal neuron synapses in the CA1 region of the hippocampus).

2. **Can the GABAA receptor be directly modulated by (endo)cannabinoids?**
(Endo)cannabinoids can directly modulate several voltage-gated and ligand-gated ion channels, independent of cannabinoid receptor activation. Ligand-gated ion channels that are directly modulated include the glycine receptor, the nicotinic acetylcholine re-
ceptor and the serotonin 5HT₃ receptor. These three receptors belong to the same receptor superfamily as the GABAₐ receptor, the most abundant inhibitory neurotransmitter receptor in the brain. However, whether the GABAₐ receptor can be directly modulated by (endo)cannabinoids has not been studied so far. In chapter 3 we have investigated (endo)cannabinoid interactions with the GABAₐ receptor using several pharmacological approaches.

3. What is the consequence of direct GABAₐ receptor modulation for synaptic transmission and neuronal microcircuit functioning?

Results from our study described in chapter 3 suggest that the GABAₐ receptor can be a direct target for (endo)cannabinoids. However, the impact of this direct GABAₐ receptor modulation for synaptic functioning was not studied yet. In chapter 4 we wanted to know what this direct receptor modulation would mean for an inhibitory synapse which does not contain the CB₁R (the fast-spiking interneuron to pyramidal neuron synapse in the neocortex). Furthermore, we investigated whether direct, CB₁R independent modulation by endocannabinoids can occur under physiological conditions, and what this means for neuronal microcircuit functioning.

The above questions have been addressed using electrophysiological techniques. We have mainly used (dual) whole-cell patch clamp recordings from neurons in acute brain slices of mice and rats. Additionally, in chapter 3 we have used different methods for studying GABAₐ receptor functioning in close detail: fast GABA application to transfected HEK293 cells, isolated neurons and nucleated patches from brain slices.

Finally, in the general discussion (chapter 5) we integrate the findings from the previous chapters, present a revised model for endocannabinoid signaling at CB₁R containing and CB₁R lacking inhibitory synapses, and discuss the relevance of these two pathways of endocannabinoid signaling for neuronal network functioning.
DAG lipase is not involved in DSI at unitary inhibitory connections in mouse hippocampus

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Endocannabinoids control hippocampal inhibitory synaptic transmission through activation of presynaptic CB₁ receptors. During depolarization-induced suppression of inhibition (DSI), endocannabinoids are synthesized upon postsynaptic depolarization. The endocannabinoid 2-arachidonoylglycerol (2-AG) may mediate hippocampal DSI. Currently, the best studied pathway for biosynthesis of 2-AG involves the enzyme diacylglycerol lipase (DAGL). However, whether DAGL is necessary for hippocampal DSI is controversial and was not systematically addressed. Here, we investigate DSI at unitary connections between CB₁ receptor-containing interneurons and pyramidal neurons in CA1. We found that the novel DAGL inhibitor OMDM-188, as well as the established inhibitor RHC-80267, did not affect DSI. As reported previously, effects of the DAGL inhibitor tetrahydrolipstatin depended on the application method: postsynaptic intracellular application left DSI intact, while incubation blocked DSI. We show that all DAGL inhibitors tested block slow self-inhibition in neocortical interneurons, which involves DAGL. We conclude that DAGL is not involved in DSI at unitary connections in hippocampus.
Introduction

Endocannabinoids mediate both short- and long-term plasticity at many synapses in the brain (Kano et al., 2009). The first form of synaptic plasticity in which endocannabinoids were shown to be involved is hippocampal depolarization-induced suppression of inhibition (DSI; Pitler and Alger, 1992; Wilson and Nicoll, 2001). Hippocampal DSI occurs at a subset of inhibitory synapses onto pyramidal neurons coming from CB₁ receptor (CB₁R)-containing interneurons. At these synapses, a postsynaptic depolarization leads to calcium-dependent synthesis of endocannabinoids, which subsequently travel to the presynaptic inhibitory terminal. Here, endocannabinoids activate the G-protein coupled CB₁R, thereby reducing presynaptic release probability for tens of seconds. The endocannabinoid 2-arachidonoyl glycerol (2-AG; Mechoulam et al., 1995; Sugiura et al., 1995) has been proposed to mediate hippocampal DSI, since inhibiting 2-AG degradation increases the duration of DSI (Kim and Alger, 2004; Makara et al., 2005; Hashimotodani et al., 2007a). However, sn-1-diacylglycerol lipase (DAGL) α, the major enzyme catalyzing 2-AG formation in the adult brain (Bisogno et al., 2003) has not been found opposite symmetrical, GABAergic synapses in hippocampus (Katona et al., 2006). Furthermore, studies in which DAGL's have been targeted pharmacologically reported conflicting results. Blocking these enzymes pharmacologically suppresses hippocampal DSI in some studies (Hashimotodani et al., 2007a) but not in others (Chevalleyre and Castillo, 2003; Edwards et al., 2006; Szabo et al., 2006). So far, the only study showing pharmacological evidence for DAGL involvement in hippocampal DSI was performed on cultured neurons. This raises the question whether DAGL is involved in hippocampal DSI at intact synapses. Here, by using three chemically different DAGL inhibitors, we have investigated DAGL involvement in hippocampal DSI at unitary connections between CB₁R-containing interneurons and CA1 pyramidal neurons.

Materials & Methods

Slice preparation

All experiments were approved by the Animal Ethical Committee of the VU University Amsterdam, in accordance with Dutch and European law. For DSI experiments, 14-21 day old wildtype C57Bl6 mice or CB1R⁻/⁻ mice (Marsicano et al., 2002) were decapitated. Transverse hippocampal slices (300 µm) were sectioned in ice-cold solution containing (mM): 126 NaCl, 3 KCl, 10 glucose, 26 NaHCO₃, 1.2 NaH₂PO₄, 1 CaCl₂, and 3 MgCl₂ (carboxygenated with 5% CO₂ / 95% O₂). For SSI experiments, sagittal slices containing somatosensory cortex were cut from 21-28 day old FVB-Tg(GadGFP)45704Swn/J mice expressing EGFP under control of the mouse Gad1 gene promoter (Jackson laboratories Stock No. 003718), thereby labeling a subset of somatostatin-positive interneurons in hippocampus and neocortex (Oliva et al., 2000). Slices were maintained at 33°C for ~30 minutes and recorded at room temperature (20-22°C) in a similar solution, but now with 2 mM CaCl₂, and 2 mM MgCl₂.
**Paired recordings of CB₁R-containing interneurons and CA1 pyramidal neurons**

Cellular recordings were performed from 2-6 neurons simultaneously using borosilicate glass pipettes with resistance of 3–5 MΩ, containing (mM): 70 K gluconate, 70 KCl, 4 Mg-ATP, 4 phosphocreatine, 0.4 GTP, 0.5 EGTA and 10 HEPES (pH 7.3, KOH). In synaptically-connected neurons, suprathreshold stimulation of the presynaptic interneuron evoked a GABA<sub>A</sub> R mediated IPSP/IPSC in the postsynaptic CA1 pyramidal neuron. Because of the high intracellular chloride concentration, IPSCs were observed as inward currents, while IPSPs were depolarizing. Presynaptic cells were stimulated with a pair of 2 suprathreshold current pulses (at ~10 Hz). Trains were delivered with intervals of 5 sec. Recording and stimulus delivery was performed using standard electrophysiological equipment.

**SSI recordings in neocortical interneurons**

For experiments on SSI, EGFP expressing interneurons in layer V of somatosensory cortex were selected using an UV light source and fluorescence imaging. Extracellular and intracellular solutions were similar as for paired recordings, but to exclude contributions from synaptic conductances DNQX (10 µM), GABA<sub>zine</sub> (100 µM) and DL-AP5 (100 µM) were added to the extracellular solution.

**Immunohistochemistry**

Some recorded interneurons were filled with biocytin (0.5%) during recording. Slices were fixed and resliced. In a first step sections were stained using the antibodies goat-anti-streptavidin Alexa Fluor 488 conjugate (1:200) and the primary antibody rabbit-anti- CB₁R (diluted 1:1000 in TBS-TX; Tsou et al., 1998). In a second step, sections were incubated with the secondary antibody Alexa Fluor 594 goat anti-rabbit IgG (1:200). Colocalization between biocytin and CB₁R was determined using a Leica confocal laserscanning microscope (Leica, Heidelberg, Germany), the Huygens system (Scientific Volume Imaging, Hilversum, The Netherlands) and the Amira™ isosurface module (Zentrum fur Information Technology, Berlin, Germany). Care was taken to prevent false positives, incomplete signal separation and crosstalk of dyes (Wouterlood et al., 1998). Neuron reconstructions were performed under an up-right microscope (Leica, Heidelberg, Germany) using a 40x objective with Neurolucida software (Neurolucida, MicroBrightField, Williston, VT).

**Chemicals**

Of tetrahydrolipstatin, RHC-80267 (Sigma-Aldrich, St. Louis, MO) and OMDM-188 (Ortar et al., 2008) appropriate stock solutions were prepared in DMSO (Final concentration <0.2%). Slice-incubation was done in the slice chamber for at least 30 minutes before recordings started, with the same compound concentration present extracellularly. For intracellular application, intracellular equilibration was allowed for at least 20 minutes after whole-cell break-in (Figure 2.4).
Results

Simultaneous whole-cell recordings were made from CA1 pyramidal neurons and interneurons located in stratum radiatum, close to the border with stratum pyramidale (Figure 2.1). Cell-bodies of selected interneurons had a multipolar appearance and their firing pattern upon current injection showed considerable spike-frequency adaptation (Figure 2.1C). To confirm the presence of CB\(_{1}\)Rs in the studied interneurons, we used biocytin staining and immunostaining for the CB\(_{1}\)R in a subset of interneurons (Figure 2.1A,B). All interneurons showed substantial colocalization of biocytin and CB\(_{1}\)R immuno-reactivity in axons (n=5/5; Figure 2.1B).

Inhibitory connections were activated by evoking two action potentials (APs) at ~10 Hz in the interneuron, while recording inhibitory postsynaptic currents (IPSCs) in the CA1 pyramidal neuron (voltage-clamped at -70 mV; Figure 2.1D). In cell pairs showing

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**Figure 2.1 Properties of CB\(_{1}\)R-containing interneuron to CA1 pyramidal neuron pairs**

(A) Reconstruction of the axonal (green) and dendritic (black) tree of a biocytin-filled CA1 basket interneuron with its soma (black) within stratum radiatum (str. rad.), close to the border with stratum pyramidale (str. pyr.). (B) Fluorescent image of part of the biocytin filled axon in green (top), CB\(_{1}\)R staining in red (middle) and merged image (bottom). All images are from the same focal plane. Note the colocalization of biocytin and CB\(_{1}\)R antibody staining (yellow spot, arrow). (C) AP firing in response to current injection of a CB\(_{1}\)R-containing interneuron (green) and a CA1 pyramidal neuron (blue). (D) Example trace of APs evoked by two short current injections into the interneuron (green) and the IPSC response in a postsynaptic pyramidal neuron voltage clamped at -70 mV (blue; average of 50 sweeps) of the same cell pair as in (C).

**Analysis**

All analysis was done using custom written procedures in Igor Pro (Wavemetrics, OR, USA). Statistical analysis was done either using two-sided student’s t-test (for single comparisons; \(\alpha=0.05\)), or ANOVA with post-hoc Dunnett’s test (for multiple comparisons; \(\alpha=0.05\)).
an inhibitory synaptic connection we tested for the presence of DSI. Two APs (at ~10 Hz) were evoked in the interneuron once every 5 seconds (0.2 Hz). After recording 50 control responses, the subsequent 50 responses were each preceded by a 1 second depolarizing step (1 sec to 0 mV) to the postsynaptic pyramidal cell (depolarization period; Figure 2.2A). For IPSCs evoked after such a depolarizing step, the IPSC amplitude was reduced to 36.0±2.6% of control (Figure 2.2A-D; p=0.0001; n=7), resulting in a DSI
Figure 2.3 DSI is not observed in CB$_1$R$^{-/-}$ mice
(A) Left, recording configuration. Middle, example traces of postsynaptic IPSC responses to presynaptic stimulation in an interneuron to CA1 pyramidal neuron pair in a CB$_1$R$^{-/-}$ mouse under control conditions (control), following depolarization of the postsynaptic pyramidal neuron (depolarization) and during recovery from this depolarization (recovery). Traces show average response of 50 subsequent responses for each condition. Right, IPSC amplitude under control conditions, following depolarization and during recovery of the same experiment as shown left, displayed as in Figure 2.2B. (B) Average normalized IPSC amplitudes of all experiments in CB$_1$R$^{-/-}$ mice (n=6), displayed as in Figure 2.2C. (C) Failure rate during control, following depolarization and during recovery in CB$_1$R$^{-/-}$ mice, displayed as in Figure 2.2D. (D) Amount of DSI observed in wildtype versus CB$_1$R$^{-/-}$ mice.

Figure 2.4 The effect of DAGL inhibitors on DSI
(A) Left, postsynaptic IPSC responses to presynaptic stimulation in a connected interneuron to CA1 pyramidal neuron pair from a slice incubated and recorded in the DAGL inhibitor OMDM-188 (2 µM). Traces show average response of 50 subsequent responses for each condition. Middle, average normalized IPSC amplitudes of all experiments in OMDM-188 (n=6), displayed as in Figure 2.2C. Right, failure rate during control, following depolarization and during recovery for experiments in OMDM-188, displayed as in Figure 2.2D. (B) Same as in (A), but now for experiments on slices incubated and recorded in the DAGL inhibitor RHC-80267 (70 µM; n=5). (C) Same as in (A,B), but now for experiments in which THL (5 µM) was added to the intracellular solution of the postsynaptic pyramidal neuron (n=15). (D) Same as in (A-C), but now for experiments on slices incubated and recorded in the DAGL inhibitor THL (10 µM; n=6). (E) The amount of DSI observed in experiments in which THL (5 µM) was added to the intracellular solution of the postsynaptic pyramidal neuron versus the time waited after whole-cell on the postsynaptic pyramidal neuron (same experiments as in (C)). Dotted line shows the amount of DSI observed under control conditions. (F) Amount of DSI observed under different conditions.
DAG lipase is not involved in hippocampal DSI

A. OMDM-188 (bath)

B. RHC-80267 (bath)

C. THL (intracellular)

D. THL (bath)

E. THL (intracellular)

F. Amount of DSI (%)

**Figure 1**: DAG lipase is not involved in hippocampal DSI.
of 64.0±2.6% at this particular synapse. Amplitude reduction was accompanied by a strong increase in synaptic failure rate (Figure 2.2D; control 30.0±4.6%; depolarization 49.4±5.1%; p=0.024). After the depolarization period, both IPSC amplitude and failure rate partially recovered, respectively to 76.3±3.1% and 37.1±5.0% (Figure 2.2A-D).

The increase in failure rate during the depolarization period is in support of a presynaptic locus of suppression of inhibition. Analysis of the coefficient of variation (CV) of the IPSC amplitudes (Faber and Korn, 1991) showed that the ratio between CV$^2$ during DSI versus CV$^2$ under control was proportional to the relative change in synaptic amplitude (Figure 2.2E; averaged CV$^2_{DSI}$/ CV$^2_{control}$: 0.38±0.03; amplitude ratio: 0.36±0.03), confirming that the modulation is presynaptic in origin.

To investigate the involvement of the cannabinoid CB$_1$R in depolarization induced IPSC suppression, we performed similar experiments in CB$_1$R knockout (CB$_1$R$^{-/-}$) mice. Here we did not observe a significant reduction in IPSC amplitude during the depolarization period (IPSC reduction: 19.6±4.6%; n=6; p=0.14; Figure 2.3A-B). Failure rate was also unaffected (control: 23.7±4.5%; depolarization: 30.3±3.9%; p=0.12; Figure 2.3C). The amount of DSI in CB$_1$R$^{-/-}$ mice was significantly different from that observed in wildtype mice (p=0.006). Therefore, we conclude that the CB$_1$R is necessary for depolarization induced IPSC suppression.

Activity-dependent endocannabinoid synthesis in several brain regions depends on DAGL enzymatic activity (Melis et al., 2004; Safo and Regehr, 2005; Szabo et al., 2006). To test for the involvement of DAGLs in hippocampal DSI, we used several DAGL inhibitors. Slices were incubated with the novel potent DAGL inhibitor OMDM-188 (2 µM; Ortar et al., 2008) for at least 30 minutes before commencing paired recordings in the presence of OMDM-188. Under these conditions, significant DSI was still observed (amount of DSI: 46.2±5.3%; n=6; p=0.016; Figure 2.4A), and was accompanied by an increase in failure rate (control: 51.7±1.6%; depolarization: 67.7±2.2%; p=0.03; Figure 2.4A). The amount of DSI did not differ significantly from control conditions (p=0.05; Figure 2.4F). Similarly, slices incubated and recorded with the established DAGL inhibitor RHC-80267 (50-70 µM) also showed significant DSI (87.5±2.4%; n=5; p=0.009; Figure 2.4B), and an increase in failure rate (control: 54.0±4.7%; depolarization: 92.0±0.6%; p=0.03; Figure 2.4B).

Recently, several conflicting studies have reported opposing effects of extracellular application of the DAGL inhibitor THL on hippocampal DSI (Szabo et al., 2006; Edwards et al., 2008; Hashimotodani et al., 2008). It has been shown that intracellular application of low concentrations of THL is very effective at inhibiting DAGL activity in striatum (500 nM; Melis et al., 2004) and cerebellum (2 µM; Safo and Regehr, 2005). Therefore, we added THL (5 µM) to the intracellular solution of the postsynaptic pyramidal neuron, without applying THL extracellularly. To allow intracellular diffusion of THL the depolarization protocol was started at least 20 minutes after establishing the whole-cell configuration at the pyramidal neuron. Intracellular application of THL did not affect DSI (70.9±1.6%; n=15; p=0.02; Figure 2.4C; compared to control DSI: p=0.05; Figure 2.4F). Accordingly, failure rate significantly increased upon depolarization (control: 24.9±1.6%; depolarization: 66.8±2.0%; p<0.001; Figure 2.4C). Even
DAG lipase is not involved in hippocampal DSI

with a 1 hour waiting period for intracellular THL diffusion, DSI was clearly observed (Figure 2.4E), suggesting that the ineffectiveness of intracellular THL was not due to incomplete intracellular diffusion. Surprisingly, when slices were incubated extracellularly with higher concentrations of THL (10 µM), DSI was abolished (17.2±2.1%; n=6; p=0.10; Figure 2.4D), and failure rate was not affected by depolarization (control: 51.8±3.8%; depolarization: 51.1±3.4%; p=0.86; Figure 2.4D).

This observation raised the possibility that the other treatments were not effective in inhibiting DAGL’s due to sub-optimal experimental conditions such as lack of cell
penetration. To exclude this possibility, we used the above procedures and inhibitors to block DAGLs in the well-established phenomenon of slow self-inhibition of neocortical interneurons (SSI; Bacci et al., 2004). SSI is induced by repetitive AP firing of somatostatin-positive low-threshold spiking (LTS) interneurons in neocortex (Figure 2.5). It is triggered by endocannabinoid-mediated activation of postsynaptic CB1Rs, which in turn activate a prolonged K+ conductance. Recently, it was shown that DAGL inhibition effectively abolishes SSI (Marinelli et al., 2008). We made whole-cell recordings of interneurons in mice expressing EGFP in somatostatin-positive interneurons (Oliva et al., 2000; Figure 2.5A). Upon hyperpolarizing current steps these neurons showed a clear rebound depolarization that regularly induced a rebound AP (Figure 2.5A), characteristic of LTS interneurons. After recording resting membrane potential for several minutes, LTS interneurons were stimulated with 10 trains of 60 APs at 50 Hz each (Marinelli et al., 2008). This stimulation induced a clear, long-lasting drop in membrane potential (SSI; ΔVm = 4.45±1.11 mV; n=7; p=0.007; Figure 2.5B). All pharmacological interventions reducing DAGL-activity effectively blocked SSI (Figure 2.5C-F). When slices were incubated and recorded in OMDM-188 (2 µM), SSI was absent (ΔVm = 0.78±0.76 mV; n=8; p=0.33; Figure 2.5C). Incubation with RHC-80267 (70 µM) also effectively blocked SSI (ΔVm = -0.63±1.34 mV; n=5; p=0.66; Figure 2.5D). Finally, intracellular application of THL (5 µM) also effectively blocked SSI (ΔVm = -0.35±0.98 mV; n=6; p=0.74; Figure 2.5E).

Therefore, we conclude that the lack of effect of DAGL inhibitors on DSI at the interneuron to CA1 pyramidal neuron unitary connections that we studied was not due to sub-optimal experimental conditions. Taken together, our data suggest that DSI at these unitary connections in hippocampus occurs without the involvement of DAGL activity.

Discussion

Hippocampal DSI was the first form of synaptic plasticity shown to be mediated by endocannabinoid signaling. However, the biosynthetic pathway controlling endocannabinoid formation in hippocampal DSI has remained unclear. We systematically addressed the involvement of DAGLs, key enzymes for the formation of the endocannabinoid 2-AG in the brain, in DSI at identified unitary synaptic connections in hippocampus. Using paired recordings, we show that several known DAGL inhibitors overall do not affect DSI at these hippocampal synapses.

Our results show that the novel DAGL inhibitor OMDM-188 has no effect on hippocampal DSI. Thus far, OMDM-188 has not been used to study endocannabinoid-mediated synaptic plasticity. However, like other DAGL inhibitors, this compound effectively reduces 2-AG levels in ionomycin-stimulated N18TG2 cells, indicating that indeed it inhibits DAGL activity in intact neurons (Bisogno et al., 2009). We confirmed the efficacy of OMDM-188 in our experiments by showing that it abolishes SSI in neocortical interneurons (Figure 2.5), a process which has recently been shown to require
DAGL activity (Marinelli et al., 2008).

Next, we tested the specific DAGL inhibitor RHC-80267 (Stella et al., 1997). This compound has been shown to block endocannabinoid mediated synaptic plasticity in several brain regions (Safo and Regehr, 2005; Straiker and Mackie, 2005; Szabo et al., 2006). Although it effectively inhibited DAGL activity in our experiments on SSI, RHC-80267 had no effect on hippocampal DSI. This finding is in line with other studies on DSI using extracellular stimulation to evoke IPSCs (Chevaleyre and Castillo, 2003; Edwards et al., 2006). Based on our results with OMDM-188 and RHC-80267 alone, it seems clear that DAGL is not involved in hippocampal DSI.

Recently, several conflicting studies have reported opposing effects of extracellular application of the DAGL inhibitor THL on DSI (Szabo et al., 2006; Edwards et al., 2008; Hashimotodani et al., 2008). Intracellular application of THL at concentrations between 500 nM and 2 µM effectively inhibits DAGL activity in VTA, cerebellum and neocortex (Melis et al., 2004; Safo and Regehr, 2005; Marinelli et al., 2008). THL was shown to be a more potent inhibitor of DAGLs than RHC-80267 (Bisogno et al., 2003). We found that intracellular postsynaptic application of THL did not affect DSI. The inefficacy of intracellular THL application in hippocampal pyramidal neurons is unlikely to be due to experimental conditions. Intracellular THL application successfully inhibited DAGL activity in neocortical interneurons and blocked SSI, as previously reported by Marinelli and colleagues (Marinelli et al., 2008). In addition, allowing up to one hour for intracellular diffusion did not show any reduction of DSI (Figure 2.4E). Therefore, our experiments with intracellular THL application seem to confirm the above findings with other DAGL inhibitors, and to rule out involvement of DAGL in hippocampal DSI. This raises the question by what mechanism extracellular application of the DAGL inhibitor THL on DSI (Szabo et al., 2006; Edwards et al., 2008; Hashimotodani et al., 2008) including the present study. Hashimotodani and colleagues (2008) and Szabo and colleagues (2006) ruled out indirect effects of THL on either CB1R signaling or on calcium influx necessary for DSI induction. At present, the mechanism by which extracellular THL blocks hippocampal DSI is unknown. Given the absence of effects of OMDM-188, RHC-80267, and of intracellular THL on DSI, we argue that extracellular THL does not act by inhibiting DAGL at this synapse.

It has long been unclear which endocannabinoid mediates DSI at inhibitory synapses in the hippocampus. However, recent studies have suggested that 2-AG, and not anandamide, is the endocannabinoid mediating this phenomenon. This conclusion was based on the ability of inhibitors of monoacylgllycerol lipase (MAGL) to prolong hippocampal DSI (Makara et al, 2005; Hashimotodani et al., 2007a). Furthermore, inhibition of fatty-acid amide hydrolase (FAAH), the enzyme responsible for the breakdown of anandamide, does not affect the timecourse of hippocampal DSI (Kim and Alger, 2004; Makara et al., 2005; Hashimotodani et al., 2007a). These findings indeed suggest that 2-AG, and not anandamide, is the endocannabinoid mediating hippocampal DSI.

Studies of DAGL distribution in hippocampus have shown the specific localization of this enzyme opposite glutamatergic terminals containing the CB1R in postsynaptic spines of principal neurons (Katona et al., 2006; Yoshida et al., 2006). In particular it
was suggested that *sn*-1-DAGL α is perfectly positioned for the production of 2-AG as a retrograde messenger at excitatory synapses. However, surprisingly, *sn*-1-DAGL α was not found opposite symmetrical, GABAergic synapses (Katona et al., 2006). In combination with our results, this suggests that *sn*-1-DAGL α is not involved in DSI at inhibitory synapses in the hippocampus. This may suggest that either 2-AG is produced by a different mechanism at these synapses, involving another, hitherto unknown, synthesis pathway, such as the lysophosphatidic acid-dependent route identified by Nakane and colleagues (Nakane et al., 2002). Alternatively, it may suggest that 2-AG is not the endocannabinoid mediating DSI at inhibitory synaptic connections in hippocampus.
Direct cannabinoid modulation of ionotropic GABA\(_A\) receptors

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Cannabinoid effects on the brain are thought to be mainly mediated by activation of the G-protein-coupled cannabinoid CB\(_1\) receptor (CB\(_1\)R). However, here we show that cannabinoids can still reduce evoked IPSC amplitude in hippocampal brain slices in the presence of a CB\(_1\)R antagonist as well as in CB\(_1\)R knockout mice. We explain this finding by showing that cannabinoids can directly modulate ionotropic GABA\(_A\) receptors (GABA\(_A\)Rs), the main receptors mediating inhibitory synaptic transmission in the mature mammalian CNS. Cannabinoids reduce the amplitude and modulate the kinetics of currents mediated by recombinant GABA\(_A\)Rs expressed in HEK293 cells. Furthermore, we show a similar modulation of native GABA\(_A\)Rs on isolated hippocampal pyramidal neurons and on nucleated patches from neocortical neurons. The modulation of GABA\(_A\)R mediated current kinetics consists of an increase in desensitization, together with a prolongation of deactivation time, suggesting that cannabinoids can interact with the transitions between channel states. Thus CB\(_1\)R-independent modulation of GABA\(_A\)Rs by (endo)cannabinoids could provide a novel mechanism for regulation of synaptic inhibition.
Introduction

Most of the effects of cannabinoids on mammalian behavior are attributed to activation of cannabinoid CB1 receptors (CB1Rs), which are abundantly expressed throughout the brain. Application of exogenous cannabinoids, or activity dependent synthesis of endogenous cannabinoids (endocannabinoids), activates presynaptic CB1Rs, which reduce neurotransmitter release through G-protein mediated inhibition of Ca2+ channels (Sullivan, 1999; Wilson et al., 2001). In doing so, CB1R mediated endocannabinoid signaling mediates several forms of short-term (Kreitzer and Regehr, 2001a,b; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) and long-term synaptic plasticity (Chevaleyre et al., 2006).

However, cannabinoids still affect animal behavior both in the presence of CB1R antagonists as well as in CB1R knockout (CB1R−/−) mice (Adams et al., 1998; Di Marzo et al., 2000; Baskfield et al., 2004). This suggests the existence of additional brain targets for cannabinoids. Indeed, several studies have suggested the existence of novel cannabinoid receptors (Breivogel et al., 2001; Hoffman et al., 2005). In line with this, it has been shown that cannabinoids may directly affect various ligand-gated ion channels of the Cys-loop receptor superfamily, namely the nicotinic acetylcholine receptor (nAChR; Oz et al., 2004b), the serotonin 5HT3 receptor (Barann et al., 2002; Oz et al., 2002), and the glycine receptor (Lozovaya et al., 2005; Hejazi et al., 2006). The physiological significance of cannabinoid interaction with these targets on brain functioning remains unrevealed.

The GABA_A receptor (GABA_A,R), the main receptor mediating inhibitory synaptic transmission in the mature mammalian brain, also belongs to the Cys-loop receptor superfamily. This would imply that the GABA_A,R might also be a target for direct cannabinoid modulation. Since activity dependent release of endogenous cannabinoids has been shown to occur at GABAergic synapses in a variety of brain regions, we wanted to test whether a direct modulation of postsynaptic GABA_A,Rs by (endo)cannabinoids plays a role in regulation of inhibitory synaptic transmission.

Here we first show that exogenous application of a synthetic cannabinoid reduces the amplitude of GABAergic inhibitory postsynaptic currents (IPSCs) in hippocampal brain slices in the absence of CB1R signaling. Next we show that cannabinoids reduce the amplitude and modulate the kinetics of GABA_A,R mediated currents independent of CB1R activation in HEK293 cells expressing recombinant GABA_A,Rs, as well as in isolated pyramidal neurons and nucleated patches from neocortical pyramidal neurons. We then substantiate the CB1R independence of this modulation by making a pharmacological fingerprint, showing that the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) are more potent than the synthetic cannabinoid WIN55,212-2 in modulating GABA_A,R properties, while at the CB1R site this order is reversed. In addition, a CB1R antagonist shows similar modulation of GABA_A,R properties as CB1R agonists. Finally, by studying cannabinoid modulation of GABA_A,Rs over a range of GABA concentrations, we show that cannabinoid modulation is GABA concentration dependent, suggesting that cannabinoids interact with the affinity of the
GABA\(_A\)R for its endogenous ligand. The aggregate of these data suggest to us that the GABA\(_A\)R can function as a novel target for (endo)cannabinoids in the brain.

**Materials & Methods**

**Brain slice recordings**

Wildtype C57Bl6 mice or CB\(_1\)R\(^{-/-}\) mice (Marsicano et al., 2002) of 14-21 days old were killed by decapitation, after which the brain was removed. Transverse hippocampal slices (300 \(\mu\)m) were sectioned in ice-cold solution containing (mM): 126 NaCl, 3 KCl, 10 glucose, 26 NaHCO\(_3\), 1.2 Na\(_2\)HPO\(_4\), 1 CaCl\(_2\), and 3 MgCl\(_2\) (carboxygenated with 5% CO\(_2\)/95% O\(_2\)). Slices were transferred to an incubation chamber containing the same solution, but now with 2 mM CaCl\(_2\), and 2 mM MgCl\(_2\), and kept at 33°C for ~30 minutes after slicing. For recording, slices were transferred to a recording chamber containing extracellular solution of the same ionic composition as in the incubation chamber. Neurons were visualized via a 40x water immersion objective using infrared differential interference contrast video microscopy. Recordings were made at room temperature (20-22°C). Whole-cell voltage and/or current clamp recordings were made from CA1 pyramidal neurons using borosilicate glass pipettes with resistance of 3-5 M\(\Omega\), containing (mM): 70 Cs gluconate, 70 CsCl, 4 Mg-ATP, 4 phosphocreatine, 0.4 GTP, 0.5 EGTA, 5 QX-314 and 10 HEPES (pH 7.3, CsOH). A monopolar glass stimulation electrode was placed in stratum pyramidale in close proximity of the cell body of the recorded neuron. Because of the high intracellular chloride concentration used, IPSCs were observed as inward currents. All stimulus delivery and data acquisition was performed using EPC-8 amplifiers with PULSE software (HEKA Elektronik, Lambrecht, Germany).

**HEK293 cells**

HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml streptomycin, and 100 U/ml penicillin. Cells were transfected with expression plasmids for GABA\(_A\)R subunits \(\alpha_1\), \(\beta_2\), and \(\gamma_2\) (Pritchett and Seeburg, 1990) in the ratios 2:2:1. pEYFP-N1 (Invitrogen) was added to the transfection mixture. Transfected cells were maintained in culture for up to 48 hours before use. For recordings cells were transferred to a recording chamber containing (mM): 126 NaCl, 3 KCl, 2 MgSO\(_4\), 2 CaCl\(_2\), 10 D(+)-glucose, 1.20 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\) (carboxygenated with 5% CO\(_2\)/95% O\(_2\)). Whole-cell recordings were made at room temperature (20-22°C), using 3-4 M\(\Omega\) borosilicate glass electrodes, containing (mM): 77 K-gluconate, 77 KCl, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, 4 K\(_2\)Phosphocreatine, 0.4 GTP (pH 7.3 with KOH). Cells were voltage clamped at -70 mV, lifted from the glass coverslip, and placed in front of a piezo-controlled (P245.70, Physik Instrumente, Waldbronn, Germany) fast application system with a double-barreled application pipette. Reliability of application was checked before experiments by measuring the open-tip response to application of diluted (10%) extracellular solution (Colquhoun et al., 1992). Current responses were
recorded upon application of GABA (1 mM) using an EPC-8 amplifier with PULSE software (HEKA Elektronik, Lambrecht, Germany).

**Nucleated Patch recordings**

Neocortical brain slices were prepared from 14-21 day old Sprague/Dawley rats as described above for hippocampus, and whole-cell voltage clamp recordings ($V_m=-70$ mV) were made from pyramidal neurons in layer 2/3 of somatosensory cortex. Nucleated outside-out patches were pulled from these neurons, and GABA was applied using the same application procedure and intra/extracellular solutions as for HEK293 cells (see above).

**Isolated hippocampal neurons**

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

**Chemicals**

WIN55,212-2, WIN55,212-3 and CP55,940 were obtained from Tocris Bioscience (Avonmouth, UK). SR141716A was a gift from Solvay Pharmaceuticals (Weesp, the Netherlands). Of these chemicals appropriate stock solutions were prepared in DMSO. Final DMSO concentration in the medium never exceeded 0.1%. Anandamide stock solution was in anhydrous ethanol (Tocris Bioscience, Avonmouth, UK). In all experiments with application of these drugs control solutions always contained an equal amount of DMSO or ethanol.
Data analysis
All data was analyzed off-line using IGOR PRO software (Wavemetrics, Lake Oswego, OR). Values shown in bar histograms and text are mean ± standard error of the means (SEM). Data was filtered at 3-5 kHz. Statistical analysis was done using two-sided student’s t-test ($\alpha=0.05$).

Kinetics of $I_{GABA}$ in isolated hippocampal neurons was fitted by single-exponential decay. Kinetics of $I_{GABA}$ in HEK293 cells and nucleated patches was analysed as follows: Current desensitization was defined as (1-([amplitude 400ms after onset of GABA application]/[peak amplitude]). Current deactivation was defined as the 90-10% decay time after GABA application was stopped.

Results

Cannabinoids reduce hippocampal inhibitory synaptic transmission independent of CB$_1$R activation
To test whether cannabinoids can modulate inhibitory synaptic transmission independent of CB$_1$R activation, we recorded CA1 pyramidal neurons in whole-cell voltage clamp in hippocampal slices. IPSCs were evoked by stimulating in close proximity (<100 µm) of the pyramidal neuron soma (Figure 3.1A) in the continuous presence of the ionotropic glutamate receptor antagonists DNQX (10 µM) and DL-AP5 (20 µM) to block AMPA/kainate and NMDA receptors, respectively. We did not observe any changes in baseline amplitude during 30 min recordings (Figure 3.1B,F). Application of the CB$_1$R agonist CP55,940 (CP; 5 µM) caused a significant reduction in IPSC amplitude to $77\pm9\%$ of the amplitude in control ($p=0.037; n=8$; Figure 3.1C,F). This IPSC reduction could not be reversed by washout of CP, as is often the case with lipophilic substances like cannabinoids (Figure 3.1C; $n=5$). The IPSC reduction was not accompanied by a change in paired pulse ratio (PPR; amplitude of IPSC$_2$/amplitude of IPSC$_1$) of two consecutive IPSCs 50 ms apart (PPR control: 0.76±0.02; PPR in CP: 0.74±0.02; $p=0.57$; $n=8$; Figure 3.1G), suggesting a postsynaptic locus of the IPSC reduction (although this finding should be interpreted with caution; see chapter 2).

When the same experiment was performed on brain slices that were incubated in the CB$_1$R antagonist SR141716A (SR; 2 µM) a significant reduction in IPSC amplitude was still observed by application of CP ($I_{norm}: 0.79\pm0.03; p=0.003; n=5$; Figure 3.1D,F). Again, this IPSC reduction was again not accompanied by a change in PPR (Figure 3.1G). Similarly, application of CP also caused IPSC reduction in brain slices from CB$_1$R$^{-/-}$ mice ($I_{norm}: 0.74\pm0.08; p=0.026; n=6$; Figure 3.1E,F) without changing PPR (Figure 3.1G). This indicates that cannabinoids can cause a CB$_1$R independent -and most likely postsynaptic- reduction of IPSC amplitude in hippocampus. This reduction could be mediated by a direct cannabinoid modulation of postsynaptic GABA$_A$Rs.
Figure 3.1 Cannabinoids reduce hippocampal IPSC amplitude in the presence of a CB, R antagonist and in CB, R-/- mice.

(A) Schematic experimental setup, showing a hippocampal brain slice with recording electrode (rec.) and stimulation electrode (stim.) located in CA1. (B) Averaged IPSC amplitude in time. Inset traces show the averaged IPSC during the first 10 minutes (Control1) and the last ten minutes (Control2) of a representative recording. (C) Averaged IPSC amplitude in time, showing the effect of washin of CP (5 µM) on IPSC amplitude. Inset traces show averaged IPSC during baseline (Control) and following CP washin (10-20 minutes after the onset of washin). (D) Same as in (C), but now for brain slices incubated and recorded in the CB, R antagonist SR (2 µM). (E) Same as in (C,D), but now for brain slices from CB, R-/- mice. (F) Bar histograms show averaged normalized IPSCs during control experiments (stability), as well as 10-20 minutes after the washin of CP for the different groups. Open circles show individual experiments. (G) Left, representative IPSC traces normalized to peak amplitude. CP did not have any significant effect on PPR. Bar histogram compares averaged PPR before and after CP application.
Cannabinoids decrease amplitude and alter kinetics of recombinant GABA<sub>A</sub>R mediated currents

To test whether cannabinoids directly modulate GABA<sub>A</sub>R mediated currents, we made whole-cell voltage clamp recordings from HEK293 cells expressing recombinant GABA<sub>A</sub>Rs. HEK293 cells do not express any known cannabinoid receptors endogenously (Griffin et al., 2000). The α<sub>1</sub>, β<sub>2</sub>, and γ<sub>2</sub> subunits of the GABA<sub>A</sub>R, which are abundantly expressed in the adult rat brain (Wisden et al., 1992), were transiently co-expressed in HEK293 cells. Rapid application of GABA (1 mM; 0.4 ms; V<sub>m</sub>=-70 mV) resulted in a fast inward GABA<sub>A</sub>R mediated current (I<sub>GABA</sub>; Figure 3.2A). When CP (1 µM) was added to the extracellular solution, the amplitude of I<sub>GABA</sub> was significantly reduced (current amplitude normalized to control; I<sub>norm</sub>: 0.85±0.05; p=0.02; n=6). This inhibition was accompanied by an enhancement of current desensitization (normalized desensitization: 1.42±0.04; p=0.0001; n=6; Figure 3.2B), and a prolongation of current deactivation (normalized deactivation: 1.86±0.17; p=0.002; n=6; Figure 3.2C). All these parameters slowly and partially recovered upon washout of CP.
Qualitatively similar results were obtained with another CB\(_1\)R agonist, WIN55,212-2 (WIN-2, 5 µM; \(I_{\text{norm}}\): 0.66±0.04; \(p=0.003; n=4\); normalized desensitization: 1.79±0.09; \(p=0.002; n=4\); normalized deactivation: 3.02±0.45; \(p=0.01; n=4\); Figure 3.2A-C). Furthermore WIN55,212-3 (WIN-3; 5 µM), an enantiomer of WIN-2 which does not activate the CB\(_1\)R, had comparable effects on \(I_{\text{GABA}}\) amplitude (\(I_{\text{norm}}\): 0.70±0.08; \(p=0.02; n=4\)) and desensitization (normalized desensitization: 1.68±0.21; \(p=0.03; n=4\); Figure 3.2A-C). Surprisingly, the CB\(_1\)R antagonist SR (5 µM) also inhibited amplitude (\(I_{\text{norm}}\): 0.60±0.10; \(p=0.02; n=4\); Figure 3.3A), increased desensitization (normalized desensitization: 1.31±0.05; \(p=0.007; n=4\); Figure 3.3B) and had a particularly strong effect on current deactivation (normalized deactivation: 6.3±1.11; \(p=0.01; n=4\); Figure 3.3C). Thus, both CB\(_1\)R agonists and a CB\(_1\)R antagonist similarly affect the amplitude and kinetics of evoked currents carried by recombinant GABA\(_A\)Rs.
Cannabinoids modulate native GABA\textsubscript{A}R mediated currents in isolated hippocampal pyramidal neurons

To determine whether native GABA\textsubscript{A}Rs are also sensitive to cannabinoids, whole-cell voltage clamp recordings were made from pyramidal neurons acutely isolated from the hippocampus of rats. Application of GABA (100 µM; \(V_m\)=-80 mV) elicited a GABA\textsubscript{A}R mediated current that was completely and reversibly blocked by the GABA\textsubscript{A}R antagonist bicuculline (20 µM, data not shown).

The amplitude of \(I_{GABA}\) was reduced by the synthetic cannabinoid WIN-2 in a concentration dependent manner (Figure 3.4A). Because of the shape of the dose-response curve a reliable estimate for the IC\textsubscript{50} of WIN-2 could not be obtained, but half maximal inhibition occurred at a concentration of approximately 10 µM. In addition to a reduction in current amplitude, WIN-2 accelerated desensitization of \(I_{GABA}\) and
Figure 3.5 Endocannabinoids modulate GABA$_A$R mediated currents in isolated hippocampal pyramidal neurons.

(A) Top, the endogenous cannabinoid AEA causes a concentration dependent decrease in $I_{GABA}$ amplitude. Traces show a representative example of the response of an isolated hippocampal pyramidal neuron to application of GABA (100 µM; $V_m$=-80 mV) in the absence (control) or presence of different concentrations of AEA. Each trace is the average of 2-4 individual sweeps. Bottom, dose-response curve showing the effect of different concentrations of AEA on $I_{GABA}$ amplitude. Values are normalized to $I_{GABA}$ amplitude under control conditions (n=5 for each data point). (B) Top, AEA causes a concentration dependent acceleration of $I_{GABA}$ desensitization. Same traces as in (A), but normalized to the peak current amplitude. Bottom, dose-response curve showing the effect of different concentrations of AEA on the desensitization time-constant ($\tau_{desens}$) of $I_{GABA}$. Values are normalized to $\tau_{desens}$ in control (n=5 for each data point). (C) Top, AEA causes a deceleration of current deactivation. Same traces as in (A), but normalized to the current amplitude at the end of GABA application. Bottom, dose-response curve showing the effect of different concentrations of AEA on the deactivation time-constant ($\tau_{deact}$) of $I_{GABA}$. Values are normalized to $\tau_{deact}$ in control (n=4 for data point). Error bars indicate ± SEM. (D) Left, the endogenous cannabinoid 2-AG causes a concentration dependent decrease in $I_{GABA}$ amplitude. Traces show a representative example of the response of an isolated hippocampal pyramidal cell to application of GABA (100 µM; $V_m$=-80 mV) in the absence (control) or presence of different concentrations of 2-AG, and after washout of 2-AG. Each trace is the average of 2-4 individual sweeps. Right, summary showing the effect of different concentrations of 2-AG on $I_{GABA}$ amplitude. Values are normalized to $I_{GABA}$ amplitude under control conditions (n=2-3 for each bar).
prolonged current deactivation following removal of GABA. Both effects on current kinetics showed concentration dependence (Figure 3.4B,C). All parameters partially recovered after WIN-2 was washed out (data not shown).

Next, we tested whether endogenously occurring cannabinoids (endocannabinoids) can have similar effects on GABA<sub>A</sub>R mediated currents as synthetic cannabinoids. The endocannabinoid AEA caused a concentration dependent reduction of I<sub>GABA</sub> amplitude (Figure 3.5A; IC<sub>50</sub>=880±210 nM; Hill coefficient=1.2±0.3; n=5). It should be noted that the effective concentration range for AEA effects on GABA<sub>A</sub>Rs, as estimated from these experiments, is comparable to that for activation of the CB<sub>1</sub>R (EC<sub>50</sub>=1.4±0.3 µM; Breivogel et al., 2001). In addition to a reduction in current amplitude, AEA also accelerated desensitization of I<sub>GABA</sub> and prolonged current deactivation following removal of GABA in a concentration dependent way (Figure 3.5B,C).

The endocannabinoid 2-arachidonoylglycerol (2-AG) caused a similar concentration dependent reduction of I<sub>GABA</sub> amplitude as AEA (Figure 3.5D; n=2-3 for each concentration), although we did not have enough data to assess effects on current kinetics or perform statistics.

Interestingly, at low concentrations of AEA and WIN-2, on average a slight potentiation of I<sub>GABA</sub> was observed. A more detailed analysis revealed that two pharmacologically distinct populations of pyramidal neurons could be identified. At 200 nM of AEA half of the neurons tested (7 out of 14 cells) showed potentiation of I<sub>GABA</sub> (Figure 3.6A), while the other half showed depression (Figure 3.6B). These populations demonstrate statistically significant differences in the effects produced by 200 nM of AEA (I<sub>norm</sub>: 1.18±0.06 (n=7) vs. 0.89±0.02 (n=7); p<0.0002). However, at higher concentrations

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**Figure 3.6 Dual effect of AEA on GABA<sub>A</sub>R mediated currents in acutely isolated hippocampal pyramidal neurons.**

(A) Left, representative traces from a neuron showing dual modulation of peak I<sub>GABA</sub> by AEA. At 200 nM of AEA there is a potentiation of I<sub>GABA</sub>, whereas at 1 µM peak current is reduced. Right, representative traces from a cell showing only a dose-dependent inhibition of I<sub>GABA</sub>. (B) Dose-response curves showing the effect of different concentrations of AEA on I<sub>GABA</sub> amplitude. The set of data (same as in Figure 3.5A) is subdivided into 2 subsets, depending on the polarity of the effect produced by 200 nM AEA. Cells showing amplitude potentiation at 200 nM AEA are shown in grey, while cells showing depression are shown in black. (C) Dose-response curves showing the effect of different concentrations of AEA on the desensitization time-constant (τ<sub>desens</sub>) of I<sub>GABA</sub> for the same two groups of cells. Values are normalized to τ<sub>desens</sub> in control. Error bars indicate ± SEM.
Figure 3.7 Cannabinoid modulation in nucleated patches from neocortical pyramidal neurons depends on GABA concentration.

(A) Representative current traces from nucleated patches activated by different GABA concentrations (1 µM, 5 µM, 100 µM and 1 mM) in the absence (black) and in the presence (grey) of the synthetic cannabinoid WIN-2 (5 µM). (B) Left, averaged amplitude for experiments in which different GABA concentrations were applied in the absence (black) and presence (grey) of WIN-2 (5 µM). Middle, averaged amount of desensitization calculated as (1-(amplitude 400ms after onset of GABA application)/[peak amplitude]) for experiments in which different GABA concentrations were applied in the absence (black) and presence (grey) of WIN-2 (5 µM). Right, averaged deactivation time (90-10%) for experiments in which different GABA concentrations were applied in the absence (black) and presence (grey) of WIN-2 (5 µM). (C) Same as in (B), but now the data in the presence of WIN-2 is normalized to control for each GABA concentration. (D) Time to peak for experiments in which different GABA concentrations were applied in the absence (black) and presence (grey) of WIN-2 (5 µM).
Direct GABA$_A$ receptor modulation by cannabinoids

(0.5-5 µM) all neurons showed depression (Figure 3.6C). Furthermore, this difference in amplitude modulation by low AEA concentrations was not accompanied by a difference in modulation of desensitization (Figure 3.6C).

Taken together, these results indicate that both synthetic and endogenous cannabinoids reduce amplitude, accelerate and increase desensitization, and prolong deactivation of GABA$_A$R mediated currents both in HEK293 cells and isolated hippocampal neurons, independent of CB$_1$R activation.

Cannabinoids modulate GABA$_A$R mediated currents on nucleated patches pulled from neocortical pyramidal neurons

Finally we wanted to have a closer look at the kinetic modulation of native GABA$_A$R properties by cannabinoids. Isolated neurons are quite large, and therefore the rise and falling phase of fast agonist application are obscured by solution dynamics around the cell. A faster agonist application can be achieved in nucleated patches pulled from the somatic membrane of pyramidal neurons. Therefore, we tested cannabinoid modulation of GABA$_A$Rs in nucleated patches pulled from layer 2/3 pyramidal neurons in slices from somatosensory cortex. We investigated the dependence of cannabinoid modulation on the GABA concentration. Rapid application of a low concentration of GABA (1 µM; 2 sec) resulted in a slowly rising current which showed almost no desensitization (Figure 3.7A). Application of WIN-2 (5 µM) did not change $I_{GABA}$ amplitude ($I_{norm}$: 1.01±0.05; p=0.80; n=5). Since desensitization was almost absent, the effect of WIN-2 on current desensitization could not be assessed. However, WIN-2 significantly increased deactivation time at this GABA concentration (Figure 3.7A-C).

Application of a slightly higher GABA concentration (5 µM; 1 sec) resulted in a current of higher amplitude, which showed more receptor desensitization (Figure 3.7A). At this GABA concentration addition of WIN-2 decreased $I_{GABA}$ amplitude, increased the amount of desensitization and slowed down deactivation (although the effect on deactivation only reached borderline significance; Figure 3.7A-C).

Further increasing the concentration of applied GABA (100 µM; 1 sec / 1 mM; 400ms) resulted in even higher $I_{GABA}$ amplitudes, and in more receptor desensitization (Figure 3.7A). At these concentrations both amplitude, amount of desensitization and deactivation time were significantly modulated by application of WIN-2 (Figure 3.7B,C). However, the normalized increase in the amount of desensitization was much smaller at these high concentrations than at a GABA concentration of 5 µM (Figure 3.7C).

Finally, we looked at the rise time of $I_{GABA}$ at these different GABA concentrations. At 1 µM the time to peak of $I_{GABA}$ was 630±83 ms. In the presence of WIN-2 the concentration. (D) Top, representative current traces from the onset of the GABA$_A$R mediated current from nucleated patches activated by different GABA concentrations (1 µM, 5 µM, 100 µM and 1 mM) in the absence (black) and in the presence (grey) of the synthetic cannabinoid WIN-2 (5 µM). Bottom, bar histograms show averaged time to peak for all different GABA concentrations in the absence (black) and presence (grey) of WIN-2.
Chapter 3

The time to peak was slightly, but not significantly, faster (510±35 ms; p=0.28; n=5; Figure 3.7D). When 5 µM of GABA was applied the time to peak was much faster, and at this concentration it was significantly accelerated by WIN-2 (control: 221±16 ms; WIN-2: 158±7 ms; p=0.01; n=7; Figure 3.7D). At a GABA concentration of 100 µM or 1 mM the time to peak was very fast, and unchanged by WIN-2 (100 µM: control: 4.1±0.4 ms; WIN-2: 3.9±0.2 ms; p=0.49; n=5; 1 mM: control: 2.1±0.3 ms; WIN-2: 2.1±0.3 ms; p=0.88; n=7; Figure 3.7D). However, here the current rise time was close to the speed of solution exchange on the nucleated patch, so we cannot be sure that we would be able to resolve changes in these kinetics.

In conclusion, cannabinoids can also modulate GABA<sub>A</sub>R mediated responses in nucleated patches from neocortical pyramidal neurons. Furthermore, the nature of this cannabinoid modulation shows a strong dependence on GABA concentration.

Discussion

Our findings imply that the GABA<sub>A</sub>R, the most abundant inhibitory ligand-gated ion channels in the mammalian CNS, is directly modulated both by synthetic and by endogenous cannabinoids. This modulation is independent of CB<sub>1</sub>R activation. Because of the ubiquitous nature of cannabinoid signaling in the brain, this direct modulation of GABA<sub>A</sub>Rs is likely to affect synaptic transmission, plasticity and microcircuit processing in many cortical and subcortical regions (see chapter 4).

The ionotropic GABA<sub>A</sub>R as a novel pharmacological target for cannabinoids

Despite the fact that a direct interaction of cannabinoids with both voltage-gated and ligand-gated ion channels has been suggested before (for review see Oz, 2006; Lozovaya et al., 2009), we are the first to describe a direct modulation of the GABA<sub>A</sub>R.

In the brain cannabinoids are known to activate G-protein coupled CB<sub>1</sub>Rs at the concentration range used in this study (Breivogel et al., 2001; Howlett et al., 2002). However, for several reasons it is highly unlikely that our observations can be attributed to the activation of any known cannabinoid receptor. First, effects on synaptically evoked responses persisted in the presence of a CB<sub>1</sub>R antagonist as well as in CB<sub>1</sub>R<sup>-/-</sup> mice. Furthermore, cannabinoids affected currents mediated by recombinant GABA<sub>A</sub>Rs expressed in HEK293 cells, which do not express any known cannabinoid receptors endogenously (Griffin et al., 2000).

Second, the pharmacological profile of cannabinoid-induced effects on GABA<sub>A</sub>Rs differs from that of the CB<sub>1</sub>R. In particular, the order of potency for different agonists differs from that observed for CB<sub>1</sub>R activation. While WIN-2 is much more potent than AEA at activating the CB<sub>1</sub>R (Breivogel et al., 2001), AEA is more potent than WIN-2 at suppressing I<sub>GABA</sub> amplitude (Figure 4.4/4.5). In addition, WIN-3, an enantiomer of the CB<sub>1</sub>R agonist WIN-2 that does not activate the CB<sub>1</sub>R, effectively reduces the GABA<sub>A</sub>R mediated current amplitude in HEK293 cells. These differences clearly indicate that the effect of cannabinoids on the GABA<sub>A</sub>R does not involve CB<sub>1</sub>R activation.
Surprising, and maybe most convincing of a CB₁R independent action of cannabinoids is the fact that the CB₁R antagonist SR modulated both I_{GABA} amplitude and kinetics in a similar way as CB₁R agonists. Again, this is a strong indication that the CB₁R is not involved in the direct GABAₐR modulation.

A recent study reported that GABAₐRs do not show modulation by cannabinoids (Hejazi et al., 2006). This study differs from ours in the concentration of GABA that was applied (3-30 µM). This difference in experimental approach is relevant since we found that the strength of cannabinoid modulation strongly depends on GABA concentration. In nucleated patches pulled from layer 2/3 pyramidal cells WIN-2 (5 µM) had no effect on current amplitude evoked by a low GABA concentration (1 µM), but significantly reduced current amplitude when higher GABA concentrations were applied (5 µM-1 mM; Figure 3.7). This shows that cannabinoid modulation of GABAₐRs may critically depend on the GABA concentration. Moreover, the study from Hejazi and colleagues (2006) differed from ours since they studied oocytes expressing α₂, β₃, and γ₂ subunits of the GABAₐR, while in our study we used HEK293 cells expressing the α₁, β₂, and γ₂ subunit of the GABAₐR. This could suggest that cannabinoid modulation of GABAₐRs shows receptor subunit specificity.

**Mechanism of cannabinoid modulation of GABAₐRs**

Although our results clearly indicate that cannabinoids can modulate GABAₐRs without activation of presynaptic CB₁Rs, the mechanism underlying this modulation remains unclear. Cannabinoids decrease GABAₐR mediated current amplitude, increase desensitization and slow down deactivation. Similar effects on current kinetics have been described for the nAChR (Spivak et al., 2007), as well as for the glycine receptor (Lozovaya et al., 2005) and the serotonin 5-HT₃A receptor (Xiong et al., 2008). Furthermore, cannabinoid modulation of voltage gated Ca²⁺ channels is also accompanied by an increase in current desensitization (for review see Lozovaya et al., 2009). It seems that the increased amount of receptor desensitization is a key observation in cannabinoid effects on ligand-gated and voltage-gated ion channels. Increased desensitization would explain the slowing of current deactivation, as these two parameters have been previously shown to be coupled: increased entry into desensitized states buffers the receptors in their agonist bound confirmation. Re-opening after receptor desensitization will therefore lengthen the deactivation time (Jones and Westbrook, 1995). Increased desensitization would also explain the decrease of GABAₐR mediated current amplitude, if we assume that more GABAₐRs may enter the desensitized state during the rising phase of the GABAₐR mediated current in the presence of cannabinoids.

The increase in GABAₐR mediated current amplitude that we observed at low AEA concentrations, as well as the decrease in current rise-time at low GABA concentrations cannot be explained by an increase in receptor desensitization, and it suggests that cannabinoids can influence the GABA affinity of the GABAₐR. Hence, single-channel analysis of GABAₐR mediated currents in the absence and presence of cannabinoids needs to be performed to pinpoint the exact kinetic mechanism underlying these observed effects.
It is important to note that benzodiazepines and neurosteroids, like cannabinoids, increase desensitization and slow down deactivation of the GABAₐR (Mellor and Randell, 1997; Zhu and Vicini, 1997; Shen et al., 2000). Interestingly these substances, which act on modulatory sites of the GABAₐR, have similar therapeutic abilities as cannabinoids, inducing anxiolytic, sedative, hypnotic and anticonvulsant effects (Franks and Lieb, 1994; Ashton et al., 2005). It would be interesting to investigate whether cannabinoids interact with the same (or similar) modulatory sites on the GABAₐR.

Because of the highly lipophilic properties of cannabinoids we cannot exclude the possibility that they alter GABAₐR gating properties not through a specific binding site on the GABAₐR, but through unspecific protein-lipid interactions. Cannabinoids will easily partition into the plasma membrane, where they may modulate the properties of the protein-lipid interface surrounding ligand-gated ion channels. In this way they could change energies associated with ligand binding and channel opening and closing.

**Direct GABAₐR modulation as a novel mechanism for (endo)cannabinoid signaling in the brain**

Thus far most of the effects of cannabinoids on mammalian behavior were attributed to activation of CB₁Rs. However, while the majority of cannabinoid effects appear to be absent in CB₁R deficient mice, AEA still induces catalepsy, analgesia and decreased spontaneous activity in these mutant mice (Di Marzo et al., 2000; Baskfield et al., 2004). Furthermore, the typical effects of AEA on spontaneous activity, body temperature, and pain perception in wild-type mice are not reversed by treatment with the selective CB₁R antagonist SR (Adams et al., 1998). Therefore, it could be that some of these behavioral effects are mediated by a direct cannabinoid interaction with ligand- or voltage-gated ion channels like the GABAₐR. In our study we show that the principal effect of cannabinoids on the GABAₐR is inhibiting: a decrease in GABAₐR mediated current amplitude together with an increase in GABAₐR desensitization. However, we show that this effect is critically dependent on GABA concentration, and that at low cannabinoid concentrations a potentiation of GABAₐR mediated current can sometimes be observed. It is at present unclear how this dependence on cannabinoid and GABA concentration shapes the properties of GABAₐRs in different locations (synaptic versus extrasynaptic) and under different conditions (high versus low endocannabinoid synthesis).

We conclude that while the search for novel cannabinoid receptors is yielding new possible targets (Hoffman et al., 2005; Lauckner et al., 2008), it is likely that at least some of the reported CB₁R-independent cannabinoid effects are mediated by a direct interaction with well established and abundant receptors, like the GABAₐR.
Suppression of inhibitory synaptic transmission by a direct action of cannabinoids on ionotropic GABA<sub>A</sub> receptors

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Cannabinoids have been shown to regulate inhibitory synaptic transmission via activation of presynaptic G-protein-coupled cannabinoid CB<sub>1</sub> receptors (CB<sub>1</sub>Rs). However, cannabinoids can also directly modulate the GABA<sub>A</sub> receptor (GABA<sub>A</sub>R), the most abundant inhibitory neurotransmitter receptor in the brain. It is unknown (A) what the impact of this direct modulation is on inhibitory synaptic transmission, (B) whether endogenous release of endocannabinoids can lead to direct GABA<sub>A</sub>R modulation, and (C) to what extent this mechanism affects input-output processing in neuronal microcircuits. Here we investigated these questions in a synapse which does not contain the CB<sub>1</sub>R: the fast-spiking interneuron to pyramidal neuron synapse in neocortex. Paired recordings from neocortical brain slices of rats and CB<sub>1</sub>R knockout mice show that exogenous cannabinoids modulate both the strength and the kinetics of inhibitory synaptic transmission. Furthermore, endocannabinoids released from pyramidal neurons by a train of depolarizing pulses transiently inhibit evoked IPSCs in a CB<sub>1</sub>R-independent manner. Suppression of GABA<sub>A</sub>Rs by cannabinoids reduces the influence of GABAergic interneurons on the firing of pyramidal neurons. Thus CB<sub>1</sub>R-independent modulation of GABA<sub>A</sub>Rs by cannabinoids provides a novel mechanism for regulation of synaptic inhibition. We hypothesize that this type of local synaptic modulation plays a role in the temporal re-assembly of neuronal microcircuits.
Introduction

The cannabinoid CB$_1$ receptor (CB$_1$R) is the most abundant G-protein-coupled receptor in the mammalian brain. Endocannabinoid mediated CB$_1$R activation is associated with several forms of short- and long-term plasticity at both inhibitory and excitatory synapses throughout the brain (Freund et al., 2003; Chevaleyre et al., 2006). Application of exogenous cannabinoids, or activity dependent synthesis and release of endogenous cannabinoids (endocannabinoids), can activate presynaptic CB$_1$Rs, which in turn reduce neurotransmitter release through G-protein mediated inhibition of Ca$^{2+}$ channels (Sullivan, 1999; Wilson et al., 2001). A well described form of short-term plasticity mediated by endocannabinoids is depolarization induced suppression of inhibition (DSI; Kreitzer and Regehr, 2001a; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). It has been postulated that in this process endocannabinoids are released from the postsynaptic neuron, and act retrogradely on presynaptic CB$_1$Rs to induce a transient suppression of synaptic efficacy, which lasts for tens of seconds.

However, we have shown that (endo)cannabinoids can also directly modulate the GABA$_A$R (Chapter 3 of this thesis). (Endo)cannabinoids reduce GABA$_A$R mediated current amplitude, increase the amount of receptor desensitization and slow down receptor deactivation independent of CB$_1$R activation. Moreover, dose-response studies suggest that the endogenous cannabinoid anandamide (AEA) inhibits GABA$_A$R mediated current amplitude with an IC$_{50}$ of 880±210 nM. Interestingly, this value is comparable to that for activation of the CB$_1$R by AEA (EC$_{50}$=1.4±0.3 µM; Breivogel et al., 2001). GABA$_A$R modulation by the endogenous cannabinoid 2-arachidonoylglycerol (2-AG) seems to occur within the same concentration range. These findings raise the question whether exogenously applied or endogenously released cannabinoids can modulate GABA$_A$R mediated synaptic transmission under physiological conditions.

In this study we show that exogenous application of cannabinoids reduces amplitude and modulates kinetics of GABAergic inhibitory postsynaptic currents (IPSCs) in pairs of connected neurons in neocortical slices treated with a CB$_1$R antagonist or prepared from CB$_1$R knockout (CB$_1$R$^{-/-}$) mice. Furthermore, we show that, in the presence of a CB$_1$R antagonist as well as in CB$_1$R$^{-/-}$ mice, activity dependent release of endogenous cannabinoids produces a transient inhibition of GABAergic synaptic transmission. Previous studies suggest that inhibitory interneurons coordinate neuronal activity in cortical networks (Jefferys et al., 1996; Galarreta and Hestrin, 2001). We show that either exogenously applied or endogenously released cannabinoids, independent of CB$_1$R activation, uncouple fast-spiking (FS) interneuron control of pyramidal neuron firing in layer 2/3 of somatosensory cortex. This suggests that direct modulation of GABA$_A$Rs by cannabinoids significantly modifies information processing in neuronal networks.

Altogether, these findings provide a new dimension in cannabinoid signaling in the brain, proposing a novel mechanism for tonic and activity-dependent regulation of synaptic inhibition in neuronal microcircuits.
Materials & Methods

Brain slice recordings
Wistar rats, Sprague/Dawley rats, wildtype CD1 mice or CB₁R⁻/⁻ mice (Ledent et al., 1999), wildtype C57Bl6 mice or CB₁R⁻/⁻ mice (Marsicano et al., 2002) of 14-17 days old were killed by decapitation, after which the brain was removed. Transverse neocortical slices (300 µm) of somatosensory cortex were sectioned in ice-cold solution containing (mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, and 1 MgCl₂ (carboxygenated with 5% CO₂ / 95% O₂). For paired recordings slices were transferred to a recording chamber containing extracellular solution of the same ionic composition as used for slicing. Neurons were visualized via a 40x water immersion objective using infrared differential interference contrast video microscopy. Recordings were made at room temperature (20-22°C). Whole-cell voltage and/or current clamp recordings were performed simultaneously from 2 neurons using borosilicate glass pipettes with resistance of 5-7 MΩ, containing (mM): 105 K gluconate, 30 KCl, 4 Mg-ATP, 10 phosphocreatine, 0.3 GTP, and 10 HEPES (pH 7.3, KOH). In synaptically connected neurons, suprathreshold intracellular stimulation of the presynaptic FS interneuron evoked a GABAₐR mediated evoked IPSCs (eIPSCs) or IPSPs in postsynaptic pyramidal neuron. In experiments on IPSPs, the intracellular solution of the pyramidal neuron contained a low chloride concentration so that the IPSP was hyperpolarizing. This solution contained (in mM): 130 K gluconate, 10 Na gluconate, 4 NaCl, 4 Mg-ATP, 4 phosphocreatine, 0.3 GTP, and 10 HEPES (pH 7.3, KOH). Presynaptic cells were stimulated with a 10 Hz train of 2 or 3 suprathreshold current pulses. Trains were delivered with intervals longer than 7 sec.

In experiments where endocannabinoid mediated suppression of inhibition was investigated, postsynaptic pyramidal neurons were kept in voltage clamp mode (-70 mV), while presynaptic FS interneurons were in current clamp mode. After recording baseline eIPSCs (50-100 sweeps at 0.1Hz), another 50-100 eIPSCs were collected during conditioning (also at 0.1Hz). In this protocol, every single eIPSC was preceded by a conditioning train (25 Hz) of depolarizing current pulses to -30 mV (16 pulses, duration of each pulse was 20 ms) in the postsynaptic neuron. To obtain a recovery curve, eIPSCs were recorded at variable time intervals after this conditioning train.

For measurement of spontaneous activity the extracellular solution contained (mM): 126 NaCl, 3 KCl, 10 glucose, 26 NaHCO₃, 1.2 NaH₂PO₄, 3 CaCl₂, and 1 MgCl₂ (carboxygenated with 5% CO₂ / 95% O₂), and DNQX (10 µM) and DL-AP5 (50 µM) were added to isolate GABAergic events. In some experiments, TTX (1 µM) was added to isolate miniature IPSCs (mIPSCs). The intracellular solution contained (mM): 77 K-gluconate, 77 KCl, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, 4 K₂Phosphocreatine, 0.4 GTP (pH 7.3 with KOH). After recording spontaneous activity for 10 minutes, cannabinoids were washed in, and activity was recorded for at least 10 more minutes.

All stimulus delivery and data acquisition was performed using EPC-8 amplifiers with PULSE software (HEKA Elektronik, Lambrecht, Germany).
Chemicals
WIN55,212-2, WIN55,212-3 and CP55,940 were obtained from Tocris Bioscience (Avonmouth, UK). SR141716A was a gift from Solvay Pharmaceuticals (Weesp, the Netherlands). Tetrahydrolipstatin (THL) was obtained from Sigma-Aldrich (St. Louis, MO). Of these chemicals appropriate stock solutions were prepared in DMSO. Final DMSO concentration in the medium never exceeded 0.1%. In all experiments with application of these drugs control solutions always contained an equal amount of DMSO or ethanol.

Data analysis
All data was analyzed off-line using IGOR PRO software (Wavemetrics, Lake Oswego, OR). Spontaneous synaptic transmission was analyzed using MiniAnalysis (Synaptosoft Inc., Decatur, GA). Values shown in bar histograms and text are mean ± standard error of the means (SEM). Data was filtered at 3-5 kHz. Statistical analysis was done using two-sided student’s t-test ($\alpha=0.05$), unless noted otherwise.

Decay of eIPSCs, mIPSCs and sIPSCs was fitted with a double-exponential decay function of the form: $I=A_1*\exp(-t/\tau_1)+A_2*\exp(-t/\tau_2)$. Values mentioned in text refer to the composite time-constant, calculated with: $\tau_{comp}=A_1/(A_1+A_2)\tau_1+A_2/(A_1+A_2)\tau_2$.

For CV analysis we calculated CV$^2$ of IPSC amplitude under control conditions and in the presence of cannabinoids. An equal amount of eIPSCs was chosen for both conditions (>40 eIPSCs). For details on analysis see (Faber and Korn, 1991).

Because of intrinsic regularity at the onset of pyramidal neuron spiking, we only analyzed pyramidal neuron APs within the boxed area in Figure 4.6A. Distribution statistics were performed using Kuiper’s test, a circular variant of the Kolmogorov-Smirnov test (Batschelet, 1981).

Results

Cannabinoids modulate evoked IPSCs independent of CB$_1$R activation
To see whether exogenous cannabinoid application can modulate the properties of inhibitory synaptic transmission independent of CB$_1$R activation, we made paired recordings in acute brain slices both in rats and in CB$_1$R$^{-/-}$ mice. Simultaneous whole-cell recordings were made from FS GABAergic interneurons and synaptically connected neighboring pyramidal neurons in layer 2/3 of the somatosensory cortex. Importantly, neocortical FS interneurons do not express the CB$_1$R on their presynaptic terminals (Bodor et al., 2005). Cell types were identified by action-potential (AP) firing pattern (Reyes et al., 1998). In cell pairs, APs were elicited in the FS interneuron while the resulting eIPSCs were recorded in the postsynaptic pyramidal neuron in voltage clamp mode ($V_m=-70$ mV). In experiments with rats we excluded CB$_1$R mediated effects on transmitter release by adding the selective CB$_1$R antagonist SR to the extracellular solution (5 µM). In contrast to our observations on recombinant GABA$_A$Rs (see chapter 3), SR by itself did not cause a significant modulation of eIPSC amplitude ($I_{norm}$: 0.89±0.08; n=5; p=0.16),
although a very small, but significant, increase in decay time was observed (normalized $\tau$, $\tau_{\text{norm}}$: 1.04±0.02; n=5; p=0.047; Table 4.1). After recording 50-100 baseline sweeps in the presence of SR, 1 µM of the synthetic cannabinoid CP was applied to the bath solution. In rats CP reduced the eIPSC amplitude to 0.62±0.06 of the amplitude in SR alone (n=8; p<0.001). Similarly in CB$_1$R$^{-/-}$ mice the eIPSC amplitude was reduced to 0.45±0.05 of control by CP (n=6; p<0.001; Figure 4.1A).

In the presence of SR, a similar reduction of the eIPSC amplitude was observed in rat brain slices using the synthetic cannabinoid WIN-2 (5 µM; $I_{\text{norm}}$: 0.51±0.12; n=5; p=0.005; Table 4.1). Furthermore, the CB$_1$R inactive enantiomer WIN-3 (5 µM) caused
a reduction in eIPSC amplitude when applied in the absence of SR ($I_{\text{norm}}$: 0.63±0.08; n=5; p=0.009; Table 4.1).

Reduction of the eIPSC amplitude by CP was accompanied by an increase in decay time constant from 17.1±0.9 ms to 19.6±0.4 ms (n=7; p=0.009) in rats and from 16.4±1.6 ms to 21.6±1.2 ms (n=6; p=0.006) in CB$_1$R$^{-/-}$ mice (Figure 4.1B). Qualitatively similar increases in the decay time constant were observed with WIN-2 and WIN-3, although the modulation by WIN-3 did not reach statistical significance ($\tau_{\text{norm}}$; WIN-2: 1.23±0.07; n=5; p=0.044; WIN-3: 1.21±0.12; n=5; p=0.16; Table 4.1).

Previous studies on cannabinoid modulation reported an increase in paired-pulse ratio (PPR) in accordance with presynaptic, CB$_1$R dependent inhibition of synaptic transmission (Kreitzer and Regehr, 2001b; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Therefore, to investigate the origin of the observed effects of CP on eIPSCs, the PPR (amplitude of eIPSC$_2$/eIPSC$_1$) of two subsequent IPSCs evoked with a 100 ms interval was calculated. The PPR was not changed by application of CP either in rats in the presence of SR or in CB$_1$R$^{-/-}$ mice (Rats SR: 0.81±0.03; SR+CP: 0.75±0.04; n=8; p=0.15; CB$_1$R$^{-/-}$ control: 0.82±0.03; CP: 0.91±0.10; n=6; p=0.25; Figure 4.1C), which is suggestive of a postsynaptic localization of eIPSC modulation. However, the absence of a change in PPR should be interpreted with caution, as a change in presynaptic release probability is not always accompanied by a change in PPR (for example see Chapter 2). Therefore, we performed an analysis of the coefficient of variation (CV) of the eIPSC amplitudes, since this can also be used to differentiate between a pre- and a postsynaptic locus of synaptic modulation (Faber and Korn, 1991). Generally, a decrease in eIPSC amplitude should be accompanied by a reduction in CV$^2$ if the modulation is presynaptic in origin. However, if the effect is postsynaptic, CV$^2$ should not change. In our experiments in rats, the ratio between CV$^2$ in CP versus CV$^2$ under control showed considerable scatter between experiments. Still, on average it was close to 1 (CV$^2_{\text{control}}$/CV$^2_{\text{(CP)}}$ = 1.03±0.21, n=8). In CB$_1$R$^{-/-}$ mice the scatter in CV$^2$ ratio was less, and the average value was 1.09±0.10 (n=6). Again, this is suggestive of a postsynaptic rather than a presynaptic locus of the eIPSC reduction (Figure 4.1D).
Cannabinoids modulate spontaneous and miniature GABAergic events independent of CB₁R activation

Strong evidence for an entirely presynaptic, CB₁R dependent modulation of inhibitory synaptic transmission is that application of CB₁R agonists has a strong impact on the frequency of spontaneous IPSCs (sIPSCs), but not on their amplitude (Hoffman and Lupica, 2000; Trettel and Levine, 2002). To assess a possible CB₁R independent modulation of spontaneous inhibitory synaptic transmission by cannabinoids we performed whole-cell recordings of sIPSCs from layer 2/3 pyramidal neurons in rat brain slices, in the presence of the CB₁ antagonist SR (1 µM). We analyzed both sIPSCs as well as AP independent miniature IPSCs (mIPSCs), recorded in the presence of tetrodotoxin (TTX; 1 µM). Figure 4.2A-C summarizes analysis of sIPSCs.

We did not observe any change in median sIPSC interevent interval (IEI; averaged IEI: control 0.41±0.13 sec. vs CP 0.51±0.09 sec.; p=0.25; n=6; Figure 4.2B), suggesting the absence of presynaptic release probability modulation. However, we observed a slight, but significant reduction of median sIPSC amplitude after application of CP (1 µM; normalized median amplitude: 0.81±0.07; p=0.04; n=6; Figure 4.2B). The amplitude distribution shows that this reduction in median amplitude is mainly caused by the disappearance of large amplitude (>100 pA) events (Figure 4.2B, see inset). We assessed modulation of decay time constant both by fitting an exponential decay function and by comparing normalized charge of the average sIPSC. Even though sIPSC decay time did not show a significant increase, the average integral of the sIPSCs was significantly larger in the presence of CP, indicating a slight slowing down of the synaptic current decay (mean τ control: 19.3±1.8 vs CP: 20.0±1.0; p=0.35; increase in normalized charge: 8±3%; p=0.03; n=6; Figure 4.2E). CP did not have a significant effect on either averaged mIPSCs median amplitude or IEI (Figure 4.2C,D). However, kinetics of the averaged mIPSCs were significantly slower in the presence of CP, reflected by a significant increase of both decay time constant and normalized charge (mean τ control: 17.3±0.9 ms vs CP: 19.7±0.9 ms; p=0.008; increase in normalized charge: 11±3%; p=0.02; n=7; Figure 4.2E).

A similar set of experiments was performed in CB₁R⁻/⁻ mice. In these experiments averaged median amplitude and averaged median IEI of both sIPSCs and mIPSCs were unchanged by CP application (Figure 4.3A-D). However, it should be noted that in these mice less high-amplitude sIPSCs were observed when compared to rats (Figure 4.3B, see inset). Both sIPSCs and mIPSCs showed a significant increase in normalized charge in the presence of CP, although prolongation of decay kinetics did not reach statistical significance (sIPSCs: mean τ control: 19.7±1.0 ms vs CP: 23.3±2.3 ms; p=0.12; increase in normalized charge: 9±3%; p=0.015; n=5; Figure 4.3C; mIPSCs: mean τ control: 16.1±1.6 ms vs CP: 17.6±1.5 ms; p=0.18; increase in normalized charge: 10±3%; p=0.02; n=4; Figure 4.3E).

Altogether, these results suggest that, in the absence of CB₁R signaling, cannabinoids have a purely postsynaptic effect on inhibitory synaptic transmission. Prolongation of decay time of eIPSCs, sIPSCs and mIPSCs in rats as well as in CB₁R⁻/⁻ mice indicates a modulation of postsynaptic GABAₐ₁R properties and is consistent with the prolongation
Figure 4.2 Cannabinoids modulate properties of spontaneous inhibitory synaptic events in the presence of a CB₁R antagonist.

(A) Example traces of sIPSCs before and after application of CP (1 µM). (B) Left: Averaged distribution of sIPSC amplitudes in control (top) and after application of CP (bottom). Insets show events with amplitude greater than 100 pA. Right top: Pairwise comparison of median amplitude under control conditions versus CP application. Open symbols show individual experiments, closed symbols show mean ± SEM. Right bottom: Averaged median IEI in control and after CP application (mean ± SEM). (C,D) Same as on A and B, but experiments were done in the presence of TTX (1 µM) to isolate mIPSCs. (E) Representative average sIPSC trace before (black) and after application of CP (1 µM; grey). Bar histograms compare changes of decay time constant (left) or normalized charge (right) after CP application with control, for both mIPSCs and sIPSCs. All experiments were performed in the presence of SR (1 µM) to eliminate presynaptic, CB₁R dependent modulation of synaptic transmission.
Figure 4.3 Cannabinoids modulate properties of spontaneous inhibitory synaptic events in CB1R−/− mice.

(A) Example traces of sIPSCs before and after application of CP (1 µM). (B) Left: Averaged distribution of sIPSC amplitudes in control (top) and after application of CP (bottom).Insets show events with amplitude greater than 100 pA. Right top: Pairwise comparison of median amplitude under control conditions versus CP application. Open symbols show individual experiments, closed symbols show mean ± SEM. Right bottom: Averaged median IEI in control and upon CP application (mean ± SEM). (C,D) Same as on A and B, but experiments were done in the presence of TTX (1 µM) to isolate mIPSCs. (E) Representative average sIPSC trace before (black) and after application of CP (1 µM; grey). Bar histograms compare changes of decay time constant (left) or normalized charge (right) after CP application with control, for both mIPSCs and sIPSCs.
Figure 4.4 Postsynaptic activity causes a CB₁R-independent suppression of inhibitory synaptic transmission.

(A) Experimental setup. Postsynaptic eIPSCs were recorded in pyramidal neurons under voltage clamp (V_m = -70 mV) upon stimulation of the FS interneuron. After recording 50 baseline eIPSCs evoked at 0.1 Hz eIPSCs were recorded 1 second following a conditioning stimulation of the postsynaptic pyramidal neuron. This stimulation consisted of a train of depolarizing pulses (20 ms to -40 mV; 16 pulses at 25 Hz; middle). This sequence of pre- and postsynaptic stimulation was repeated 50 times at 0.1 Hz. (B) eIPSC suppression in wildtype mice. Left top, representative averaged eIPSC under control conditions and upon conditioning. Traces show the average response of 50 subsequent responses for each condition. Left bottom, eIPSC amplitude under control conditions, during conditioning and during recovery of the same experiment as shown on top. Each dot represents a single eIPSC amplitude. Closed circles show the average amplitude of the 10 preceding timepoints. Lines show the average of 50 control responses, 50 responses during conditioning and 50 responses during recovery. Right, same as left, but now for a representative
of GABA\textsubscript{A}R deactivation in isolated hippocampal neurons, HEK293 cells and nucleated patches as described in chapter 3. Additionally, cannabinoids cause a strong reduction in the amplitude of eIPSCs in paired recordings as well as in large amplitude sIPSCs recorded in rats. However, in apparent contrast to this observation, there is a lack of effect on mIPSC amplitude, as well as on sIPSC amplitude in CB\textsubscript{1}R\textsuperscript{-/-} mice (see discussion).

**Release of endocannabinoids modulates eIPSCs independent of CB\textsubscript{1}R activation**

In many synapses, activity dependent release of endocannabinoids modulates inhibitory synaptic transmission, inducing DSI by activation of presynaptic CB\textsubscript{1}Rs (Kreitzer and Regehr, 2001a; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Hence, the question arises whether endogenously released cannabinoids can affect GABAergic synaptic transmission independent of CB\textsubscript{1}R activation via direct inhibition of postsynaptic GABA\textsubscript{A}Rs and, if so, what the lifetime of this suppression is. To address these issues paired whole-cell recordings were made from FS interneurons and synaptically connected pyramidal neurons in brain slices from wildtype mice in the presence of SR (5 µM) as well as in CB\textsubscript{1}R\textsuperscript{-/-} mice. APs were evoked in the FS interneuron, while resulting baseline eIPSCs in the postsynaptic pyramidal neuron were recorded in voltage clamp mode ($V_{m}$=-70 mV; eIPSC frequency: 0.1Hz). Next, a conditioning protocol was applied to the postsynaptic neuron. During conditioning eIPSCs were measured again at 0.1Hz, but now each eIPSC was preceded (1 second) by a single conditioning train of depolarizations in the pyramidal neuron (Figure 4.4A). In wildtype mice in the presence of SR the amplitude of eIPSCs recorded after the conditioning depolarization was reduced to 0.67±0.06 of the baseline (control) value (p<0.001; n=5, Figure 4.4B,D). This reduction in IPSC amplitude was not associated with a change in PPR (control: 0.79±0.05; conditioning: 0.81±0.02; p=0.68; n=5; Figure 4.4D). In some, but not all, experiments, eIPSC amplitude returned to control after the conditioning period.

To prove that the observed reduction of eIPSC amplitude was due to the release of endocannabinoids from the postsynaptic neuron, and not due to other processes, we pharmacologically manipulated endocannabinoid synthesis using the endocannabinoid synthesis inhibitor tetrahydrolipstatin (THL). THL blocks DAG lipase (DAGL), a key enzyme involved in the biosynthesis of the endocannabinoid 2-AG (Bisogno et al., 2003). THL has been shown to effectively inhibit endocannabinoid synthesis when included into the postsynaptic recording pipette (Melis et al., 2004; Safo and Regehr, 2005). Therefore, we included THL (5 µM) into the recording pipette of the postsynaptic pyramidal neuron. Under these conditions the conditioning train no longer caused a significant reduction in eIPSC amplitude (normalized amplitude: 1.06±0.1; experiment in which THL was included into the postsynaptic recording pipette. (C) Same as in (B), but now for experiments in CB\textsubscript{1}R\textsuperscript{-/-} mice. (D) Left, summary of the eIPSC suppression during conditioning for wildtype mice. Middle, example trace of the averaged normalized eIPSC response during control and conditioning (average of 50 traces for each condition). Right, paired pulse ratio during control and during conditioning. (E) Same as in (D), but now for experiments in CB\textsubscript{1}R\textsuperscript{-/-} mice.
p=0.29; n=4; Figure 4.4B,D). We performed the same experiment in CB$_1$R$^{-/-}$ mice, and observed a reduction in normalized eIPSC amplitude to 0.67±0.1 during conditioning (p<0.001; n=5; Figure 4.4C,E). This again was not accompanied by a change in PPR (control: 0.73±0.03; conditioning: 0.75±0.03; p=0.24; n=3; Figure 4.4E). Postsynaptic addition of THL abolished the conditioning induced eIPSC reduction in CB$_1$R$^{-/-}$ mice (normalized eIPSC: 0.95±0.07; p=0.18; n=5; Figure 4.4C,E).

This shows that endocannabinoids mediate a CB$_1$R independent depolarization induced suppression of inhibitory synaptic transmission at neocortical FS interneuron to pyramidal neuron pairs.

**CB$_1$R independent endocannabinoid mediated suppression of inhibition is transient**

Next we wanted to investigate the duration of this CB$_1$R independent suppression of eIPSC amplitude. To do this we varied the time interval (Δt) between postsynaptic depolarization and presynaptic stimulation during the conditioning protocol (Figure 4.5A). In rats suppression of the eIPSC amplitude in the presence of SR was observed as early as 180 ms after postsynaptic depolarization ($I_{\text{norm}}$: 0.61±0.08; n=5; p=0.005), and the eIPSC amplitude recovered to the control value within approximately 5 seconds after depolarization ($I_{\text{norm}}$: 0.97±0.09; n=5; p=0.77; Figure 4.5B). The same experiments were performed in the absence of SR. In this case eIPSC amplitude was still significantly reduced 5 seconds after postsynaptic depolarization ($I_{\text{norm}}$: 0.79±0.07; n=4; p=0.03; Figure 4.5B), although there was no significant difference from data obtained at 5 seconds in the presence of SR (p=0.15).

Synthesis of the endocannabinoid 2-AG can also be partially blocked by the DAGL inhibitor RHC80267 (Stella et al., 1997; Safo and Regehr, 2005), although this is a weaker and less specific inhibitor than THL (Szabo et al., 2006). Indeed, when brain slices were treated with RHC80267 (30 µM; no SR present), reduction of eIPSC amplitude was less pronounced at 800 ms after postsynaptic depolarization ($I_{\text{norm}}$: RHC80267: 0.85±0.04 vs control, no SR: 0.59±0.07; p=0.01) and no longer observed after a 2.6 second delay ($I_{\text{norm}}$: 1.14±0.29; n=5; p=0.39; Figure 4.5B). The opposite effect on endocannabinoid signaling can be achieved by blocking the degradation of endocannabinoids. The monoacylglycerol lipase (MAGL) inhibitor URB602 has been shown to prolong the timecourse of CB$_1$R dependent DSI in hippocampus (Makara et al., 2005). Application of 100 µM of URB602, in the presence of SR (5 µM) did not change initial level of eIPSC suppression ($I_{\text{norm}}$: 0.69±0.13; n=4), but caused a prolongation of this suppression, with eIPSC amplitude not even fully recovered after 20 seconds ($I_{\text{norm}}$: 0.82±0.02; n=5; p<0.001; Figure 4.5C).

Finally we performed the same experiment in wild type (WT) vs CB$_1$R$^{-/-}$ mice, in the absence of CB$_1$R antagonists. We found that depolarization of the postsynaptic cell again induced a short-lived reduction of eIPSC amplitude in WT and CB$_1$R$^{-/-}$ mice. Initial suppression of eIPSC amplitude was 0.56±0.08 (n=5; p=0.007) and 0.65±0.07 (n=5; p=0.008) for WT and CB$_1$R$^{-/-}$ respectively (WT vs CB$_1$R$^{-/-}$: p=0.44; Figure 4.5D). eIPSC amplitudes recovered within around 5 seconds after postsynaptic depolarization
Figure 4.5 The timecourse of CB1R-independent suppression of inhibitory synaptic transmission.
(A) Experimental setup. Experiments were similar as in figure 4.4, but the delay between the conditioning train and the eIPSCs (Δt) was varied between experiments. (B) Timecourse of stimulation induced suppression of eIPSC amplitude in rats, normalized to the averaged baseline eIPSC amplitude. Connected symbols show experiments performed in the absence of SR (open squares), in the presence of SR (5 µM; filled circles) or after treatment with RHC80267 (30 µM; open triangles). (C) Timecourse of stimulation induced suppression of eIPSC amplitude either in the presence of SR (5 µM; filled circles; same data as in B) or in the presence of both SR and URB602 (100 µM; open squares). (D) Comparison of stimulation induced suppression of IPSC amplitude in WT (open squares) and CB1R-/- mice either under control conditions (filled circles) or after treatment with RHC80267 (30 µM; open triangles). SR was not used in these experiments on mice. Each point shows the average of 4-5 cell pairs. (E) Experimental setup. Paired current clamp recordings were made in rats, in the presence of SR (5 µM). IPSPs were evoked at 0.1 Hz under control conditions and 2 seconds after the conditioning AP-train (700 ms current injection to the pyramidal neuron, causing a train of APs). (F) Effect of conditioning AP-train on IPSP amplitude in the absence or presence of BAPTA (5 mM) in the postsynaptic pyramidal neuron.

(\(I_{\text{norm}}\): WT: 0.83±0.14; n=5; p=0.29; CB1R-/-: 0.96±0.07; n=6; p=0.57; Figure 4.5D). In CB1R-/- mice, eIPSC suppression was reduced by DAGL inhibition with RHC80267, with full recovery occurring at a 2.6 second delay (\(I_{\text{norm}}\): 1.15±0.12; n=5; p=0.35), again confirming that suppression is mediated by endocannabinoid synthesis (Figure 4.5D).
One may argue that the conditioning protocol applied in Figures 4.4 and 4.5A-D provides an unnatural stimulation of the postsynaptic neuron. Therefore, we tested whether a milder, physiologically relevant stimulation was able to reduce synaptic transmission. In this experiment both the pre- and the postsynaptic neuron were held under current-clamp. Endocannabinoid release was induced by a conditioning action potential (AP) train in the pyramidal neuron (Figure 4.5E). In rats, in the presence of SR, the amplitude of IPSPs evoked 2 seconds after this conditioning AP-train was significantly reduced (Figure 4.5F; 36.7±13.1% suppression; n=5; p=0.035). Since endocannabinoid release requires elevation of the postsynaptic Ca\textsuperscript{2+} concentration, it can be blocked by the fast Ca\textsuperscript{2+} buffer BAPTA (Kreitzer and Regehr, 2001b; Wilson and Nicoll, 2001). Indeed, when BAPTA (5 mM) was added to the intracellular solution of the pyramidal neuron, the IPSP amplitude was not affected by the conditioning AP-train (9.9±10.7% suppression; n=5; p=0.36; Figure 4.5F).

The aggregate of these results is that endocannabinoids released from the postsynaptic pyramidal neuron induce a CB\textsubscript{1}R-independent suppression of inhibition at synapses between neocortical FS interneurons and pyramidal neurons. Furthermore, the fact that IPSC suppression is similar in the absence or presence of SR as well as in CB\textsubscript{1}R\textsuperscript{-/-} mice, suggests that CB\textsubscript{1}R mediated signaling does not occur at this synapse, consistent with earlier reports (Bodor et al., 2005).

**Modulation of GABA\textsubscript{A}Rs by cannabinoids has a strong impact on functioning of neuronal microcircuits**

Our previous experiments show that cannabinoids can affect both amplitude and kinetics of GABA\textsubscript{A}R mediated synaptic currents independent of CB\textsubscript{1}R activation. However, while the amplitude of the eIPSC appears to be reduced, the eIPSC decay time is increased. These two effects might contribute differently to the net inhibition in neuronal networks. To assess the impact of this cannabinoid modulation of GABA\textsubscript{A}Rs on local neuronal network activity, we again performed experiments on FS interneuron to pyramidal neuron pairs. The pyramidal neuron received a constant current injection (700 ms), causing it to fire a train of APs. Simultaneously, the presynaptic FS interneuron received pulsed current injections at 20 Hz (Figure 4.6A). Experiments were performed in the presence of SR (5 µM). An overlay of 30 consecutive sweeps of pyramidal firing from a representative experiment shows that under control conditions the pyramidal neuron firing was time locked to FS interneuron stimulation. After application of CP (1µM) synchrony of firing between the FS interneuron and the pyramidal neuron was greatly reduced (Figure 4.6B). To quantify this effect, the probability of AP appearance within the 52 ms window between the onsets of two consecutive FS interneuron stimulations was plotted as a function of time relative to the onset of FS interneuron stimulation. Under control conditions, probability of AP firing in the pyramidal neuron was the lowest at about 20 ms after the onset of FS interneuron stimulation (Figure 4.6C; pooled data, n=8). This time point corresponds roughly to the peak of the IPSP resulting from a single FS interneuron stimulation (Figure 4.6C, inset).
Figure 4.6 Exogenous cannabinoids reduce the influence of FS interneuron stimulation on pyramidal neuron firing in a CB1R-independent manner.

(A) Experimental setup. A FS interneuron (FS) and a connected pyramidal neuron (P) were kept in current clamp mode. The postsynaptic pyramidal neuron was injected with a 700 ms current pulse to trigger AP firing, while the presynaptic FS interneuron was stimulated with a train (20 Hz) of stimuli. Pyramidal neuron APs within the area marked by dashed lines were used for further analysis. (B) Representative example showing the firing of a pyramidal neuron (overlay of 30 traces) during simultaneous stimulation of the presynaptic FS interneuron under control conditions (with 5 µM SR; top) and in the presence of both SR and CP (1 µM; bottom). Lines under the traces represent FS interneuron stimulation pulses. (C) AP distribution of a pyramidal neuron under control conditions (with 5 µM SR; left graph; n=8) and in the presence of both SR and CP (1 µM, middle graph; n=8). Right graph shows the cumulative spike probability plot before and after CP application. Distributions differ significantly (Kuiper’s test: p<0.001). Inset shows an example of the IPSP response of a pyramidal neuron at resting potential (bottom) to one pulse of FS interneuron stimulation (top). (D) Pyramidal neuron AP distribution without FS interneuron stimulation. Histograms show AP distribution both under control conditions (with 5 µM SR; left graph; n=6) and in the presence of both SR and CP (1 µM, middle graph; n=6). Right graph shows the cumulative spike probability plot. Distributions do not differ significantly (Kuiper’s test: p>0.2). Analysis was identical to (C).
In the presence of CP the AP probability distribution was still not uniform. However, the decrease in the AP probability caused by FS interneuron stimulation was significantly less pronounced when compared to control conditions (Kuiper’s test: p<0.001). When the data were plotted in a cumulative spike probability plot, a clear shift towards a uniform distribution was observed in the presence of CP (Figure 4.6C).

The change in AP probability distribution after CP application could be due to a reduction of inhibitory strength, but it might also be explained by effects of CP on intrinsic pyramidal neuron properties. To exclude the latter possibility, the same experiment was performed in the absence of FS interneuron stimulation. Without inhibitory stimulation, spikes remained uniformly distributed both in control conditions as well as in the presence of CP (Figure 4.6D; pooled data, n=6; Kuiper’s test: p=0.2). These results indicate that exogenously added cannabinoids are able to suppress inhibition of
pyramidal neurons by FS interneurons independent of CB₁R activation.

Modulation of GABAₐRs by activity dependent release of endocannabinoids has a strong impact on functioning of neuronal microcircuits
Finally, we wanted to test whether endocannabinoid mediated CB₁R-independent suppression of inhibition could have a similar impact on neuronal microcircuits as exogenously applied cannabinoids. Similar experiments as in figure 4.6 were performed in rats in the presence of SR (5 µM). However, the current injection to the pyramidal neuron that was synchronized with pulsed FS interneuron stimulation was preceded by a conditioning AP-train to the postsynaptic pyramidal neuron alone (Figure 4.7A). Conditional current injection caused a clear reduction in the influence of FS interneuron firing on the firing of the pyramidal neuron compared to control conditions (Figure 4.7B). However, loading of BAPTA (5 mM) into the postsynaptic pyramidal neuron restored FS interneuron control over pyramidal neuron spiking (Figure 4.7B,C). These results show that endocannabinoids, released in an activity- and Ca²⁺-dependent manner, have similar effects on postsynaptic GABAₐRs and microcircuit functioning as exogenously applied cannabinoids.

Discussion
The results described in this study show that direct GABAₐR modulation by (endo)cannabinoids can have strong effects on inhibitory synaptic transmission in the neocortex. Experiments performed either in the presence of a CB₁R antagonist, or in CB₁R⁻/⁻ mice allow us to exclude involvement of presynaptic CB₁Rs in the observed synaptic modulation. Furthermore, we show that this modulation has dramatic consequences for inhibitory control in layer 2/3 of somatosensory cortex, by greatly reducing strength of FS interneuron input to pyramidal neurons. Because of the ubiquitous nature of cannabinoid signaling in the brain, this is likely to affect synaptic transmission, plasticity and microcircuit processing in many cortical and subcortical regions (see chapter 5).

CB₁R independent modulation of synaptic transmission
In agreement with our findings on recombinant and native GABAₐRs (chapter 3), we show that cannabinoids suppress GABAₐR mediated IPSCs at FS interneuron to pyramidal neuron synapses through postsynaptic mechanisms. Our findings are in apparent contrast to previous studies reporting that cannabinoids have a purely presynaptic, CB₁R dependent action on inhibitory synaptic transmission.

The fact that in our experiments PPR remained unchanged suggests that cannabinoids act postsynaptically. However, the absence of a change in PPR cannot rule out a reduction in presynaptic release probability (for example see chapter 2). Therefore, we also performed an analysis of the coefficient of variation(CV; Figure 4.1). This analysis also indicates that cannabinoids act postsynaptically. Moreover, we did not observe
modulation of sIPSC or mIPSC frequency, suggesting the absence of modulation of presynaptic release probability. Finally, the prolongation of IPSC decay kinetics most probably reflects the prolongation of deactivation kinetics also observed in isolated cells, and therefore underscores the likelihood of postsynaptic modulation of GABA_ARs.

Earlier studies have shown that mIPSC amplitude is not affected by WIN-2 or other CB_1R agonists (Hoffman and Lupica, 2000; Trettel and Levine, 2002). In our experiments we did not observe an effect of CP on mIPSC amplitude in the presence of SR. However, sIPSCs in rats, which can be AP driven, did show a cannabinoid agonist mediated reduction in amplitude, with a particularly profound effect on large amplitude events. In addition, CP application significantly prolonged decay time of mIPSC in rat and CB_1R^/-/- mice, indicating the alteration of biophysical properties of postsynaptic GABA_ARs.

The lack of effect of CP on mIPSCs amplitude could be explained in at least two ways. First, several lines of evidence indicate that the spatiotemporal GABA concentration transient during AP independent neurotransmitter release is different from that during AP evoked synaptic transmission (for review see Mozrzymas, 2004). Thus, it has been argued that spontaneous GABA release of a single quantum, perhaps in kiss-and-run fusion mode, might not cause saturation of postsynaptic GABA_ARs (Hajos et al., 2000). APs on the other hand are likely to trigger full fusion and/or even multiquantal release (Bisogno et al., 2001), resulting in a higher GABA concentration at the individual synaptic cleft, thereby bringing postsynaptic receptors closer to saturation. This could explain why some pharmacological properties of mIPSCs differ from those of sIPSCs and eIPSCs (Mozrzymas, 2004). As mentioned in chapter 3, cannabinoid modulation of GABA_ARs critically depends on agonist concentration and on the amount of postsynaptic receptor saturation by agonist. The fact that modulation of mIPSC kinetics was observed, while amplitude was unchanged, suggests that even at low receptor occupancy cannabinoids can alter GABA_AR gating properties. Similarly, in chapter 3 we showed that at a very low GABA concentration the GABA_AR mediated current amplitude was unchanged, while receptor deactivation was slowed down (Figure 3.7). Second, modulation of GABA_ARs by cannabinoids might be receptor-subunit specific. Synapses might differ in their sensitivity to cannabinoids, depending on the subunit composition of the GABA_ARs they contain. Since mIPSCs are mediated by synapses from several interneuron subtypes, the effect of cannabinoids on FS interneuron synapses could be masked by mIPSCs originating from other synapses, which could contain GABA_AR subunits insensitive to cannabinoid modulation.

**DSI versus CB_1R independent suppression of inhibition by endocannabinoids**

Synthesis of endocannabinoids can be triggered by a rise in intracellular Ca^{2+} in the postsynaptic neuron. The proposed subsequent retrograde CB_1R-dependent modulation of inhibitory synapses by endocannabinoids (e.g. DSI) has been extensively studied in a variety of preparations (Kreitzer and Regehr, 2001a; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Our findings show that postsynaptic depolarization induced IPSC suppression at FS interneuron to pyramidal neuron synapses in L2/3 of somatosensory
cortex still occurs in the presence of a CB$_1$R antagonist and in CB$_1$R$^{-/-}$ mice. Like DSI, this CB$_1$R independent IPSC suppression was prevented by loading the postsynaptic neuron with the fast Ca$^{2+}$ buffer BAPTA, showing involvement of postsynaptic Ca$^{2+}$ signaling in this process. To confirm involvement of endocannabinoids in this process we used blockers of endocannabinoid synthesis and degradation. Indeed, the postsynaptic application of the endocannabinoid synthesis inhibitor THL, as well as treatment with RHC80267, significantly reduced IPSC suppression. Accordingly, the endocannabinoid degradation inhibitor URB602, which has previously been shown to prolong the duration of conventional hippocampal DSI (Makara et al., 2005) also prolonged the duration of CB$_1$R independent IPSC suppression. Since these substances interact with the synthesis and degradation machinery associated with the endocannabinoid 2-AG, it is likely that this endocannabinoid is responsible for direct GABA$_A$R modulation.

**Direct GABA$_A$R modulation: implications for microcircuit functioning**

It is generally accepted that the balance between synaptic excitation and synaptic inhibition is essential for network activity, and therefore is important for information processing (Jefferys et al., 1996; Galarreta and Hestrin, 2001). We show that cannabinoids reduce the influence of FS interneurons on pyramidal neuron firing, in the presence of CB$_1$R antagonists and in CB$_1$R$^{-/-}$ mice. This shows that endocannabinoids can modulate microcircuit functioning at synapses lacking the CB$_1$R.

Interneurons which do not express the CB$_1$R on their presynaptic axon terminals are thought to play very different roles in microcircuit processing than interneurons which do express the CB$_1$R. In particular, CB$_1$R lacking FS interneurons and CB$_1$R containing CCK positive interneurons, which both target the perisomatic region of pyramidal neurons, are thought to have fundamentally different functions. We show now that both types of interneuron synapses can be modulated by endocannabinoid signaling, either through direct GABA$_A$R modulation (FS interneurons), or through activation of presynaptic CB$_1$Rs (CCK interneurons). These two different forms of modulation occur on slightly different timescales (tens of seconds for CB$_1$R mediated DSI vs ~5s for CB$_1$R independent suppression), and have a different synaptic expression locus (presynaptic vs postsynaptic). Therefore, they can have fundamentally different effects on microcircuit processing (see chapter 5). It would be interesting to study the composite contribution of these two processes when simultaneously occurring at inhibitory terminals onto the same pyramidal neuron.

In conclusion, we describe a novel endocannabinoid mediated, CB$_1$R independent short-term plasticity mechanism at inhibitory synapses. While we do not yet know whether a similar process occurs at different inhibitory synapses in the brain, our findings underline the importance of studying endocannabinoid signaling beyond the CB$_1$R.
The studies described in this thesis have been aimed at increasing our knowledge about (endo)cannabinoid signaling in the mammalian brain. In this respect we present two main novel findings. In chapter 2 we have shown that diacylglycerol lipase, a key enzyme involved in the formation of the endocannabinoid 2-arachidonoyl glycerol (2-AG) is not involved in hippocampal depolarization induced suppression of inhibition (DSI). In chapters 3 and 4 we have described a novel, CB1 receptor (CB1R) independent pathway by which endocannabinoids that are synthesized by the postsynaptic cell can affect inhibitory synaptic transmission in a DSI-like manner, most likely through direct modulation of postsynaptic GABAA receptors (GABAA Rs). The conclusions from these studies force us to revise our current models of (endo)cannabinoid mediated modulation of inhibitory synaptic transmission. In this chapter we will shortly discuss these conclusions, and their significance for microcircuit functioning in the mammalian brain.

**Endocannabinoids: How are they formed?**

Endocannabinoid signaling is involved in a variety of neurological disorders, which makes the endocannabinoid system an attractive target for pharmacological intervention (for review see Pacher et al., 2006). However, for selective pharmacological intervention in specific endocannabinoid pathways it is essential that the molecular mechanisms underlying endocannabinoid signaling are well understood. The first step in any endocannabinoid mediated signaling cascade is the biosynthesis of the endocannabinoids. Several biochemical studies have addressed pathways of the synthesis of the main endocannabinoids anandamide (AEA) and 2-AG at synapses in the brain (see Figure 1.5). For 2-AG, the current consensus was that the enzyme diacylglycerol lipase (DAGL) is essential for its formation.

In chapter 2 we have addressed the synthesis pathway for endocannabinoids in hippocampal DSI, the first described and most-studied form of endocannabinoid signaling in the brain (Pitler and Alger, 1992; Wilson and Nicoll, 2001). It is thought that 2-AG is the endocannabinoid mediating hippocampal DSI, since blockade of its degradation prolongs the duration of DSI (see Chapter 1, Figure 1.6; Kim and Alger, 2004; Makara et al., 2005; Hashimotodani et al., 2007a). However, concerning the involvement of DAGL, contradicting results have been published (Chevaleyre and Castillo, 2003; Edwards et al., 2006; Szabo et al., 2006; Hashimotodani et al., 2007a; Hashimotodani et al., 2008). In chapter 2 we have systematically addressed the involvement of DAGL in
hippocampal DSI in paired recordings from CB₁R containing interneuron to CA1 pyramidal neuron pairs. We show that DAGL is not involved in hippocampal DSI (Figure 5.1A).

This finding raises the important question which signaling cascade then is responsible for the formation of endocannabinoids in hippocampal DSI. There are alternative pathways for the biosynthesis of 2-AG, which do not involve DAGL activity (for review see Sugiura et al., 2006). It should be noted, however, that this alternative route of 2-AG production should be Ca²⁺ sensitive, since an elevation of the intracellular Ca²⁺ concentration is the necessary trigger for DSI. Another option is that not 2-AG, but another endocannabinoid mediates hippocampal DSI. AEA is not likely to be involved, since inhibition of AEA degradation does not influence the timecourse of DSI. Future studies should be performed to tease out the molecular machinery necessary for endocannabinoid formation in hippocampal DSI.

It is interesting to discuss our findings in chapter 2 in light of forms of endocannabinoid signaling occurring at other synapses in the brain. The fact that DAGL is not involved in hippocampal DSI, while 2-AG is thought to mediate this form of plasticity, questions the importance of DAGL for 2-AG formation. However, there is a multitude of forms of endocannabinoid signaling in which the evidence for DAGL involvement is strong. First of all, at the same inhibitory synapses as studied in chapter 2, DAGL does seem to be necessary for both carbachol induced IPSC suppression (Edwards et al., 2006; Edwards et al., 2008), as well as for long term depression (LTD; Chevaleyre and Castillo, 2003). This suggests that DAGL is present and functional at these synapses, even though it does not play a role in DSI.

In cerebellum, DAGL inhibition is very effective in inhibiting LTD as well as associative short-term endocannabinoid signaling at excitatory synapses onto Purkinje cells (Safo and Regehr, 2005). Furthermore, DAGL has been localized to the base of the postsynaptic spine necks in cerebellum (Yoshida et al., 2006). However, it is important to note that at these same synapses onto Purkinje cells, non-associative short-term endocannabinoid signaling evoked by a postsynaptic depolarization, seems to be insensitive to DAGL inhibition (Safo and Regehr, 2005). Therefore, endocannabinoid signaling at these synapses seems to be very similar to endocannabinoid signaling at CB₁R containing inhibitory synapses in hippocampus: DAGL is necessary for LTD or metabotropic receptor dependent synaptic depression, but is not involved in depolarization induced endocannabinoid signaling.

The above described findings in hippocampus and cerebellum would suggest that endocannabinoid synthesis evoked purely by a cellular depolarization is never dependent on DAGL activity. However, a DAGL dependent form of depolarization induced suppression of excitation (DSE) has been described at excitatory synapses in the ventral tegmental area (Melis et al., 2004). Furthermore, DSE in hippocampal autaptic cultures also seems to depend on DAGL activity (Straiker and Mackie, 2005). Finally, a non-synaptic form of endocannabinoid signaling in the brain termed sustained self-inhibition is clearly also mediated by DAGL mediated 2-AG formation (Marinelli et al., 2008; see chapter 2). Like DSE, this process can be activated by pure postsynaptic depolarization.
Therefore, endocannabinoid signaling evoked solely by postsynaptic depolarization can also depend on DAGL activity in some synapses.

In conclusion, there is clear evidence for DAGL mediated endocannabinoid signaling in several forms of synaptic plasticity in the brain. However, there are also some forms of plasticity in which DAGL does not seem to be involved, although they seem to be mediated by 2-AG. Future studies should be aimed at dissecting out this alternative pathway of endocannabinoid formation. The formation of a genetically modified mouse lacking DAGL's would be an important step forward to study the exact importance of this enzyme for brain functioning. This might lead to important new targets for pharmacological intervention in endocannabinoid signaling cascades.

The **GABA$_A$ receptor: a novel cannabinoid receptor?**

In chapter 3 of this thesis we have described a novel mechanism of cannabinoid modulation of synaptic transmission. First, we show that the GABA$_A$R, the most abundant inhibitory neurotransmitter receptor in the brain, can be directly modulated by exogenous cannabinoids as well as by endocannabinoids independent of CB$_1$R activation. Cannabinoids decrease the amplitude of GABA$_A$R mediated currents, while changing current kinetics. These results imply that inhibitory synapses which lack the CB$_1$R can still be modulated by cannabinoids. In chapter 4 we test this implication, and show that neocortical inhibitory FS interneuron to pyramidal neuron synapses, which lack the CB$_1$R, indeed can still be directly modulated by cannabinoids. First, we show that application of exogenous cannabinoids modulates postsynaptic GABA$_A$Rs. Second, we show that depolarization induced synthesis of endocannabinoids in a postsynaptic
pyramidal neuron causes a transient reduction in IPSC amplitude. Finally, we address the importance of this direct GABA$_A$R modulation for microcircuit functioning. We show that both exogenously applied as well as endogenously released cannabinoids reduce inhibitory control of FS interneurons over pyramidal neuron action potential firing.

These findings have profound implications for the way in which we think about endocannabinoid signaling in the brain. The existence of novel cannabinoid receptors has long been hypothesized (Breivogel et al., 2001; Pistis et al., 2004; Begg et al., 2005), but research has mainly focused on finding novel G-protein-coupled cannabinoid receptors (see for example Lauckner et al., 2008). Although it has been known for some time that different voltage- and ligand-gated ion channels can be directly modulated by cannabinoids (for review see Oz, 2006), the functional significance of such direct effects has remained elusive. We are the first to show that endogenous release of cannabinoids can modulate synaptic transmission independent of CB$_1$R activation, most likely through direct GABA$_A$R modulation (Figure 5.1B).

Future studies should be aimed at investigating how widespread direct endocannabinoid-mediated modulation of GABA$_A$R function is, as a mechanism for short-term synaptic plasticity. Questions that should be answered are whether direct GABA$_A$R modulation is receptor-subunit specific, and what the exact mechanism of this modulation is (i.e. do cannabinoids bind to a specific site on the receptor or do they modulate channel gating through protein-membrane interactions?). Furthermore, it would be important to study the functional consequences of this direct modulation. For this, behavioral experiments on the effects of endocannabinoid synthesis and breakdown modulators in the CB$_1$R$^{-/-}$ mouse would be valuable.

**CB$_1$R dependent versus CB$_1$R independent endocannabinoid signaling**

In this thesis, we studied two different forms of synaptic plasticity at different inhibitory synapses in the brain. It is interesting to note the differences between these forms of synaptic plasticity. One important difference is the formation of endocannabinoids in the two processes. While endocannabinoid formation in hippocampal DSI surprisingly is DAG lipase independent, direct modulation of GABA$_A$Rs in neocortex depends on DAG lipase activity (Figure 5.1A,B). Another difference is the site of endocannabinoid action. In hippocampal DSI endocannabinoids are thought to act as retrograde messengers, and the final reduction in synaptic efficacy takes place through activation of presynaptic CB$_1$R activation. In contrast, at neocortical FS interneuron to pyramidal neuron synapses, endocannabinoid formation takes place in close proximity of the postsynaptic GABA$_A$R, with 2-AG acting as an intracellular or membrane-delimited messenger, modulating postsynaptic GABA$_A$R properties (Figure 5.1A,B). One could imagine that such different processes would take place on a different timescale. Direct ion channel modulation occurring at the same side of the synapse as where endocannabinoid formation takes place could be very fast, while a G-protein mediated signaling cascade at the opposite synaptic side could be slower. Indeed, direct GABA$_A$R modulation seems to take place on a slightly faster timescale as hippocampal DSI (~5 sec. vs tens of seconds
respectively). However, this difference is minor, which might indicate that the main determinants of the timescale of endocannabinoid mediated synaptic plasticity are the speed of endocannabinoid synthesis and degradation.

As mentioned above, it is interesting to see whether other synapses than the FS interneuron to pyramidal neuron synapse studied in chapter 4 show direct GABA\(_A\)R modulation by endocannabinoids. In this respect, it is important to note that in chapter 2 we showed that hippocampal DSI is not significantly observed in CB\(_1\)R\(^{-/-}\) mice (Figure 2.3). This implies that hippocampal CB\(_1\)R containing interneuron to pyramidal neuron synapses do not show a significant direct GABA\(_A\)R modulation by endocannabinoids. A possible explanation for this observation could be that there is a difference in GABA\(_A\)R subunit expression at these synapses, as compared to the neocortical FS interneuron to pyramidal neuron synapses studied in chapter 4. However, an extension of our investigation of direct GABA\(_A\)R modulation by endocannabinoids to other inhibitory synapses in the brain would be very interesting.

**Modulation of microcircuitry processing**

Interneurons which do not express the CB\(_1\)R on their presynaptic axon terminals are thought to play very different roles in microcircuit processing compared to interneurons which do express the CB\(_1\)R. The main group of CB\(_1\)R containing interneurons in hippocampus and neocortex are basket cells positive for the neuropeptide cholecystokinin (CCK). CB\(_1\)R lacking FS cells and CB\(_1\)R containing CCK positive interneurons, which both target the perisomatic region of pyramidal neurons, are thought to have fundamentally distinct functions in microcircuitry processing. Because of their somatic targeting, both interneuron types are able to efficiently control pyramidal neuron AP firing. These perisomatic interneurons are particularly important during fast network oscillations, when the precise regulation of pyramidal neuron ensembles is required (Mann et al., 2005). However, while FS interneurons are considered to be essential in the generation of this precise timing in cortical circuits, CCK positive interneurons are considered to act more like modulators of such cortical activity (Freund and Katona, 2007). In agreement with this, FS interneurons receive strong, precisely timed and persistent excitation, allowing them to faithfully report the timing of ongoing neuronal activity (Galarreta and Hestrin, 2001; Glickfeld and Scanziani, 2006). In contrast, CCK positive interneurons receive weaker excitatory inputs showing strong synaptic depression (Glickfeld and Scanziani, 2006). Therefore, CCK positive interneurons are efficiently activated by the sequential activation of independent excitatory inputs. Furthermore, their membrane constant is slower than that of FS interneurons, thereby increasing their ability to integrate excitatory inputs over long time ranges (Glickfeld and Scanziani, 2006). The synapses formed by CCK positive interneurons are unreliable and do not show exact timing, but rather release neurotransmitter asynchronously (Hefft and Jonas, 2005). Finally, while FS interneurons hardly express neuromodulator receptors on their presynaptic terminals, CCK positive interneurons are sensitive to a variety of neuromodulators. For these reasons, FS interneurons have been referred to as the ‘clockwork’ of cortical networks, while CCK positive interneurons form a ‘fine tuning mechanism’
We show now that both types of interneuron synapses can be modulated by endocannabinoid signaling, either through direct GABA_A receptors (FS interneurons), or through activation of presynaptic CB_1 receptors (CCK positive interneurons). Although mediated by the same messenger, these two types of modulation have fundamentally distinct implications for network functioning. CB_1 signaling powerfully inhibits neurotransmitter release from CB_1-containing interneuron terminals, thereby making synaptic release less reliable, or even completely muting synapses by increasing the failure-rate of vesicle release (Losonczy et al., 2004; Figure 5.2). In contrast, postsynaptic GABA_A modulation by endocannabinoids does not change the reliability of synaptic signaling, but rather affects synaptic strength. (Figure 5.2).

(Freund and Katona, 2007).

Figure 5.2 Different effects of pre- and postsynaptic synaptic modulation on synaptic transmission
Schematic representation highlighting the different modulation of CCK interneuron and FS interneuron synapses. Lines represent presynaptic action potentials, circles represent synaptic release. Top, under control conditions CCK interneurons show less reliable synaptic transmission than FS interneurons. Bottom, when endocannabinoid synthesis is initiated through postsynaptic depolarization, this causes an even further decrease in the synaptic reliability of CCK interneurons through presynaptic CB_1 activation. In contrast, while the synaptic efficacy of FS interneurons is reduced because of direct postsynaptic GABA_A modulation, their reliability is not affected by endocannabinoids.
These two modes of action will have dramatically different effects on information processing in the brain, causing a different form of microcircuit reassembly. The first mechanism will mainly affect the reliability of the CCK positive interneuron input, thereby working as a presynaptic filter. When the postsynaptic neuron is stimulated enough to cause endocannabinoid signaling at this input, weak activity in the presynaptic CCK positive interneuron will no longer result in synaptic transmission. Only very strong, repetitive CCK positive interneuron firing will result in inhibition of the postsynaptic neuron. In contrast, the second mechanism will not induce presynaptic filtering (or in any other way affect presynaptic processing) of the input. The FS interneuron will transmit information upon the same stimuli as in the absence of endocannabinoid signaling. But now the endocannabinoids act as a postsynaptic filter, which reduce the impact of inhibitory transmission, while keeping its timing intact (Figure 5.2).

It is important to note that both endocannabinoid mediated forms of synaptic plasticity described in this thesis cause a transient reduction in the strength of inhibitory synaptic transmission. Therefore, both mechanisms allow pyramidal neurons to ‘disinhibit’ themselves when they are excited, by the activity dependent synthesis of endocannabinoids. In this way, endocannabinoids would increase postsynaptic excitability even further. This could for example lead to a transient facilitation of the induction of long-term potentiation at such excitatory synapses (Wigstrom & Gustafsson, 1983).

**Challenges to come**

Constituents of the endocannabinoid system (the enzymatic machinery for endocannabinoid synthesis and breakdown and the CB\(_1\)R) are present in brain areas involved in learning and memory like the hippocampus, amygdala, neocortex, striatum and cerebellum. Consequently, the effects of cannabinoids on memory functions are well documented (for review see Lutz, 2007). Furthermore, the endocannabinoid system plays a role in neuroprotection (Marsicano et al., 2003; Melis et al., 2006; Monory et al., 2006). However, until now most studies on the role of endocannabinoid signaling in the brain have focused on CB\(_1\)R activation. The findings described in this thesis make it clear that endocannabinoids can strongly influence neuronal microcircuits in the absence of CB\(_1\)R signaling. A challenge for the future is to elucidate the functional relevance of this CB\(_1\)R independent endocannabinoid signaling, and to pharmacologically and genetically segregate the contributions of CB\(_1\)R-dependent and independent endocannabinoid signaling to behavior.


Summary

Revised models of endocannabinoid signaling at inhibitory synapses in the brain

The research described in this thesis focuses on how endocannabinoids influence the communication between brain cells. Here, I will first describe what endocannabinoids are and why it is interesting to study them. Then I will shortly explain how brain cells communicate, and how we can record this. Finally, I will shortly describe the main findings from this thesis.

Endocannabinoids are substances that naturally occur in the brain. Their name is a concatenation of the words ‘endogenous’ (meaning occurring within the body) and ‘cannabinoid’. The second part of the name derives from the similarities between endocannabinoids and the active component from the Cannabis sativa plant (marijuana), Δ9-THC. The Cannabis sativa plant has been known for thousands of years for its psychoactive and medicinal properties. Both Δ9-THC and endocannabinoids affect the brain by activating the same target, a so-called cannabinoid receptor. This cannabinoid receptor is very abundant in the brain, which suggests that it plays an important role in normal brain functioning.

Recent studies have shown that endocannabinoids in the brain are important for learning and memory, that they play a role in the processing of pain, and that they are important for the regulation of food intake. Pharmacological intervention in endocannabinoid signaling is seen as a promising treatment for a multitude of problems like pain, nausea, obesity and depression. Furthermore, a malfunctioning endocannabinoid system seems to play a role in several neurological diseases, including schizophrenia and epilepsy. Therefore, it is important to have a better understanding of the exact way in which endocannabinoids work. I will now shortly describe how brain cells communicate, and what role endocannabinoids play in this.

The principle task of the brain is to translate sensory information that it receives from the senses into proper behavioral output. To do this, the billions of brain cells (neurons) in the brain assemble into complex neuronal networks. These neurons communicate by means of so-called synapses. A synapse is the contact point between a sending (presynaptic) neuron and a receiving (postsynaptic) neuron. When a synapse is active, the presynaptic neuron releases a substance called a neurotransmitter. This neurotransmitter activates and opens ionic channels on the postsynaptic neuron. The opening of these ionic channels permits the flow of electrically charged ions in and
out of the postsynaptic neuron, which causes an electrical current in this neuron. This current can either activate or inhibit the postsynaptic neuron (depending on the nature of the neurotransmitter and the properties of the ionic channels). Every neuron in the brain receives thousands of synapses, while also sending thousands of synapses to other neurons.

In most experiments described in this thesis we have recorded the electrical activity of synapses. To do this, we use brain slices of mice or rats. In such slices a part of the neuronal network remains intact. By contacting a neuron in this network with a tiny glass electrode, it is possible to record the electrical activity in this neuron. It is possible to simultaneously record the activity of two neurons that make a synaptic contact. By stimulating the presynaptic neuron while recording activity in the postsynaptic neuron, we can map the properties of the synapse between these neurons.

In order to properly process sensory information, it is necessary that synapses can temporarily change their strength. The property of synapses to temporarily strengthen or weaken is termed ‘synaptic plasticity’. Synaptic plasticity can occur on both sides of the synapse: the presynaptic neuron can change the amount of neurotransmitter release, and the postsynaptic neuron can change the amount or properties of the receiving ionic channels. In this way a synapse can temporarily strengthen (more neurotransmitter release or a stronger response of the ionic channels) or weaken (less neurotransmitter release or a weaker response of the ionic channels).

Endocannabinoids play a role in several forms of synaptic plasticity. The classical way in which endocannabinoids work is as follows: strong activity in the postsynaptic neuron triggers the formation of endocannabinoids. These endocannabinoids subsequently travel to the presynaptic neuron, where they activate the cannabinoid receptor. Activation of this receptor causes a reduction in neurotransmitter release, resulting in a weakening of the synapse. This weakening can last for tens of seconds, until the endocannabinoids are degraded.

Until now it was assumed that an enzyme termed DAG lipase is involved in the formation of endocannabinoids in many neurons. In chapter 2 of this thesis we study a brain region which contains many cannabinoid receptors, and which is important for memory formation: the hippocampus. We study synapses in the hippocampus which show the above described form of endocannabinoid mediated synaptic plasticity. We show that blocking the activity of DAG lipase does not affect this form of synaptic plasticity. This means that, surprisingly, DAG lipase is not involved in the formation of endocannabinoids in this form of plasticity. Future research is needed to find out in which alternative way endocannabinoids are formed in this process.

In chapters 3 and 4 we describe an entirely novel way in which endocannabinoids can mediate synaptic plasticity. Until now it was thought that cannabinoids only influence synaptic transmission by activation of presynaptic cannabinoid receptors. In chapters 3 and 4 we show that endocannabinoids can also modulate synapses that lack this receptor.
Using several different approaches, we show that cannabinoids can directly change the properties of some postsynaptic ion channels. This means that synapses which lack the cannabinoid receptor can still show endocannabinoid mediated synaptic plasticity.

The research described in this thesis shows that endocannabinoids are versatile substances, which can change brain functioning not only through activation of specialized cannabinoid receptors, but also by direct modulation of unexpected targets. Hopefully, the increased understanding of these signaling processes will further our understanding of how the brain works, and enable more specific pharmacological intervention in brain diseases involving endocannabinoid signaling.
Nederlandse samenvatting

Herziene modellen voor endocannabinoïd signaal transductie in inhibitoire synapsen in het brein

In dit proefschrift wordt onderzocht hoe endocannabinoïden de communicatie tussen hersencellen beïnvloeden. Ik zal eerst uitleggen wat endocannabinoïden zijn, en waarom het interessant is ze te bestuderen. Vervolgens zal ik uitleggen hoe communicatie tussen hersencellen plaatsvindt, en hoe we dat kunnen meten. Tot slot zal ik de belangrijkste bevindingen uit dit proefschrift samenvatten.

Endocannabinoïden zijn stoffen die van nature voorkomen in de hersenen. De naam is een samentrekking van de woorden ‘endogene’ (lichaamseigen) en cannabinoïd. Dit tweede deel van de naam danken endocannabinoïden aan hun gelijkenis met de actieve component van de Cannabis sativa plant (marihuana), Δ9-THC. De Cannabis sativa plant staat al duizenden jaren bekend om zijn psychoactieve en medicinale eigenschappen. Δ9-THC en endocannabinoïden oefenen beiden hun effect op de hersenen uit door activatie van een zogenaamde cannabinoïd receptor. Deze cannabinoïd receptor komt buitengewoon veel voor, wat suggereert dat hij een belangrijke rol speelt in het functioneren van de hersenen.

Recent onderzoek heeft laten zien dat endocannabinoïden in de hersenen belangrijk zijn voor leren en geheugenfuncties, dat ze een rol spelen bij het verwerken van pijn, en dat ze belangrijk zijn voor regulatie van eetlust. Het farmacologisch ingrijpen in het endocannabinoïd systeem wordt gezien als een veelbelovende behandeling voor uiteenlopende problemen waaronder pijnklachten, misselijkheid, overgewicht en depressie. Bovendien lijkt een verkeerd functionerend endocannabinoïd systeem een rol te spelen bij een aantal neurologische aandoeningen, waaronder schizofrenie en epilepsie. Daarom is het belangrijk dat de precieze werking van endocannabinoïden beter begrepen wordt. Ik zal nu kort beschrijven hoe hersencellen met elkaar communiceren, en op welke manier endocannabinoïden daar een rol in spelen.

De belangrijkste taak van de hersenen is het vertalen van informatie afkomstig van de zintuigen in gepast gedrag. Om deze taak te vervullen rangschikken de miljarden hersencellen (neuronen) zich in ingewikkelde netwerken. Deze neuronen communiceren met elkaar door middel van zogenaamde synapsen. Een synaps is het contactpunt tussen een verzendend (presynaptisch) neuron en een ontvangend (postsynaptisch) neuron. Bij synaptische communicatie tussen neuronen geeft het presynaptische neuron een stofje af. Dit stofje wordt een neurotransmitter genoemd. Na afgifte activeert en opent
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dezo neurotransmitter ionkanalen op het postsynaptische neuron. Het openen van deze ionkanalen zorgt ervoor dat ionen met een elektrische lading het postsynaptische neuron in of uit kunnen stromen. Dit veroorzaakt een elektrische stroom. Een dergelijke stroom kan ervoor zorgen dat het ontvangende neuron geactiveerd, of juist afgeremd wordt (afhankelijk van de aard van de neurotransmitter en de eigenschappen van de ionkanalen). Ieder neuron ontvangt duizenden synapsen, en verzendt op zijn beurt ook weer duizenden synapsen naar andere neuronen.

Bij de experimenten die in dit proefschrift beschreven worden hebben we de elektrische activiteit van synapsen bestudeerd. Hiervoor gebruiken we hersenplakjes van muis of rat. In zulke plakjes is een deel van het neuronale netwerk nog intact. Door een neuron in dit netwerk aan te prikken met een minuscule glazen elektrode kunnen we de elektrische activiteit van dit neuron meten. Het is mogelijk tegelijkertijd twee neuronen in het netwerk aan te prikken die een synaptisch contact met elkaar maken. Door dan het presynaptische neuron te stimuleren terwijl we de activiteit in het postsynaptische neuron meten kunnen we de eigenschappen van de synaps tussen deze twee neuronen gedetailleerd in kaart brengen.

Voor een goede verwerking van zintuiglijke informatie is het noodzakelijk dat synapsen tijdelijk sterker of zwakker kunnen worden. Het vermogen van synapsen om tijdelijk hun sterkte te reguleren wordt ‘synaptische plasticiteit’ genoemd. Synaptische plasticiteit kan aan beide kanten van de synaps plaatsvinden: het presynaptische neuron kan de hoeveelheid neurotransmitter die het afgeeft veranderen, en het postsynaptische neuron kan de hoeveelheid of de eigenschappen van de ontvangende ionkanalen veranderen. Op deze manier kan een synaps dus tijdelijk sterker (meer neurotransmitter afgifte of een sterkere reactie van de ionkanalen) of zwakker worden (minder neurotransmitter afgifte of een zwakkere reactie van de ionkanalen).


Tot nu toe dacht men dat een bepaald eiwit, genaamd DAG lipase, betrokken was bij de vorming van endocannabinoïden in veel neuronen. In hoofdstuk 2 doen we onderzoek naar een hersengebied waarin veel cannabinoid receptoren aanwezig zijn, en dat belangrijk is voor geheugenvorming; de hippocampus. We onderzoeken synapsen in de hippocampus die de hierboven beschreven vorm van synaptische plasticiteit vertonen. We laten zien dat als we de activiteit van het DAG lipase eiwit blokkeren, deze vorm van synaptische plasticiteit nog gewoon aanwezig is. Dit geeft aan dat het DAG lipase eiwit verassend genoeg niet betrokken is bij de aanmaak van endocannabinoïden in deze
vorm van synaptische plasticiteit. Toekomstig onderzoek zal moeten uitwijzen op welke alternatieve manier endocannabinoïden hier worden aangemaakt.

In hoofdstuk 3 en 4 beschrijven we een geheel nieuwe manier waarop endocannabinoïden synaptische plasticiteit kunnen veroorzaken. Tot nu toe werd gedacht dat endocannabinoïden synapsen alleen kunnen beïnvloeden zoals hierboven beschreven: via activatie van de presynaptische cannabinoïd receptor. In hoofdstuk 3 en 4 laten we zien dat endocannabinoïden ook in afwezigheid van deze receptor synapsen kunnen beïnvloeden. Op een aantal verschillende manieren tonen we aan dat endocannabinoïden direct de eigenschappen van sommige postsynaptische ionkanalen kunnen beïnvloeden. Dit betekend dat ook synapsen zonder de cannabinoïd receptor een vorm van endocannabinoïd afhankelijke synaptische plasticiteit kunnen laten zien.

Het onderzoek dat wordt beschreven in dit proefschrift laat zien dat endocannabinoïden veelzijdige stoffen zijn. Ze beïnvloeden de hersenen niet alleen via activatie van de gespecialiseerde cannabinoïd receptor, maar ook door onverwachte directe effecten op ionkanalen. Deze resultaten dragen bij aan een beter begrip van hoe de hersenen werken. Uiteindelijk zullen we dan misschien in staat zijn gerichter in te grijpen bij hersenziekten waarin endocannabinoïden een rol spelen.
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About the Author

Rogier Min was born on the 14th of July 1980 in Hoorn, the Netherlands. From 1993 onwards he attended secondary school ‘Oscar Romero’ in Hoorn, from which he graduated in 1998. In that same year he started his biology studies at the VU University in Amsterdam.

On the first day of this study, during his first lecture, he sat next to his future wife, Janneke.

During his biology studies, Rogier specialized in Neuroscience. He did his first internship at the VU University in Amsterdam under supervision of Prof. Dr. Arjen Brussaard, on retrograde signaling by the hormone oxytocin in the rat brain. Next, he did an internship with Prof. Dr. Wytse Wadman at the University of Amsterdam, where he used a computer modeling approach to understand the interactions of an anti-epileptic drug with neuronal sodium channels. After finishing his studies in 2003, he started a PhD project under supervision of Prof. Dr. Nail Burnashev and Prof. Dr. Arjen Brussaard at the Department of Integrative Neurophysiology of the VU University in Amsterdam. The results of this project are described in this thesis.

In the summer of 2008 Rogier and Janneke moved to Switzerland. Here, Rogier works as a postdoctoral researcher in the group of Dr. Thomas Nevian, at the Department of Physiology of the University of Bern. The main focus of his current research is to understand the mechanisms underlying long-term changes in synaptic efficacy, using a combination of electrophysiology and 2-photon laser microscopy.
List of Publications

Chapter 2:
Min, R, Testa-Silva, G, Heistek, TS, Canto, CB, Lodder, JC, Bisogno, T, Di Marzo, V, Brussaard, AB, Burnashev, N, Mansvelder, HD. DAG lipase is not involved in DSI at unitary inhibitory connections in mouse hippocampus. (Under Revision)

Chapters 3 & 4:
Golovko, T, Min, R, Lozovaya, N, Yatsenko, N, Lozovaya, N, Mack, V, Ledent, C, Rozov, A, Burnashev, N. Suppression of inhibition by the direct action of cannabinoids on postsynaptic GABA_A receptors. (Submitted; *equal contribution)

Publications not in this thesis:


Wadman, WJ, Min, R, Sun, GC (2005). Sodium current properties in different models of epilepsy. In: Kindling 6 (Corcoran, ME, Ed.) 61(10)

De Kock, CPJ, Wierda, KDB, Bosman, LWJ, Min, R, Koksma, JJ, Mansvelder, HD, Verhage, M, Brussaard, AB (2003). Somatodendritic secretion in oxytocin neurons is upregulated during the female reproductive cycle. Journal of Neuroscience 23(7): 2726-2734