Modulation of Synaptic Transmission by Metabotropic Glutamate Receptors and Endocannabinoid Signaling

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The cover shows a leaf from the plant *Cannabis sativa* from which the psychoactive compound cannabinoid is derived.

Modulation of Synaptic Transmission by Metabotropic Glutamate Receptors and Endocannabinoid Signaling

Modulatie van synaptische transmissie door metabotrope glutamaat receptoren en endocannabinoiden

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Asatoma Sadgamaya
Thamaso Maa Jyothir Gamaya
Mrithyor Maa Amrutham Gamaya
Aum Shanti Shanti Shantihi

(From the unreal lead me to the Real
From darkness lead me to light
From death lead me to immortality
Om. Peace, peace, peace.)

Brihadaranyaka Upanishad 1.3.28

For my parents
and brother…

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Chapter 1

General Introduction

1.1. Synaptic plasticity

Neurons express a wide variety of physiological mechanisms capable of changing synaptic strength. The capacity of the nervous system to adaptive alterations, generally referred to as neural plasticity, is obvious during the development of neural circuits. However, the adult brain must also possess at least some plasticity to learn new skills, establish new memories, and respond to injury throughout life by the modification of synaptic strength, and is responsible for much of the ongoing change in synaptic function in adults. Long-lasting activity-dependent changes in the efficacy of synaptic transmission in the mammalian brain are considered to be of fundamental importance for the development of neural circuitry and for the storage of information. Many synapses in the mammalian central nervous system (CNS) exhibit long-lasting forms of synaptic plasticity, which are plausible substrates for enduring changes in brain function. Because of their duration, these forms of synaptic plasticity in mammals are widely believed to be cellular correlates of learning and memory. Thus, a great deal of effort has gone into understanding how they are generated. An enormous effort has been channeled into understanding the mechanisms by which strengthening of synaptic connections can be achieved and, in this effort, long-term potentiation (LTP) and long-term depression (LTD) are the most compelling and reliable models studied. These two forms of plasticity have been extensively studied in the mammalian CNS, especially in the hippocampus and cerebellum.

1.2. Hippocampus: organization and function

The hippocampus is one of the major components of the limbic system, along with the amygdala, the cingulate gyrus and the prefrontal cortex. Different components of the limbic system have been shown to play a critical role in all aspects of emotions, fear, learning and memory. The hippocampal formation is an area of the primitive cortex (i.e., allocortex) connected to the adjacent entorhinal cortex and other associative cortical areas (Figure 1.1). The hippocampal formation is composed of the subiculum, the hippocampus proper, and the dentate gyrus. Based on cytoarchitecture the hippocampus proper is further subdivided into four regions designated CA1-CA4 (cornu ammonis, or Ammon's horn), where CA1 is located proximal to the subiculum and CA4 close to the dentate gyrus. The neuronal patterning is the same through CA1-CA3 with a single layer of soma from pyramidal neurons whose dendrites protrude and extend in one direction.

The hippocampal formation is composed of three major excitatory pathways forming a trisynaptic cascade (Figure 1.2). The first major excitatory pathway is called the perforant pathway. This input to the hippocampal formation originates from layers two and three of the entorhinal cortex and passes through the subiculum to terminate in the molecular layer of the dentate gyrus. Granule cells in the dentate gyrus form the second excitatory pathway, the mossy fiber pathway, which projects to the proximal dendrites of pyramidal cells in the CA3 region of the hippocampus. In addition to sending recurrent collaterals to cells within CA3 and collaterals back to the granule cells, CA3 pyramidal neurons also send an excitatory projection, comprising the third excitatory pathway, to the pyramidal cells in the CA1 hippocampal region (i.e., Schaffer collateral fibers). The CA1 neurons, in turn, provide input to the subiculum as well as layers four and five of the entorhinal cortex. This form of unidirectional synaptic flow is unusual in cortical structures, and has been shown to be very important in the consolidation of memories.

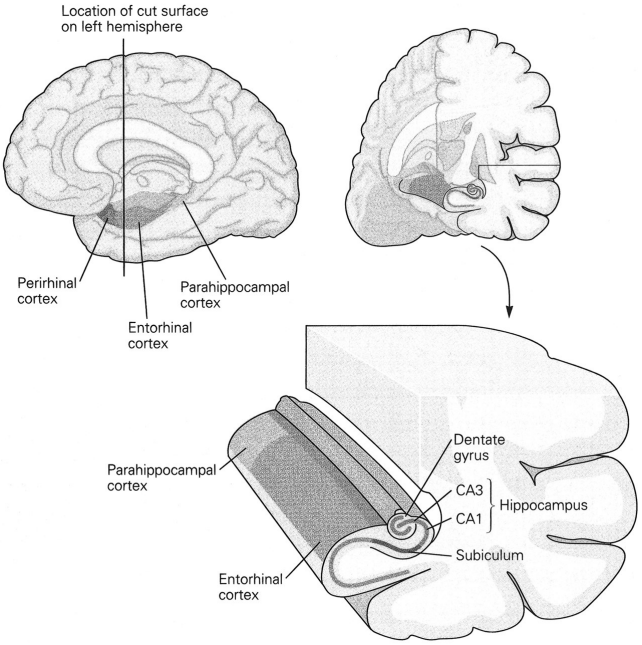

Figure 1.1.
Anatomical organization of the hippocampal formation. Medial (left) and ventral (right) surfaces depicting key components of the medial temporal lobe important for memory storage. Enlargement (below) shows the location of the CA1 and CA3 hippocampal regions, the dentate gyrus, and the entorhinal cortex (from Kandel et al., 2000).

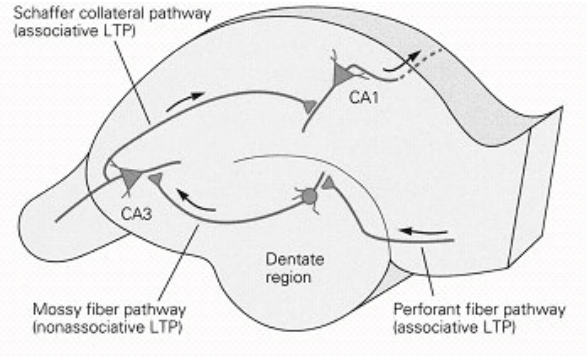

Figure. 1.2.
Afferent pathways in the hippocampus. The perforant fiber pathway from the entorhinal cortex forms excitatory connections with granule cells in the dentate gyrus. The axons of these cells in turn form connections with CA3 pyramidal neurons. Their axons comprise the Schaffer collateral pathway via connections with CA1 pyramidal neurons (from Kandel et al., 2000)

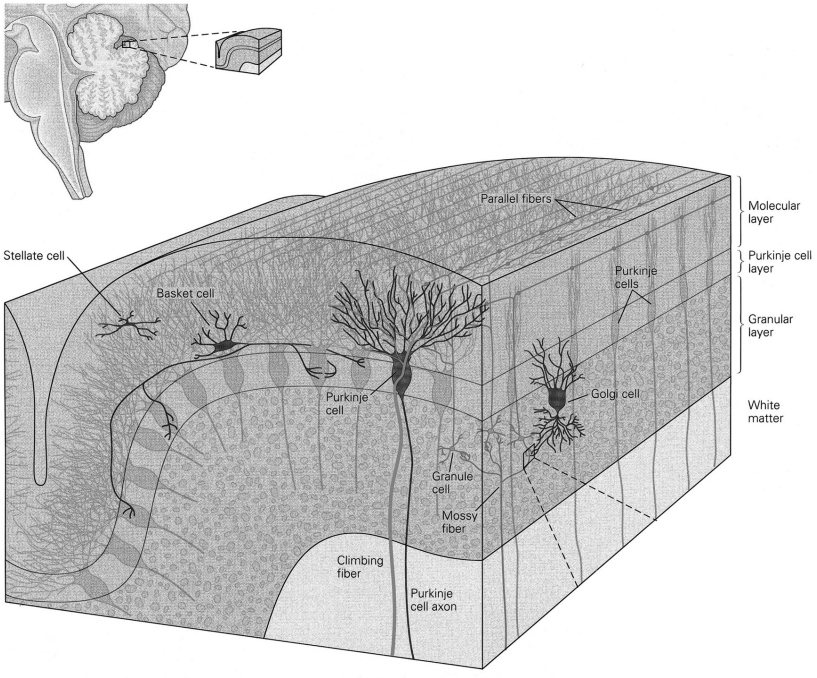

Figure 1.3.
Anatomical organization of the cerebellar cortex. Enlargement of a vertical section of the cerebellar cortex in both longitudinal and transverse planes. Note that the granule cells are found in the granular layer, the Purkinje cell (PC) soma are found in the molecular layer, and the PC dendrites are located in the molecular layer (from Kandel et al., 2000).

1.3. Cerebellum: organization and function

The cerebellum encompasses the highly organized cerebellar cortex and the cerebellar nuclei. The main functions of the cerebellum include fine-tuning of motor coordination and posture from input received via most of the major sensory and motor systems. The Purkinje cells are inhibitory and form the sole output of the cerebellar cortex. These large neurons directly innervate both inhibitory and excitatory neurons in the cerebellar and vestibular nuclei so as to ultimately alter motor behaviour. A single Purkinje cell is thought to contact about 30-50 deep cerebellar nucleus cells and one DCN cell receives input from 20-30 Purkinje neurons (Chan-Palay, 1977). The deep cerebellar and the vestibular nuclei in turn project to motor areas in the cerebral cortex, and the brainstem (Rand, 1954; Sastry et al., 1997).

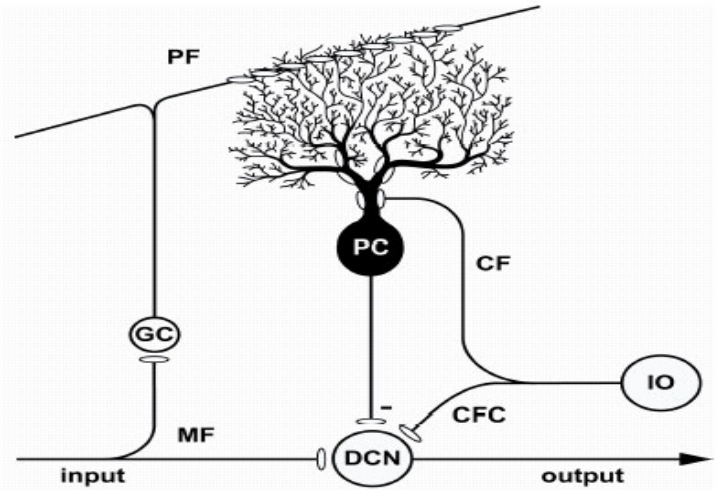

Figure 1.4. Simplified diagram of cerebellar circuitry. The main relay pathway: mossy fiber (MF) input from the precerebellar nuclei projects to the deep cerebellar nuclei (DCN), and DCN excitatory output targets premotor centers. Synapses are excitatory except when indicated (-). GC, granule cell; PF, parallel fiber; PC, Purkinje cell; IO, inferior olive; CF, climbing fiber; CFC, climbing fiber collateral.

Despite its relatively small size, the cerebellum contains more neurons than the whole cerebral cortex (Korbo et al., 1993). Of the seven cell types found in the mammalian cerebellar cortex (i.e. Purkinje cells, granule cells, brush cells, Golgi cells, stellate cells, basket cells and Lugaro cells) granule cells are the most abundant, estimated at about one trillion cells (10^{11}). The cerebellar cortex, which contains the somata of all these neurons, is a relatively simple three-layered structure (Figure 1.3). The axons of the granule cells, the Purkinje cell dendrites and the basket and stellate cells together form the outer layer, known as molecular layer. The Purkinje cell somata form the second layer, and the third layer is called the granule cell layer, containing all the small somata of the granule cells. These layers together form the gray matter. The collateral forming axons of the sole cortical output, the Purkinje cells, leave the gray matter of the cortex to form, together with the climbing fiber and mossy fiber axons, the cerebellar white matter (Ghez and Thach, 2000). The inputs to the cerebellum carry extensive internal feedback information about goals, commands and programming of movements, as well as external feedback information associated with the execution of movement, which allows the cerebellum to compare the intended movement with the reported actual movement. The cerebellar circuitry is essentially composed of a relay station in the deep cerebellar and vestibular nuclei (DCN and VN) and a cortical 'side-loop' (Figure 1.4). Cerebellar output to premotor centers originates from the DCN and VN and is driven by direct excitatory input from the mossy fibers. The DCN and VN output is modulated by inhibitory input from Purkinje cell axons, which convey the computations and interactions in the Purkinje cell. Two main types of afferents, the mossy fiber afferent/parallel fiber system and the inferior olive/climbing fiber system, project to the cerebellar cortex, and

produce different firing patterns in the Purkinje neurons, which provide the only output from the cerebellar cortex.

1.4. Excitation and Inhibition in the brain

Studies in the past decade have provided evidence on the prominent role of central excitatory amino acids (EAAs) in neurodegenerative diseases, including ischemia and seizure-related brain damage. It is widely accepted that excitatory amino acid transmitters such as glutamate are involved in a variety of pathological conditions. On the other hand, EAAs were found to be involved in processes of synaptic plasticity, such as LTP and LTD.

While most of our knowledge of these activity-dependent changes in synaptic efficacy is derived from studies at excitatory synapses, little is known about synaptic plasticity at inhibitory synapses. Because excitability in the brain is highly dependent on the level of inhibition set by GABAergic interneurons, synaptic plasticity at inhibitory synapses should have important consequences for neural function and pathological states of neuronal excitability such as epilepsy.

1.5. Glutamate-mediated synaptic transmission

Glutamate is the principal excitatory neurotransmitter mediating fast synaptic transmission in the mammalian CNS. When glutamatergic terminals are depolarised, vesicular glutamate is released into the synaptic cleft in a Ca^{2+}-dependent manner. In general, this is triggered by action potential invasion of the presynaptic terminal with consequent depolarization and influx of Ca^{2+} through non-inactivating voltage-sensitive Ca^{2+}-channels. Once released into the synaptic cleft, glutamate interacts with several distinct families of receptors located principally on postsynaptic membranes. Glutamate acts through both ligand-gated ion channels (ionotropic receptors) and G-protein-coupled (metabotropic) receptors. Activation of these receptors is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity such as LTP and LTD, which are thought to be cellular mechanism underlying learning and memory (Bliss and Collingridge, 1993; Anwyl, 1999).

1.5.1. Ionotropic glutamate receptors

Ionotropic glutamate receptors (iGluRs) which mediate fast excitatory synaptic transmission are ligand-gated ion channels composed of multimeric transmembrane proteins. The ionotropic receptors are composed of various subunits that form membrane channels, allowing a flux of ions into the cell from the extracellular space.

These receptors are segregated based on sequence homology and agonist selectivity into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate preferring subtypes (table 1.1). Most attention has been directed to synapses using NMDA receptors, but more recent evidence indicates potential roles for ionotropic non-NMDA (AMPA/kainate) and metabotropic glutamate receptors as well. NMDA receptors are well distributed in the brain, and activation of this receptor subtype causes an influx of Ca^{2+} into cells. The integral channel of the NMDA receptor-channel complex is highly permeable to Ca^{2+}, Na^{2+} and K^{+} and the resultant increase in postsynaptic

calcium concentration is thought to contribute to the induction of neuronal plasticity. This receptor is distinguished from all others by the voltage-dependent channel block by Mg^{2+}.

Receptor	NMDA	AMPA	Kainate
Subunits	NR1, NR2A, NR2B, NR2C, NR2D, NR3A, NR3B	GLUR1, GLUR2, GLUR3, GLUR4	GLUR5, GLUR6, GLUR7, KA1, KA2
Agonists	trans-ACBD cis-ACPD L-Glutamic acid NMDA Quinolinic acid	AMPA Cl-HIBO (S)-CPW 399	ATPA Domoic acid Kainate
Antagonists	D(-)-AP-7 CGS 19755 (R)-CPP LY 235959 MK-801	CNQX DNQX NBQX GYKI 52466 dihydrochloride	CNQX DNQX UBP 301 GYKI52466 dihydrochloride

Table. 1.1. Ionotropic Glutamate receptor classification and selective compounds.

AMPA and kainate receptor activation leads primarily to Na^+ influx, although some types of these receptors are also permeable to Ca^{2+} depending on their subunit combination (AMPA receptor subunit GluR2 and the kainate receptor subunit GluR6). AMPA receptor activation can also indirectly lead to Ca^{2+} influx by causing membrane depolarization which activates voltage-gated Ca^{2+} channels (VGCCs), as well as by increasing the activation of NMDA receptors, which are sensitive to the membrane potential of the cell. The roles of these receptors in various neurodegenerative syndromes have been extensively reviewed (Chapmann, 1998; Meldrum et al., 1999). It is well documented that the overactivation of iGluRs with their specific subtype agonists NMDA, kainate, and AMPA leads to seizures and excitotoxic injury throughout the CNS and, in particular, in the hippocampus, one of the most vulnerable cerebral regions to injury after seizures (Meldrum and Garthwaite, 1990; Choi, 1994).

1.5.2. Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are G-protein linked and operate by releasing second messengers. mGluRs have been implicated in different forms of synaptic plasticity, such as LTP and LTD, memory formation (Conn and Pin, 1997; Nakanishi, 1994; Riedel, 1996; Riedel et al., 1996) and changes associated with epilepsy (Chapman, 1998). An interesting feature of mGluR-mediated effects is that they are often long lasting. To date, eight subtypes of metabotropic glutamate receptors (mGluRs) have been cloned and divided into three groups according to their sequence homology, pharmacological characterization, and coupling to second messenger pathways. The first group includes mGluR1 and mGluR5 receptors, which are concentrated in the postsynaptic membrane at the periphery of synaptic junctions (Lujan et al., 1996, Shigemoto et al., 1997).

Group I mGluRs (mGluR1 and mGluR5) are linked through G-proteins which are coupled to phospholipase C (PLC). PLC cleaves phosphatidylinositol-bi-phosphate (PIP_2) from the cell membrane, producing diacylglycerol (DAG), which activates the enzyme protein kinase C (PKC), and inositol 1,4,5-triphosphate (IP_3), which binds to IP_3 receptors on intracellular Ca^{2+} stores located on the endoplasmic reticulum (ER), resulting in a release of Ca^{2+} from the stores and an elevation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) (Conn and Pin, 1997; Nakanishi, 1994). Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) mGluRs are also G-protein linked receptors that inhibit adenylate cyclase (AC) activity, which leads to a decrease in the second messenger, cyclic adenosine monophosphate (cAMP). In general, activation of group II and III mGluRs leads to a decrease in $[Ca^{2+}]_i$. mGluRs can also have other types of modulatory effects on membrane ion channels. For example, some studies have shown that activation of group I mGluRs can enhance the elevation of $[Ca^{2+}]_i$ mediated by NMDA receptors (Bruno et al., 1995; Pisani et al., 2001). Group II and III mGluRs are believed to localize in the presynaptic nerve terminal, and their activation leads to the inhibition of neurotransmitter release (Cartmell and Schoepp, 2000; Schoepp, 2001). In contrast to iGluRs, mGluRs play a modulatory role in CNS function, regulating both neuronal excitability (Conn and Pin, 1997; Anwyl, 1999) and the release of neurotransmitters (Cartmell and Schoepp, 2000). Activation of all three groups is known to be involved in the induction of synaptic plasticity in several brain regions (Anwyl, 1999). Indeed, mGluRs may have evolved as a primary mechanism to modulate the ongoing and delicate balance between excitation and inhibition in the CNS (Schoepp, 2001).

Receptor	**Group I**	**Group II**	**Group II**
Subtype	mGlu1, mGlu5	mGlu2, mGlu3	mGlu4, mGlu6, mGlu7, mGlu8
Transduction mechanism	η PLC η [Ca2+]	ιAC ιcAMP	ιAC ιcAMP
Agonists	(1S,3R)-ACPD tADA CHPG (S)-3,5-DHPG Quisqualate	(2R,4R)-APDC L-CCG-I (S)-3C4HPG DCG-IV LY354740	L-AP4 ACPT (R,S)-PPG L-SOP
Antagonists	AIDA CPCCOEt 4-CPG LY 367385 MPEP	LY 341495 MCCG MCPG MTPG	CPPG MAP4 MPPG MSOP

Table. 1.2. Metabotropic Glutamate receptor classification and selective compounds.

1.6. GABA-mediated Synaptic Transmission

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian CNS and, like glutamate and other transmitters, acts via both ligand-gated ion channels ($GABA_A$ receptors) and G-protein coupled ($GABA_B$) receptors. Activation of GABA receptors in the

CNS produces inhibitory hyperpolarizing responses on neurons under most conditions. The hyperpolarizing response is due to an increase in the chloride conductance of the neuronal membrane allowing chloride ions to flow down their electrochemical gradient into the cell. $GABA_A$ receptors are the most prevalent in the mammalian brain. The $GABA_A$ receptor has four basic subunits, 2 alpha and 2 beta peptides which surround a chloride channel. A number of drugs bind to the $GABA_A$ receptor. They bind at sites different from the spot where GABA itself binds, but increase the strength of GABA's binding to its site. Thus they enhance the inhibitory effect of GABA in the CNS. These drugs include sedatives, anti-anxiety drugs and anticonvulsants. Just like glutamatergic excitation, impairment of GABAergic inhibition is also thought to play a crucial role in the processes underlying neurodegenerative diseases, including ischemia and epilepsy (Bradford, 1995).

Receptor subtype	**$GABA_A$**	**$GABA_B$**
Agonists	Muscimol	Baclofen
Antagonists	Bicuculline Picrotoxin	CGP35348 CGP55845A

Table. 1.3. GABA receptor classification and selective compounds.

1.7. Glutamate excitotoxicity

Excessive glutamate-mediated excitation, often referred to as excitotoxicity, stands out as a critical factor common to a variety of neurological disorders, including stroke, epilepsy, traumatic brain injury and neurodegenerative disorders (Meldrum and Garthwaite, 1990; Bradford, 1995; Zauner and Bullock, 1995). Excitotoxicity involves the activation of glutamate receptors in the CNS. It is generally accepted that excitotoxic injury to neurons results from excessive inward currents of Ca^{2+} and Na^+ through glutamate-operated ion channels leading to increased $[Na^+]_i$, $[Cl^-]_i$ and $[H_2O]_i$, inducing cell swelling (Rothman, 1985; Choi, 1994; Limbrick et al., 1995). This increased Ca^{2+} is supplemented by release of Ca^{2+} from intracellular stores subsequent to metabotropic glutamate receptor activation, leading to intracellular Ca^{2+} overload (Mody and MacDonald, 1995). High intracellular Ca^{2+} concentration is potentially damaging because it can overstimulate Ca^{2+}-dependent enzymes such as phospholipases, proteases and endonucleases inducing degradative and apoptotic cell death pathways (Trump and Berezesky, 1995). The mechanisms involved in glutamatergic transmission lead to a number of abnormalities, either presynaptic or postsynaptic. This can be excitotoxic when there is enhanced presynaptic glutamate release, increased glutamate receptor sensitivity, etc. However, high extracellular glutamate is the prevalent trigger of excitotoxicity associated with neurological disorders like epilepsy (Bradford, 1995; Zauner and Bullock, 1995; Obrenovitch and Urenjak, 1997). In general, excitotoxicity is linked to over-stimulation of NMDA receptors in most areas of the brain, for example, in the hippocampus, and in particular, in the CA1 region. In addition to studies using cortical or hippocampal neurons, cerebellar neurons have also been extensively used as models of excitotoxicity.

Purkinje cells (PCs) present a unique cellular profile in both the cerebellum and the brain. Because they represent the only output cell of the cerebellar cortex, they play a vital

role in the normal function of the cerebellum. Interestingly, PCs are highly sensitive to a variety of pathological conditions that may involve glutamate-mediated 'excitotoxicity'. Mature PCs, however, lack functional NMDA receptors, the means by which Ca^{2+} enters the cell in classic hippocampal and cortical models of excitotoxicity. In PCs, glutamate predominantly mediates its effects, first via a rapid influx of Ca^{2+}through voltage-gated calcium channels, caused by the depolarization of the membrane after AMPA receptor activation (and through Ca^{2+}-permeable AMPA receptors themselves), and second, via a delayed release of Ca^{2+} from intracellular stores.

1.8. The Epilepsies

Epilepsy is a diverse disorder characterized by periodic and unpredictable seizures, caused by the recurrent hyper-synchronous discharges of cortical neurons that reside within a localized area of one cerebral hemisphere (focal seizures) or appear throughout the forebrain (generalized seizures) (McCormick and Contreras, 2001; Morimoto et al., 2004). It is estimated that 0.5-2% of the human population is afflicted with epilepsy (Morimoto et al., 2004). The abnormal activity associated with epilepsy generates plastic changes in cerebral circuitry that play a part in the pathogenesis of the disease. Left untreated, several types of epilepsies invariably result in characteristic behavioral syndromes, cognitive decline and neuromorphological alterations. These neuromorphological alterations are considered a result of glutamate excitotoxicity. Alterations in glutamate-mediated excitatory neurotransmission has long been a candidate as a central factor in the aetiology of at least some forms of human and experimental epilepsy.

1.8.1. Epileptic process

Most forms of epilepsy develop over a defined time period. That is, at some point in time, the brain functions normally, but either after a specific developmental sequence or in response to injury, a new state develops in which the neuronal circuits become hyperexcitable, leading to spontaneous recurrent seizures. This process is called epileptogenesis. Between the occurrence of injury and the emergence of epilepsy there is a latent period, which may range from weeks to several years.

A dynamic and evolving process takes place during this latent period and progressively alters neuronal excitability and establishes critical interconnections and perhaps requires critical structural changes before the first clinical seizure appears. The likelihood that epilepsy will develop after an epileptogenic insult depends on the area of the brain damaged, the type of damage, the age at which damage occurred and the genetic predisposition (Engel et al., 1997). The development of epilepsy is a progressive phenomenon, and it is reasonable to assume that this process continues after initiation of seizures, at least in some cases, perhaps making seizures more severe or more difficult to treat, or leading to other aberrations in neuronal function that give rise to interictal behavioral disturbances (Engel et al., 1997). During an epileptic seizure, large populations of neurons in selected portions of the central nervous system abandon their normal activity and begin to fire in periodic, synchronous discharges. This pathological synchronized activity is transmitted from one neuron to the next primarily through excitatory glutamatergic and inhibitory GABAergic synapses, although distinct sets of intrinsic membrane conductances in different neuronal populations also shape seizure-related hyperexcitability.

1.8.2. Animal models of epilepsy

In the past decades, many scientists have focused on the basic neurobiology behind seizures and epilepsy. As a prerequisite, animal models that correlate to specific types of seizures and epilepsy in humans have been developed. The investigation of such animal models and human studies suggested that both a decrease in GABAergic inhibition and an increase in glutamatergic excitation are critically involved in the cellular mechanisms underlying the initiation and spread of epileptic seizures and the processes that lead to epileptogenesis and, as a consequence, chronic epilepsy (Loscher, 1993; Bradford, 1995).

The seizure-inducing potential of focal electrical stimulation of the brain was recognized by numerous investigators in the 1950s and 1960s. It was observed that repeated administration of low levels of electrical current to the hippocampus induced progressive intensification of stimulus-induced seizure activity. The potential importance of this observation was recognized by Goddard (Goddard, 1967) and the progressive changes that resulted from repeated electrical stimulation were referred to as the 'kindling effect'. Kindling can be induced by repetitive electrical or chemical stimulation. Different modes of induction result in specific alterations which might reflect the variety of different epileptic syndromes.

1.8.3. Kindling as a model of epilepsy

Kindling refers to the phenomenon whereby repeated administration of an initially subconvulsive electrical stimulus results in the appearance and progressive intensification of convulsion activity (Goddard et al., 1969). Accordingly, progressive development of seizures can be induced by repeated systemic injection of a subconvulsive dose of pentylenetetrazole (PTZ), a blocker of the $GABA_A$ receptor-associated Cl^- channel (Pinel and Van Oot 1975; Squires et al., 1984).

Chemically induced kindling in rats was first described by Mason and Cooper (1972) using PTZ as convulsant. However, the question whether the mechanisms underlying electrically and chemically induced kindling are similar or different remains open. Once established, such decrease in seizure threshold appears to be permanent (Mason and Cooper, 1972). This sort of models, therefore, allows for evaluating neuronal plasticity associated with long-term alterations in neural excitability (Cain, 1989) and it has been associated with an increase in susceptibility of the glutamatergic systems (Schroeder et al., 1994) resulting in a enhancement of density of glutamate receptors (Schroeder et al., 1993; 1998).

1.8.4. Properties of kindling

Kindling is the repetition of stimuli that initially evoke afterdischarges (AD) but not seizures (Goddard, 1967; McNamara et al., 1993). Repetition of the same stimuli results in a gradual lengthening of the afterdischarges, eventually leading to progressively more severe seizures and finally convulsions. Once an animal has been kindled, the heightened response to the stimulus seems to be permanent and spontaneous seizures occur (McNamara et al., 1993). This experimental paradigm has been crucial to our understanding of the epileptogenic process. It is apparent from the definition; kindling simultaneously refers to both a procedure and a process. As a procedure kindling uses stimuli that must meet the criterion of being sufficient to evoke an AD and as a process, kindling is monitored in terms of changes in AD

and the accompanying convulsive response. The most theoretically significant property of kindling is its relative permanence. If a kindled animal is left unstimulated for an extended period of time (e.g. several months), there are substantial savings in the number of stimulations required to elicit a fully generalized seizure once stimulations are resumed. Often a fully generalized motor seizure is elicited by the second or third stimulation following the stimulation-free period (Goddard et al., 1969; Dennison et al., 1995). Whatever changes in the brain underlie kindling, they are enduring, if not permanent. This is also same for the kindling caused by PTZ injections (Becker et al., 1992, 1997).

Goddard et al., (1969) were the first to point out the similarities between kindling and the progressive development of human epilepsy after head injury. Many investigators have subsequently tried to understand the mechanisms of kindling to shed light on human epileptogenesis. Over the years the involvement of the various neurotransmitter systems has been worked out, as well as much of the intracellular mechanisms. Some of the research strives to understand the genetic mechanisms underlying kindling, through genetic expression (McNamara, 1995; Liang and Seyfried, 2001). Although a lot of work has gone into understanding the underlying mechanisms of kindling, much remains unclear. Kindling can be thought of as a model of 'use dependent' or 'activity dependent' plasticity, which results in alterations at both single cellular and neuronal network levels. Because of its long-lasting, transsynaptic, pathway-specific plastic changes in brain function, it has been proposed also as a model of neural plasticity and learning and memory.

1.8.5. Kindling and Learning

One of the most fascinating functions of the brain is the ability to store information provided by experience and to retrieve much of it at will. Without this ability, many of the cognitive functions cannot operate. Learning is the name given to the process by which new information is acquired by the nervous system, and memory to the mechanism of storage and/or retrieval of that information.

Kindling was also the first neuroplasticity phenomenon suggested to be useful for studying memory processes (Goddard and Douglas, 1975). It is possible that the separate cascade of mechanisms constituting kindling and learning intersect and share a number of common mechanisms. For this reason both kindling and learning are viewed as phenomena of neuroplasticity. Evidently, some of the research examines the effects of kindling on learning and memory (Leung and Shen, 1991; McNamara et al., 1993). Previous studies have shown that kindling of fully generalized seizures produces subsequent deficits in spatial tasks such as the radial-arm maze and Morris water maze (Leung and Shen, 1991; Gilbert et al., 1996; Leung et al., 1996). A dramatic learning impairment of active-avoidance acquisition in PTZ-kindled rats was reported (Becker et al., 1992). It has been shown that learning and memory deficits consequent to PTZ-kindling in rats have been differently affected by anticonvulsant agents (Becker et al., 1995).

In the process of understanding neuronal plasticity of learning, kindling was then surpassed by LTP as the neuroplasticity phenomena of choice for modeling memory processes, largely because LTP more closely approximated normal neural activity. This succession of neuroplasticity phenomena has made it important to understand how the various forms of plasticity like LTP and kindling are related (Cain, 1989). Much of the understanding provided by kindling has led to direct advances in the development of effective treatments for

the neurological disease of epilepsy. As such, the study of kindling continues to play one of the more important roles in neuroplasticty research.

1.9. Long-term changes of synaptic strength

Long-lasting synaptic gain changes in the CNS are generally thought to form the information storage mechanism involved in learning and memory. The distributed nature of the memory trace makes studying the physiology of memory difficult. Model phenomena that may utilize the same underlying mechanisms provide an alternative approach. Currently, the most heavily utilized models focus on activation-induced changes in synaptic function of known synaptic pathways. The most popular of these models are long-term potentiation (LTP) and long-term depression (LTD).

The LTP phenomenon is a model for the use-dependent and enduring increase in synaptic strengthening and is widely used because its general properties show a close correspondence with the theoretical assertions of information storage. LTP and its counterpart, LTD are persistent changes in synaptic efficacy induced by tetanic afferent stimulation and are considered models of the cellular mechanisms underlying learning and memory (Bliss and Lomo, 1973; Bliss and Collingridge, 1993). Thus, the regulation of synaptic strength by activity is bi-directional (Shouval et al., 2002). There is a wide agreement that both forms of plasticity, LTP and LTD, are triggered by different Ca^{2+} levels (Bear and Malenka, 1994). Calcium threshold mechanisms controlling LTD vs. LTP induction have been studied extensively in the hippocampus and neocortex. Early papers suggested a high calcium threshold for LTP and a lower threshold for LTD induction (Bienenstock et al., 1982; Bear et al., 1987; Lisman, 1989). This hypothesis, which is best known as the Bienenstock, Cooper and Munro (BCM) model (Bienenstock et al., 1982), was verified by experiments in which the postsynaptic calcium load was modified (Cummings et al., 1996; Hansel et al., 1997), or in which the calcium signal amplitude was measured using fluorescence imaging techniques (Hansel et al., 1997; Connor et al., 1999; Cormier et al., 2001). Experiments, in which LTP and LTD induction were achieved by photolytic release of calcium from caged calcium compounds, confirmed and extended the two threshold-model by showing that, in hippocampal pyramidal cells, LTP was best induced by brief, high amplitude calcium transients, whereas LTD was more easily obtained by prolonged, lower amplitude calcium signals (Yang et al., 1999; Zucker, 1999). Thus, large calcium rises in a narrow time-window are associated with LTP; whereas a smaller calcium rise in a wider time-window is required for the induction of LTD (Cho and Bashir, 2002). Moderate rises in Ca^{2+} should lead to predominant activation of phosphatases, while stronger increases would favour activation of kinases.

1.9.1. Long-term potentiation at hippocampal synapses

LTP is an experimental model of synaptic plasticity typically expressed as a long-lasting enhancement of the efficacy of synaptic transmission within activated pathways. This increased efficacy is reflected as an increase in the amplitude of an evoked post-synaptic response following intense, repetitive synaptic activation, and it can be induced by application of brief episodes of high frequency electrical stimulation (Bliss and Lomo, 1973; Bliss and Collingridge, 1993). LTP has been most thoroughly studied in the mammalian hippocampus,

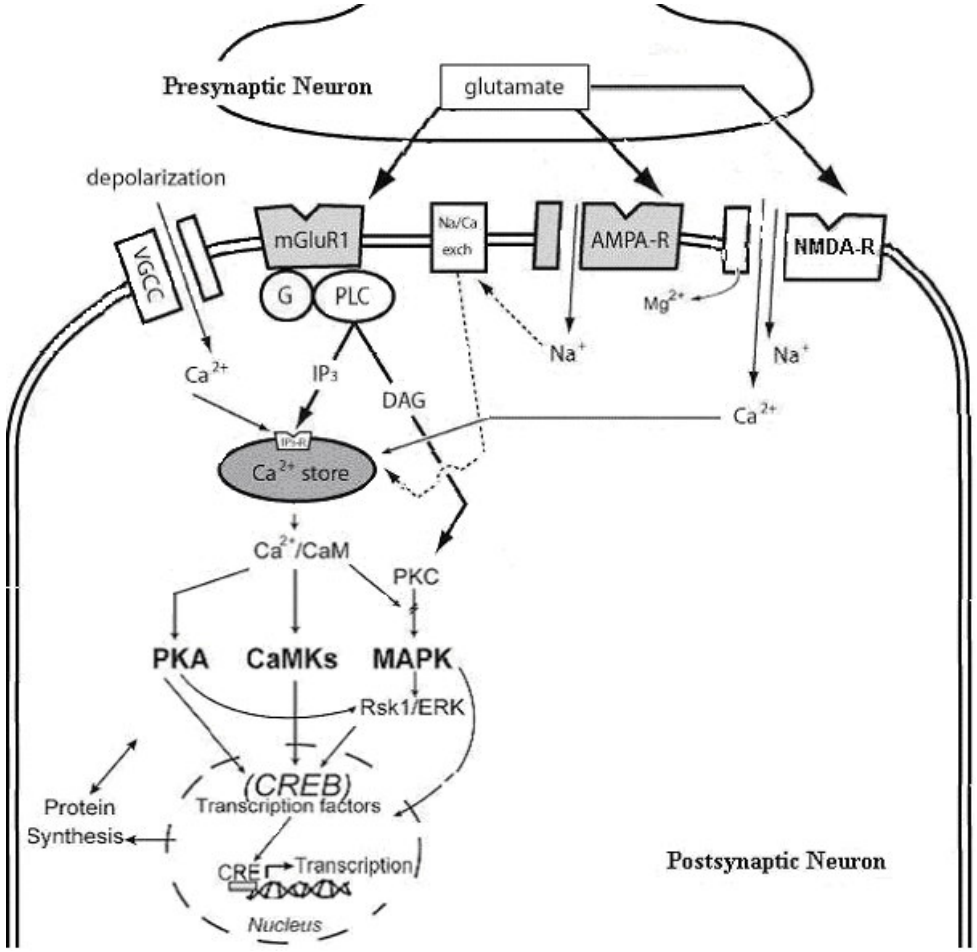

Figure 1.5. Model of CA1 hippocampal LTP induction. Tetanization of the presynaptic neuron causes glutamate to be released from the presynaptic bouton that acts on both AMPA and NMDA receptors. The NMDA receptor admits Ca^{2+} only after sufficient depolarization removes the Mg^{2+} block, the subsequent rise in $[Ca^{2+}]$ is the critical trigger for the induction of LTP. mGluRs facilitate induction and maintenance of LTP. Various sources of Ca^{2+} in the postsynaptic spine are shown. The activation of second messenger cascades acting through their respective protein kinases affect multiple cellular processes such as the phosphorylation of CREB and result in the enhancement of synaptic transmission. Long-term alterations in synaptic strength (i.e., L-LTP) are achieved through regulation of protein synthesis. G (G-protein); mGluR (metabotropic glutamate receptor); PLC (phospholipase C); VGCC (voltage gated Ca^{2+} channel). *(Adapted from Coesmans, Cerebellar Plasticity in Health and Disease, PhD thesis, Rotterdam 2004).*

an area of the brain that is especially important in the formation and/or retrieval of memory. In humans, functional imaging shows that the human hippocampus is activated during certain kinds of memory tasks, and that damage to the hippocampus results in an inability to form certain types of new memories. In rodents, hippocampal neurons fire action potentials only when an animal is in certain locations. Such "place cells" appear to encode spatial memories, an interpretation supported by the fact that hippocampal damage prevents rats from

developing proficiency in spatial learning tasks. Although many other brain areas are involved in the complex process of memory formation, storage, and retrieval, these observations have led many investigators to study this particular form of synaptic plasticity in the hippocampus. Pharmacological studies of LTP in the hippocampus have provided a molecular understanding of synaptic plastic processes, which are believed to be important for learning and memory in vertebrates.

The famous postulate of synaptic modification by the Canadian psychologist Donald Hebb suggests that the efficacy of synaptic transmission between two neurons strengthens when neuron A repeatedly or persistently takes part in firing neuron B. A cellular correlate of this 'strengthening of synaptic transmission' is provided by LTP, which, in many experimental paradigms, indeed requires correlated pre- and postsynaptic activity. The NMDA receptor, which is a glutamate receptor subtype, has key molecular properties that convey 'Hebbian' synaptic plasticity. NMDA receptors are permeable to Ca^{2+}, the critical trigger for the induction of LTP, and its permeability depends on both pre- and postsynaptic events. Opening of the channel for the influx of Ca^{2+} and Na^{+} ions requires the binding of the neurotransmitter glutamate, which is released from the presynaptic cell. In addition, coincident and sufficient depolarization of the postsynaptic cell is needed to disassociate Mg^{2+} from its binding site within the NMDA receptor channel before allowing Ca^{2+} and Na^{+} entry into the cell. The depolarization is largely mediated by the activation of AMPA receptors, which are co-localized with NMDA receptors on dendritic spines and are also activated by the binding of presynaptically released glutamate. However, it is the properties of the NMDA receptor that account for the induction of LTP through the influx of Ca^{2+} into the dendritic spine, which in turn activates Ca^{2+} dependent enzymes responsible for the induction of LTP (Figure 1.5). The synaptic activation of mGluRs is also needed for the induction of LTP and the role of mGluRs in the induction of LTP is fundamentally different from that of NMDA receptors (Bliss and Collingridge, 1993; Anwyl, 1999). The Hebbian properties of LTP can be fully explained by the behavior of the NMDA receptor voltage dependence, which allows for Ca^{2+} entry into the cell only when the presynaptic release of glutamate is coupled with postsynaptic depolarization. Accordingly, pharmacological studies have shown that this distinct form of LTP in the CA1 area is selectively prevented after the addition of competitive antagonists of the NMDA receptor site such as 2-amino-5-phosphonovaleric acid (APV). The same is true for general blockade of mGluRs, leading to the blockade of LTP expression. AMPARs are also important for the induction of LTP and are thought to involve an increase in functional AMPAR number. In cultured hippocampal neurons, enhanced miniature excitatory postsynaptic potentials following brief activation of NMDARs reflect a rapid insertion and increased clustering of AMPARs at the surface of the dendritic membrane (Lu et al., 2001).

Phases of LTP

LTP is often divided into two phases, an early, protein synthesis-independent phase (E-LTP) that lasts between one and five hours, and a late, protein synthesis -dependent phase (L-LTP) that lasts from days to months (Lu et al., 1999). Broadly, E-LTP produces a potentiation of a few hours duration. It does so by making the postsynaptic side of the synapse more sensitive to glutamate by adding additional AMPA receptors into the postsynaptic membrane. Conversely, L-LTP results in a pronounced strengthening of the postsynaptic response largely through the synthesis of new proteins. These proteins include glutamate receptors (e.g. AMPAR), transcription factors, and structural proteins that enhance existing synapses and form new connections. There is also considerable evidence that L-LTP prompts the

postsynaptic synthesis of a retrograde messenger that diffuses to the presynaptic cell increasing the probability of neurotransmitter vesicle release on subsequent stimuli. The E-LTP is transient, stable for up to 2-3 h, induced by second messenger cascades activated by Ca^{2+} influx, and maintained by activated kinases like calcium/calmodulin-dependent protein kinase II (CaMKII), PKC, protein kinase A (PKA), mitogen-activated protein kinase (MAPK), and tyrosine kinases. (see Malenka and Nicoll, 1999; Soderling and Derkach, 2000 for reviews). L-LTP begins gradually during the first 1-3 h and can last for 6-10 h in hippocampal slices in vitro or days to months in vivo. The early and late phases of LTP involve different signaling pathways. Unlike E-LTP, L-LTP requires gene transcription and protein synthesis, making it an attractive candidate for the molecular analog of long-term memory. The synthesis of gene products is driven by kinases which in turn activate transcription factors that mediate gene expression. cAMP response element binding protein-1 (CREB-1) is thought to be the primary transcription factor in the cascade of gene expression that leads to prolonged structural changes to the synapse enhancing its strength (Poser and Storm, 2001).

1.10. Long-term changes in cerebellar plasticity

The cerebellum is involved in motor learning and fine-tuning of motor coordination. Long-lasting synaptic gain changes in the cerebellar circuitry are generally thought to form the information storage mechanism involved in cerebellar motor learning. Various forms of synaptic and non-synaptic plasticity might interact to provide this 'cerebellar memory' (see Hansel et al., 2001 for review). Common wisdom among neuroscientists holds that cerebellar learning and synaptic plasticity are ''somehow'' different from their counterparts in other brain areas. This notion is largely based on the assumption that forms of cerebellar motor learning are mediated by LTD of synaptic transmission at parallel fiber (PF)–Purkinje cell (PC) synapses, whereas in other brain areas, such as the hippocampus, long-LTP is seen as the cellular learning correlate. In several aspects, cerebellar plasticity provides a mirror image of hippocampal plasticity: hippocampal LTP induction requires large calcium transients that promote the activation of CaMKII and PKC; whereas hippocampal LTD relies on protein phosphatase (PP) activation after lower calcium transients (note that most PPs are not directly calcium-activated). In cerebellar plasticity, the kinase/phosphatase dependencies are in inverse to the hippocampal ones. For example, LTD can be induced by paired PF and climbing fiber activity and requires a larger calcium transient than LTP induction (Coesmans et al., 2004).

1.10.1 Parallel and climbing fiber synapses to Purkinje cells

Two forms of postsynaptic plasticity have been described at the parallel fiber-Purkinje cell synapse, namely parallel fiber long-term depression (PF-LTD) and parallel fiber long-term potentiation (PF-LTP). Electrophysiologically, these effects can be recorded as a reduction or increase in the parallel fiber EPSC amplitude recorded under voltage-clamp in whole-cell patch-clamp experiments. In slices or in situ, PF-LTD is typically induced by pairing PF and climbing fiber (CF) stimulation at low frequencies. This paired stimulation results in an attenuation of the PF-PC synapse, typically to about 50-80% of its baseline synaptic strength (Ito et al., 1982; Ekerot and Kano, 1985). The most appropriate timing interval between PF and CF stimulation for the induction of PF-LTD is still under debate (see Bear and Linden, 2000; Ito, 2001 for reviews).

Three initial signals are required for PF-LTD induction: activation of AMPA and mGluR1 receptors by glutamate release from the PF, and depolarization of the PC by CF activity (Figure 1.6) (see Bear and Linden, 2000; Ito, 2001 for reviews). CF activation evokes a Ca^{2+} transient in PC dendrites, which is needed for PF-LTD induction (Sakurai, 1990; Konnerth et al., 1992; Shibuki and Okada, 1992). Glutamate release from the PF activates AMPA and mGluR1 receptors, both of which need to be activated in order for PF-LTD to occur (see Bear and Linden, 2000; Ito, 2001 for reviews). AMPA receptor activation causes a local depolarization in PF spines, leading to some influx of Ca^{2+} through VGCCs, which in turn enhances IP_3-mediated Ca^{2+} release from intracellular stores (Okubo et al., 2001). This local depolarization and Ca^{2+} rise combines with the massive complex spike-mediated depolarization to cause a supralinear Ca^{2+} influx (Wang et al., 2000). Furthermore, the AMPA receptor appears to contribute to PF-LTD induction through a specific effect of Na^{+} influx (Linden et al., 1993) which has not yet been fully elucidated (Bear and Linden, 2000). Activation of mGluR1 results in G-protein coupled activation of PLC and the consequent production of two second messengers: IP_3 and DAG. IP_3 binds to intracellular IP_3 receptors, resulting in a release of Ca^{2+} from intracellular stores (Takechi et al., 1998; Finch and Augustine, 1998). DAG and Ca^{2+} are both required for the activation of PKC, which is thought to underlie the final pathway for expression of PF-LTD. Inhibition of PKC blocks PF-LTD induction (Crépel and Jaillard, 1990; Linden and Connor, 1991; De Zeeuw et al., 1998), while exogenous PKC activation induces PF-LTD (Crépel and Krupa, 1988; Linden and Connor, 1991). PKC acts on AMPA receptors, phosphorylating a serine-residue on the carboxy-terminus of the GluR2 subunit (Matsuda et al., 1999; 2000), which has been shown to be critical for PF-LTD induction (Chung et al., 2003). This serine-residue is located in a PDZ domain-recognition site, by which AMPA receptors interact with the PDZ domain-containing proteins glutamate receptor interacting protein (GRIP) and protein interacting with c-kinase (PICK). GRIP stabilizes the AMPA receptor in the postsynaptic membrane, whereas binding to PICK triggers internalization (Xia et al., 2000). Phosphorylation of the PDZ domain by PKC disrupts the binding to GRIP (Matsuda et al., 1999; Chung et al., 2000) and promotes the binding to PICK (Chung et al., 2000), which finally results in clathrin-mediated endocytosis of the AMPA-receptor (Wang and Linden, 2000). Thus, internalization of AMPA receptors from the postsynaptic membrane enables the PC to reduce the contribution of selected PFs by reducing their synaptic weight.

In addition to PKC activation, a second pathway, mediated by release of nitric oxide (NO), has been implicated in PF-LTD induction (Figure 1.5). NO release from PF terminals and/or interneurons activates a postsynaptic cascade consisting of soluble guanylyl cyclase, cGMP and cGMP-dependent protein kinase G (PKG). PKG activation results in phosporylation of G-substrate, which inhibits protein phosphatases, thus increasing the levels of phosphoproteins generated by the action of protein kinases. It has been hypothesized that the mGluR1-PKC and the NO-cGMP cascade converge on the same molecular alteration, such as equilibrium between PKC and phosphatase activity (Daniel et al., 1998; Feil et al., 2003; Launey et al., 2004). Although many studies have shown an involvement of NO in PF-LTD, the reports on this pathway are rather contradictory (see Daniel et al., 1998; Bear and Linden, 2000 for reviews). It has recently been shown that NO might indeed be involved in LTD induction, but that it is not released from PF terminals, but rather from interneurons (Shin and Linden, 2005).

PF-PC synapses cannot only be depressed, but can also be potentiated (PF-LTP). The first reports on PF-LTP were merely side observations in studies focusing on PF-LTD (Sakurai, 1987; Sakurai, 1990; Hirano, 1990; Crépel and Jaillard, 1991; Shibuki and Okada,

1992). Systematic characterization revealed a presynaptic form of PF-LTP, induced by stimulating the PFs 120 times at 4-8 Hz. This form of PF-LTP depends on presynaptic influx of Ca^{2+}, activation of adenylyl cyclase I and the subsequent cAMP-dependent activation of protein kinase A (Salin et al., 1996; Storm et al., 1998).

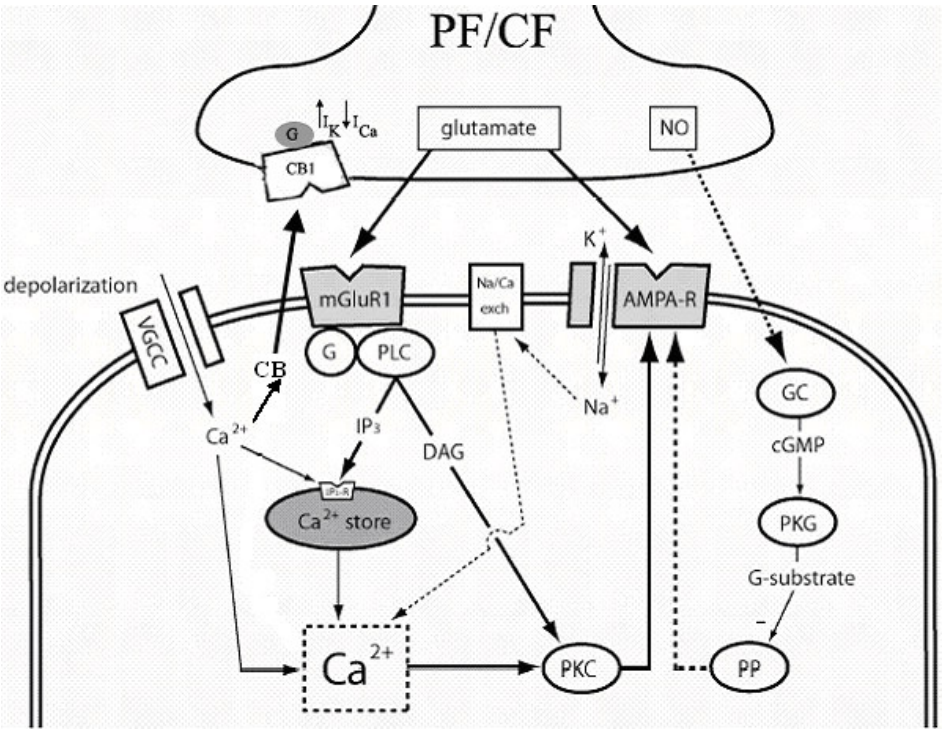

Figure 1.6: Model of the parallel and climbing fiber synapse to Purkinje cell. Phosphorylation of the AMPA receptor by PKC and its subsequent internalisation leads to a reduction of synaptic strength. Well-established mechanisms are indicated with solid lines whereas other suggested pathways are indicated using dotted lines. CB: Cannabinoid, CB1: Cannabinoid receptor type 1, NO: nitric oxide, VGCC: voltage-gated sodium channels, mGluR1: metabotropic glutamate receptor type 1, AMPA-R: AMPA receptor, G: G protein, PLC: phospholipase C, IP3: inositol 1,4,5-triphosphate, DAG: 1,2-diacylglycerol, PKC: protein kinase C, PKA: protein kinase A, PKG: protein kinase G, GC: guanylyl cyclase, PP: phosphatase, c-GMP: cyclic guanyl monophosphate, CF: climbing fiber, PF: parallel fiber. *(Adapted from Coesmans, Cerebellar Plasticity in Health and Disease, PhD thesis, Rotterdam 2004).*

However, this presynaptic PF-LTP is not an appropriate mechanism for reversing postsynaptic PF-LTD: a downregulation of postsynaptic AMPA receptors cannot be balanced by an increase of transmitter release alone, without saturating the synapse. A recently described, different form of PF-LTP (Lev-Ram et al., 2002; 2003), provides a much better candidate for such a reversal mechanism. This cAMP independent PF-LTP is induced by stimulating the PF alone for a prolonged period at a low frequency, and paired pulse facilitation (PPF) analysis suggests a postsynaptic expression site. PPF is a short-term enhancement in synaptic efficacy attributed to residual presynaptic Ca^{2+} facilitating more transmitter release (Atluri and Regehr, 1996; Salin et al., 1996). Changes in PPF during PF-LTP would argue that PF-LTP is expressed presynaptically, whereas unchanged PPF would

suggest a postsynaptic expression site. The molecular pathways of postsynaptic PF-LTP have been shown to differ from those of presynaptic PF-LTP (Lev-Ram et al., 2002), but remain to be characterized in detail. It was shown that the induction of postsynaptic PF-LTP requires lower calcium transients than PF-LTD induction (Coesmans et al., 2004) and that PF-LTP depends on the activation of protein phosphatases PP1, PP2A and PP2B rather than kinase activity (Belmeguenai and Hansel, 2005). Recently, Kakegawa and Yuzaki (2005) demonstrate that AMPA receptor delivery into synapses is involved in this form of PF-LTP. Kakegawa and Yuzaki present evidence that plasticity at cerebellar PF–PC synapses relies on GluR2 subunit trafficking. They demonstrate that, during cerebellar LTP, GluR2 subunits are delivered to synapses and that this GluR2 insertion is an activity-dependent process that involves NO-mediated *N*-ethylmaleimidesensitive factor (NSF) binding to GluR2. These findings provide evidence of activity-dependent GluR2 synapse delivery underlying LTP induction. The LTD induction cascade needs large calcium transient (resulting from paired PF and CF activity) promotes PKC activation, which phosphorylates GluR2 at Ser-880. GluR2 endocytosis requires binding of GluR2 to protein interacting with C-kinase1 (PICK1). The LTP cascade needs lower calcium transients (resulting from PF activity) promote phosphatase activation (only PP2B is directly calcium-regulated). GluR2 insertion requires GluR2 binding to NSF (see Hansel, 2005). It is obvious from the LTP data presented by Kakegawa and Yuzaki, and from previous work on GluR2 endocytosis in LTD (Wang and Linden, 2000) that GluR2 subunit trafficking mediates bidirectional cerebellar plasticity.

The contribution of CF signals to PF-LTD induction has long been appreciated. However, the CF-PC synapse itself was generally considered invariant, although the molecular machinery required for PF-LTD (mGluR1, AMPA-receptors, voltage-gated calcium channels, PKC) is also present at the CF-PC synapse. It was not until recently that LTD of the CF-PC synapse was observed after CF-only stimulation at 5 Hz for 30 seconds (Hansel and Linden, 2000; Weber et al., 2003). CF-EPSCs are typically reduced by approximately 20 %, and slow complex spike components, presumably reflecting Ca^{2+} conductances, are selectively reduced. This CF-LTD does not spread to neighboring PF synapses, is saturated after repeated tetanization, and does not show a change in paired-pulse ratio, suggesting a postsynaptic expression site (Hansel and Linden, 2000; Shen et al., 2002). Similar to PF-LTD, CF-LTD induction depends on an increase of postsynaptic Ca^{2+}, activation of mGluR1 and PKC (Hansel and Linden, 2000). The functional meaning of LTD at the CF-PC synapse is less apparent than at PF-PC synapses. CF-LTD might alter the probability of complex spike firing and/or the subsequent climbing fiber pause. In addition, the fact that CF-LTD results in a decrease of CF-evoked Ca^{2+} transients in the PC (Weber et al., 2003) might have several important functional implications (Hansel and Linden, 2000; Hansel et al., 2001; Weber et al., 2003). For instance, the CF-evoked Ca^{2+} transient has been shown to contribute to PF-LTD induction (Sakurai, 1990; Konnerth et al., 1992; Shibuki and Okada, 1992). Buffering of postsynaptic Ca^{2+} blocks PF-LTD induction (Sakurai, 1990; Linden and Connor, 1991; Konnerth et al., 1992; Shibuki and Okada, 1992), and facilitates postsynaptic PF-LTP induction (Lev-Ram et al., 2002). This suggests that the direction of the postsynaptic gain change might be determined by the amplitude of the postsynaptic Ca^{2+} transient: high Ca^{2+} for PF-LTD induction and low Ca^{2+} for PF-LTP induction (Coesmans et al., 2004). A reduction of CF-evoked Ca^{2+} transients might therefore affect normal PF-LTD induction. Similar Ca^{2+} threshold mechanisms controlling LTD vs. LTP induction have been described in the hippocampus and neocortex. However, in these areas the Ca^{2+} thresholds regulating LTD vs. LTP appear to operate in an inverse manner: low postsynaptic Ca^{2+} concentrations induce LTD, whereas a higher Ca^{2+} influx leads to LTP (see Bear and Linden, 2000).

1.10.2. mGluRs in cerebellar plasticity

mGluR1 has been shown to play a role in several forms of long-term changes in synaptic transmission. First it was shown that induction of PF-LTD required activation of metabotropic glutamate receptors (Kano and Kato, 1987; Linden et al., 1991), which were later specified as mGluR1 (Shigemoto et al., 1994). Activation of mGluR1 results in production of the second messengers DAG and IP_3, both of which are thought to be essential for PF-LTD induction (Figure 1.5). DAG activates PKC directly, whereas IP_3 is thought to contribute to PKC activation through release of Ca^{2+} from intracellular stores (see Bear and Linden, 2000; Ito, 2001 for reviews). The contribution of mGluR1 to PF-LTD was further confirmed and extended by studies in mGluR1-knockout mice (Aiba et al., 1994; Conquet et al., 1994). The fact that these mGluR1-lacking mice showed no PF-LTD made them interesting for studying the link between PF-LTD and cerebellar motor learning. However, several major problems complicated the behavioral analysis of these mice. First, mGluR1 is normally present in various cell types in the CNS, so effects on PF-LTD and motor behavior that were reported in mGluR1 knockout mice cannot be reliably linked to the specific absence of mGluR1 at the PF-PC synapse. Second, mGluR1 knockout mice show characteristic cerebellar symptoms such as ataxic gait, intention tremor, and motor coordination problems (Aiba et al., 1994; Conquet et al., 1994), which complicates specifically testing their motor learning behavior. Third, these mice have PCs that fail to undergo the normal developmental conversion from multiple to mono climbing fiber innervation in early postnatal life (Kano et al., 1997; Levenes et al., 1997). The cell-specificity issue was largely resolved by the fact that Purkinje cell-specific reintroduction of mGluR1 in these mice rescues PF-LTD as well as the developmental and behavioral phenotype (Ichise et al., 2000). However, the fact remains that mGluR1-knockout mice have cerebellar developmental abnormalities as well as severe ataxia, which makes linking PF-LTD to motor learning problematic in these mice. Experiments in which mGluR1 was acutely blocked by anti-mGluR1 antibodies in normally developed wild-type mice, showed that the mGluR1 block by itself leads to reversible ataxia (Sillevis Smitt et al., 2000) presumably by directly affecting basal PC firing rate (Coesmans et al., 2003). In addition to its contribution to PF-PC synaptic transmission, mGluR1 is also present at CF-PC synapses, as well as the rest of the molecular machinery thought to be necessary for PF-LTD induction. Like PF-LTD, CF-LTD induction has also been shown to be dependent on postsynaptic Ca^{2+} elevation, mGluR1 activation, and activation of PKC (Hansel and Linden, 2000). This suggests that mGluR1 might play a similar role in the induction of CF-LTD as it does in PF-LTD.

In the cerebellum, blocking mGluR1 function has not only been described to affect Purkinje neuronal spontaneous action potential firing (Yamakawa and Hirano, 1999; Coesmans et al., 2003; however see Neale et al., 2001) and induction of PF-LTD (Linden et al., 1991; Shigemoto et al., 1994) and CF-LTD (Hansel and Linden, 2000), a chronic mGluR1 block has also been reported to affect the development (Catania et al., 2001) and survival of Purkinje cells (Coesmans et al., 2003).

Group I mGluRs are reported to influence both presynaptic and postsynaptic functions. For example, activation of mGluR1 in PCs and hippocampal neurons causes a reversible suppression of excitatory transmission that is presumably of presynaptic origin. mGluR1 activation has been reported to have a short-term retrograde inhibitory effect on presynaptic transmitter release from the PF and CF (Glaum et al., 1992). This mGluR1-mediated retrograde inhibition uses endogenous cannabinoids as a retrograde messenger (Maejima et al., 2001; Brown et al., 2003). These findings suggest that mGluR1 does not only play a role

in PC excitability (Netzeband et al., 1997; Yamakawa and Hirano, 1999), but also dynamically modulates synaptic strength and fine-tunes the properties of synaptic integration in the short term (Brown et al., 2003).

1.12. Endocannabinoids in synaptic signaling

Exogenous cannabinoid ligands have been shown to be involved in physiological processes as diverse as food intake, anxiety, learning, and memory, but much less is known about the function of endogenous cannabionoids. Cannabinoid receptors (CB) consist of two subtypes, CB1 and CB2 (Matsuda et al., 1990; Munro et al., 1993). While the CB2 is expressed mostly in the immune system, CB1 is rich in various regions of the CNS, including the cerebellum (Egertova and Elphick 2000; Herkenham et al., 1990). Derivatives of the cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC), the primary psychoactive component of the Cannabis plant, are powerful modulators of synaptic transmission. In the brain, the endocannabinoids anandamide and 2-arachidonylglycerol (2-AG) function as modulators of transmitter release by binding to G-protein-coupled CB receptors, which are located on synaptic terminals (Figure 1.5). CB1 receptor activation can both inhibit adenylyl cyclases and modify K^+- and Ca^+-selective channels (Ameri, 1999).

So far endocannabinoids have been found to act primarily or exclusively as retrograde messengers in the mammalian brain. This special adaptation could be due to the fact that, following synthesis from membranous, lipidic precursors (Di Marzo et al., 1998; Piomelli et al., 2000), they are not stored in vesicles like the other transmitters, but rather released presumably by diffusing across membranes. Moreover, they have recently been discovered to play a prominent role both in short-term and in long-term synaptic plasticity, as well as to directly control the rate of firing of presynaptic cells. Cannabinoids cause retrograde, short-term inhibitory actions on afferent GABAergic and glutamatergic transmission, which are respectively known as depolarization-induced suppression of inhibition, or DSI, and depolarization-induced suppression of excitation, or DSE. DSI/DSE are two closely related forms of short-term plasticity, which share the same modes of induction, the same type of retrograde messengers (i.e. endocannabinoids) and similar mechanisms of expression. The activation of cannabinoid receptors induces a variety of actions on neural functions (Di Marzo et al., 1998; Felder and Glass, 1998). Cannabinoid agonists inhibit adenylate cyclase (Howlett and Flemming, 1984) and N- and P/Q-type Ca^{2+} channels, and activate inwardly rectifying potassium channels (Mackie and Hille, 1992; Fleder et al., 1995; Mackie et al., 1995; Twitchell et al., 1997). They also suppress the release of neurotransmitters, such as glutamate, GABA, acetylcholine, and noradrenaline, probably via inhibition of presynaptic Ca^{2+} channels and upregulation of K^+ channels (Shen et al., 1996; Katona et al., 1999; Hoffman and Lupica, 2000).

The induction of cerebellar LTD is similar in many ways to a transient form of retrograde inhibition that also occurs at PF synapses (Brenowitz and Regehr, 2005). Stimulation of PFs causes PCs to release endocannabinoids that activate type 1 cannabinoid receptors (CB1Rs) on PF boutons, thereby inhibiting neurotransmitter release for many seconds (Brown et al., 2003). Activation of mGluR1 and the elevation of postsynaptic calcium trigger endocannabinoid release from PCs (Brenowitz and Regehr, 2005; Maejima et al., 2001). Similar to the induction of cerebellar LTD, the associative release of endocannabinoids is more pronounced when PF stimulation precedes CF stimulation (Brenowitz and Regehr, 2005). Thus, both synaptically evoked endocannabinoid release from PCs and LTD induction are mGluR1 dependent and involve the associative interaction of PFs and the CF. These

similarities raise the possibility that endocannabinoids may play a role in the induction of cerebellar LTD. Furthermore, endocannabinoids are involved in the induction of LTD at other synapses in the brain (Chevaleyre and Castillo, 2003; Freund et al., 2003; Gerdeman et al., 2002; Marsicano et al., 2002; Robbe et al., 2002; Sjostrom et al., 2003).

1.13. Scope of this thesis

The primary aim of this research was to investigate the function of mGluRs and endocannabinoid signaling in the modulation of synaptic transmission. mGluRs are distributed throughout the central nervous system and play important roles in the modulation of synaptic transmission. The mGluRs regulate LTP/LTD and learning and memory in hippocampus. In addition, mGluR1 has been shown to play a role in long-term changes in synaptic transmission of cerebellum and motor learning. LTD at cerebellar PF-PC synapses requires activation of group I mGluRs. The introduction of this thesis started with a description of the structure and functions of the hippocampus and the cerebellum (*paragraphs 1.2 and 1.3*). An attempt was made to define the morphological and electrophysiological properties of the hippocampus and the cerebellum.

This thesis addresses the function of group I mGluRs during the initiation of synaptic plasticity and memory formation. It is widely accepted that excitatory amino acid transmitters such as glutamate are involved in the initiation of seizures and their propagation. In PTZ-kindled animals a significant increase in mGluR binding was seen. Group I mGluRs (mGluR1 and mGluR5) were reported to exert anticonculsive and neuroprotective efficacy by number of studies. Motivated by the growing evidence of the anticonvulsive and neuroprotective efficacy of group I mGluRs, special attention was given to group I metabotropic glutamate receptor antagonists. In the present study, efforts were made to see whether inhibition of group I mGluRs given in the course of PTZ-kindling would interfere with kindled seizure development and shuttle-box learning in rats. It was checked additionally whether inhibition of group I mGluRs after PTZ-kindling would improve shuttle-box learning in rats. The effect of mGluR antagonists on learning is discussed in *chapter 2*. Efforts were also made to find out whether inhibition of group I mGluRs either in the course of PTZ-kindling or shortly before evoking an ex-vivo LTP might counterbalance putative kindling-induced aberrations in LTP. The effect of mGluR antagonists on the induction and maintenance of LTP is discussed in *chapter 3.*

Group I mGluRs are reported to influence both presynaptic and postsynaptic functions. For example, activation of mGluR1 in PCs and hippocampal neurons causes a reversible suppression of excitatory transmission that is presumably of presynaptic origin. Activation of postsynaptic mGluR1 in PCs causes presynaptic inhibition that is mediated by cannabinoid receptors. At cerebellar and hippocampal synapses, endocannabinoids can bind to presynaptically located CB1 receptors. Endocannabinoid signaling has been demonstrated to mediate DSE at CF and PF synapses onto cerebellar PCs. We studied whether CF-evoked cannabinoid signaling additionally suppresses a presynaptic form of LTP at PF synapses. In *chapters 4* and *5* we describe experiments that investigate the requirements for induction of PF-LTP in rat cerebellar slices. In *chapter 6* the experimental data presented in chapters 2, 3, 4 and 5 are summarised and discussed.

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Chapter 2

Group I metabotropic glutamate receptors interfere in different ways with pentylenetetrazole seizures, kindling, and kindling-related learning deficits.

Abstract

LY 367385 (mGluR1) and MPEP (mGluR5), which are group I metabotropic glutamate receptor (mGluR) antagonists, were used to investigate their effects on pentylenetetrazole (PTZ) seizures, kindling, and kindling-related learning deficits. Both substances showed anticonvulsant efficacy against seizures induced by lower doses of PTZ (40 mg/kg), but they were ineffective in counteracting seizures evoked by higher PTZ doses. When these substances were given in the course of kindling induction, LY significantly depressed the progression of kindled seizure severity. In contrast, MPEP was ineffective in this experiment. Treatment with either LY or MPEP did not modify the reaction to challenge dose of PTZ. Kindling results in a worsening of shuttle-box learning. LY improved shuttle-box learning when administered in the course of kindling development or when given prior to the learning experiment. This suggests protective and restorative effectiveness. In contrast, MPEP was only effective on the learning performance of kindled rats when given prior to the shuttle-box experiment, which demonstrates restorative effectiveness. Kindling is associated with an increase in glutamate binding. LY counteracted this increase whereas MPEP was ineffective. It was concluded that mGluR1 and mGluR5 play a specific role in the convulsive component of kindling. The beneficial action of the antagonists on kindling-induced impairments in shuttle-box learning may be associated with their effect on glutamatergic synaptic activity.

Introduction

Kindling is a validated model in epilepsy research. This model permits investigations into the convulsive component of epilepsy, epilepsy-related alterations in behavior as well as neuromorphological, neurophysiological, and neurochemical determinants of this complex disease. Kindling can be induced by repetitive electrical or chemical stimulation. Different modes of induction result in specific alterations which might reflect the variety of different epileptic syndromes.

Repeated injections with the convulsant pentylenetetrazole (PTZ) lead to an intensification in seizure severity and seizure duration. In rats which acquired the kindling syndrome we found a dramatic impairment in shuttle-box learning which is still persistent 4 weeks after kindling completion (Becker et al., 1992). Neurophysiological investigations revealed a greater population spike potentiation induced by moderate tetanization of the perforant pathway (Becker et al., 1997a; Ruethrich et al., 1996). These alterations were accompanied by a number of significant dynamic changes in the glutamatergic neurotransmission system, e.g., aspartate release and glutamate binding (Schroeder et al., 1993, 1999). Glutamate receptors are crucial for fast synaptic signal transduction in the central nervous system and play an important role in processes of neuronal plasticity such as long-term potentiation, learning and memory, as well as neurodegenerative diseases including ischemia and seizure-related brain damage (Aarts and Tymianski 2003; Bliss and Collingridge 1993; Bordi and Ugolini 1999; Meldrum 1994). In PTZ-kindled animals a significant increase in quisqualate and kainate binding sites was found (Schroeder et al., 1999). Glutamate binding in the hippocampus was still increased by about 50% compared to controls 9 weeks after kindling completion (Schroeder et al., 1998b).This increase in glutamate binding is associated with an increase in metabotropic glutamate receptor (mGluR) binding. Most prominent was an increase in group I mGluR (mGluR1 and mGluR5) binding (Schroeder et al., 1999). This group of glutamate receptors was reported to exert anticonvulsive and neuroprotective efficacy (Aarts and Tymianski 2003; Bordi and Ugolini

1999; Chapman et al., 2000; Doherty and Dingledine 2002; Flor et al., 2002; Opitz et al., 1995; Riedel et al., 1996). To study the role of these receptors in PTZ kindling and kindling-related learning deficits we injected the mGluR1 antagonist LY 367385 or the mGluR5 antagonist MPEP in the course of kindling. After kindling completion, the animals were tested for their learning performance in a two-way active avoidance shuttle-box. In a second attempt kindled animals were injected with the substance prior to each learning session to investigate possible restorative effects.

Materials and methods

Ethical approval was sought according to the requirements of the National Act on the Use of Experimental Animals (Germany).

Animals

Experiments were carried out with male Wistar rats [Shoe:Wist(Shoe), DIMED Schoenwalde GmbH] aged 8 weeks at the beginning of the kindling experiments. The rats were kept under controlled laboratory conditions (light regime of 12 h light/12 h dark, light on at 6:00 a.m., temperature 20±2°C, air humidity 55–60%). They had free access to commercial rat pellets (Altromin 1326) and tap water. The rats were housed in groups of five per cage.

Surgery

LY 367385 and MPEP were injected intracerebroventricularly (i.c.v.) via a microcannula chronically implanted into the right lateral ventricle. For implantation, each rat was deeply anesthetized with Nembutal (40 mg/kg, i.p.) and a cannula (outer diameter 0.6 mm) was inserted according to the coordinates AP 0.25 mm, lateral 1.6 mm, ventral 4.0 mm (related to bregma) and bregma 1.0 mm above lambda (Paxinos and Watson 1997). The cannula was fixed to the skull with dental cement and was fixed with two screws to ensure stability. The behavioral experiments were carried out after a recovery period of 1 week.

Experimental protocol

Substances

LY 367385 [(S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid] and MPEP hydrochloride [2-Methyl-6-(phenylethynyl)pyridine hydrochloride] (Biotrend-Tocris, Cologne, Germany) were described as selective antagonists at the mGluR1 or mGluR5 (Bortolotto et al., 1999; Bruno et al., 1999; Gasparini et al., 2001; Kuhn et al., 2002; Lee et al., 2002; Mills et al., 2002). The substances and PTZ (Roth, Karlsruhe, Germany) were freshly dissolved in sterile physiological saline each time before use.

LY and MPEP were injected i.c.v. over a period of 30 s. Injection volume was 5 µl per animal. The injectors were withdrawn 20 s after the injection. Control animals were given saline (sal, i.c.v.).

PTZ was intraperitoneally (i.p.) given and injection volume was 1 ml/100 g.

Acute effects of LY and MPEP

In the first experiment, the acute effectiveness of LY (0.40 µMol) and MPEP (0.06 µMol) on seizures induced by a single injection of different doses of PTZ (40, 50 and 60 mg/kg) was compared in different groups of animals. The dosage for the LY experiments was calculated according to the concentrations used in vitro (Mannaioni et al., 2001). The dosage of MPEP was the same as employed by Balschun and Wetzel (2002) for examining the effects of the drug on spatial alteration learning and long-term potentiation (LTP) in freely-moving animals. Resultant seizures were scored as described below for a period of 20 min.

Per group 8–9 animals were used.

Kindling and learning experiment

In the second experiment, the same doses of LY and MPEP were used to test the development of kindling and related learning deficits in the shuttle-box. Four groups of animals were maintained to test the effect of LY or MPEP, i.e., sal/sal, antagonist/sal, sal/PTZ, antagonist/PTZ (the first substance was given prior to PTZ or sal in the course of kindling induction).

For the kindling experiment a dose of 37.5 mg/kg body weight PTZ (ED_{16} related to clonic seizures, this dose was determined in a separate groups of rats) was injected once every 48 h. After each injection the convulsive behavior was observed for 20 min. The resultant seizures were classified as follows:

Stage 0: no response
Stage 1: ear and facial twitching
Stage 2: myoclonic jerks without rearing
Stage 3: myoclonic jerks, rearing
Stage 4: turn over into side position, clonic-tonic seizures
Stage 5: turn over into back position, generalized clonic-tonic seizures

In total, rats received 13 kindling injections. Control animals received the same number of saline injections. LY, MPEP or sal were given i.c.v., 30 min before giving saline or PTZ.

Testing of learning performance in the shuttle-box was started twenty-four hours after kindling completion.

The apparatus was enclosed in a sound-attenuating box ventilated by an extractor fan. The automatic shuttle-box (0.25×0.25×0.6 m) was divided into two identical compartments separated by a 5 cm hurdle. The conditioned stimuli were light (40 W bulbs located on the central ceiling of each compartment) and a sound produced by a buzzer. The unconditioned stimulus was an electric foot shock (maximum 0.8 mA, adjusted according to the rat's individual susceptibility and below vocalization threshold, calibration of shock intensity was carried out in the course of each session, 50 Hz, impulse widths 10 ms, pulsatile direct current) delivered through stainless steel rods covering the floor. The conditioned stimuli and the unconditioned stimulus interval was 4 s. One trial was limited to 20 s if the animal failed to react before. If the animal did not avoid or escape from the shock as described, the trial was

repeated after a 30 s interval. Intertrial intervals lasted, randomized, for 15–45 s. There was a difference in the performance within the animals treated with LY and MPEP. Each session consisted of 20 trials (avoidance reactions or escape reactions) for the animals treated with LY and 30 trials for the animals treated with MPEP. In pre-tests it was found that both batches of rats differed in their learning ability. The trials were repeated on four consecutive days. Sessions were performed during the light part of the 12:12 h cycles at about the same time ± 1 h. Prior to the first session, the rats were allowed to explore the box for 5 min, and on the following days 1 min was provided.

The shuttle-box was connected to a computer that controlled the training schedule and scored the number of escapes (4 s> reaction time <20 s), conditioned reactions (reaction time <4 s), interval activity, and reaction times for further evaluation.

The number of animals used is described in the legends to the illustrations.

Reaction to challenge

One characteristic feature of kindling is the lowered seizure threshold. To test the effect of PTZ on kindling success, the rats received a challenge dose of PTZ (defined as 5 mg/kg PTZ less than the dose used in the kindling experiment, i.e., 32.5 mg/kg) which did not evoke clonic seizures in controls after completion of the learning experiment, i.e., 8 days after finishing the kindling procedure.

Effects of LY and MPEP on shuttle-box performance in kindled rats

Rats were kindled with PTZ injections as described above. Only rats which acquired the kindling syndrome were implanted with the cannula as described in the surgery and were allowed to recover. Rats were defined as having obtained the kindling syndrome when they showed stage 4/5 seizures for three consecutive times and after having received 13 PTZ injections. Seven days after the completion of kindling, learning performance was tested in the shuttle-box as described above. The rats were given LY and MPEP injections, 30 min before they were introduced into the shuttle-box. The control rats underwent the same test after the saline injection. The following groups were tested: sal/sal, sal/antagonist, PTZ/sal, PTZ/antagonist (first injection was given during kindling induction, second injection prior to the learning session).

Binding assay

One week after the final PTZ or MPEP or LY injection, rats were decapitated, the hippocampi were dissected out and crude membrane fractions were prepared (Zukin et al., 1974). At this time PTZ was expected to have been cleared (Grecksch et al., 1997).

^{3}H-L-glutamate (50 nM, specific activity: 832.5 GBq/mmol, PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany) binding was assayed as described by (Schroeder et al., 1998a). For the binding assay the membranes were incubated in 30 mM Tris-HCl buffer (pH 7.4) containing 2.5 mM $CaCl_2$ for 40 min at 37°C. The unspecific binding was

determined by adding 100 µM unlabelled L-glutamate to parallel probes (total glutamate binding sites).

The metabotropic glutamate 5 binding sites were determined using 1 nM (^{3}H)-MPEP (specific activity: 900 GBq/mmol, Biotrend-Tocris) and 10 µM unlabelled MPEP for unspecific binding.

^{3}H-trans-ACPD ((+)-1-aminocyclopentane-trans-1,3-dicarboxylic acid) (specific activity: 1.2 TBq/mmol, Biotrend-Tocris) were incubated alone or with 10 µM trans-ACPD as well as with 10 µM AIDA ((RS)-1-aminoindan-1,5-dicarboxylic acid) demonstrating the metabotropic glutamate I/II and mGluR1 binding sites, respectively. In all cases the same incubation procedure described for glutamate was used.

The reaction was terminated by rapid filtration under reduced pressure through 0.1% PEI treated GF 10 glass-fiber filters using an Inotech harvester (Berthold, Bad Wildbad, Germany). Filters were washed with buffer and taken for liquid scintillation counting in a solvent containing toluene. The data were determined as fmol bound radioligand per mg protein.

Statistical analysis

The statistical analysis (SPSS+ software) for acute effects of PTZ was based on the Mann-Whitney *U*-test. Reaction to challenge was analyzed by two-way ANOVA with pretreatment (sal vs. PTZ) and treatment (sal vs. LY and MPEP, respectively) being the independent variables. Group differences were calculated with one-way ANOVA and Bonferroni post hoc test. The statistical analysis of binding data was carried out using the Mann-Whitney *U*-test.

To compare kindling development and shuttle-box performance, repeated measure ANOVA was employed. A p value ≤ 0.05 was considered significant.

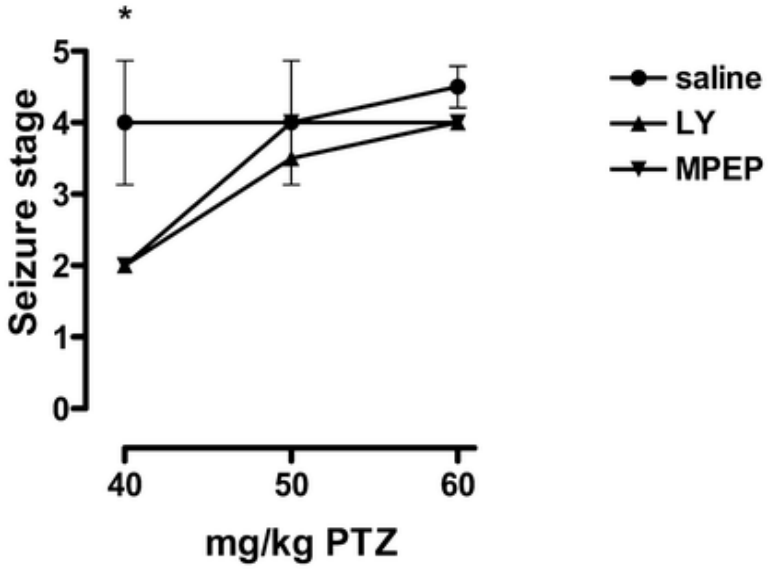

Figure 2.1. The effect of LY and MPEP (0.40 µMol and 0.06 µMol) on severity of seizures induced by a single injection of different doses of pentylenetetrazol (40, 50, and 60 mg/kg). Per group 8–9 animals were used. Median seizure score ± standard error of the median, *p<0.05. *sal* saline, *PTZ* pentylenetetrazol

Results

Acute effects of LY and MPEP on PTZ seizures

As shown in Figure 2.1, LY ($U_{9,9} = 18$, z = –2.153, $p = 0.031$) and MPEP ($U_{9,9} = 16.5$, z = -2.276, $p = 0.023$) exerted anticonvulsant efficacy against seizures induced by 40 mg/kg PTZ. When the convulsant was administered in higher doses the seizure scores from the control group and the groups treated with the antagonists did not differ (50 mg/kg PTZ: LY $U_{8,8} = 25$, z = –0.764, $p = 0.445$, MPEP $U_{8,8} = 32$, z = –0.404, $p = 0.686$; 60 mg/kg PTZ: LY $U_{8,9} = 32$, z = –0.432, $p = 0.666$, MPEP $U_{8,8} = 18$, z = –1.585, $p = 0.113$). This indicates that suppression of PTZ-induced seizures is mediated via both mGluR1 and mGluR5 receptors.

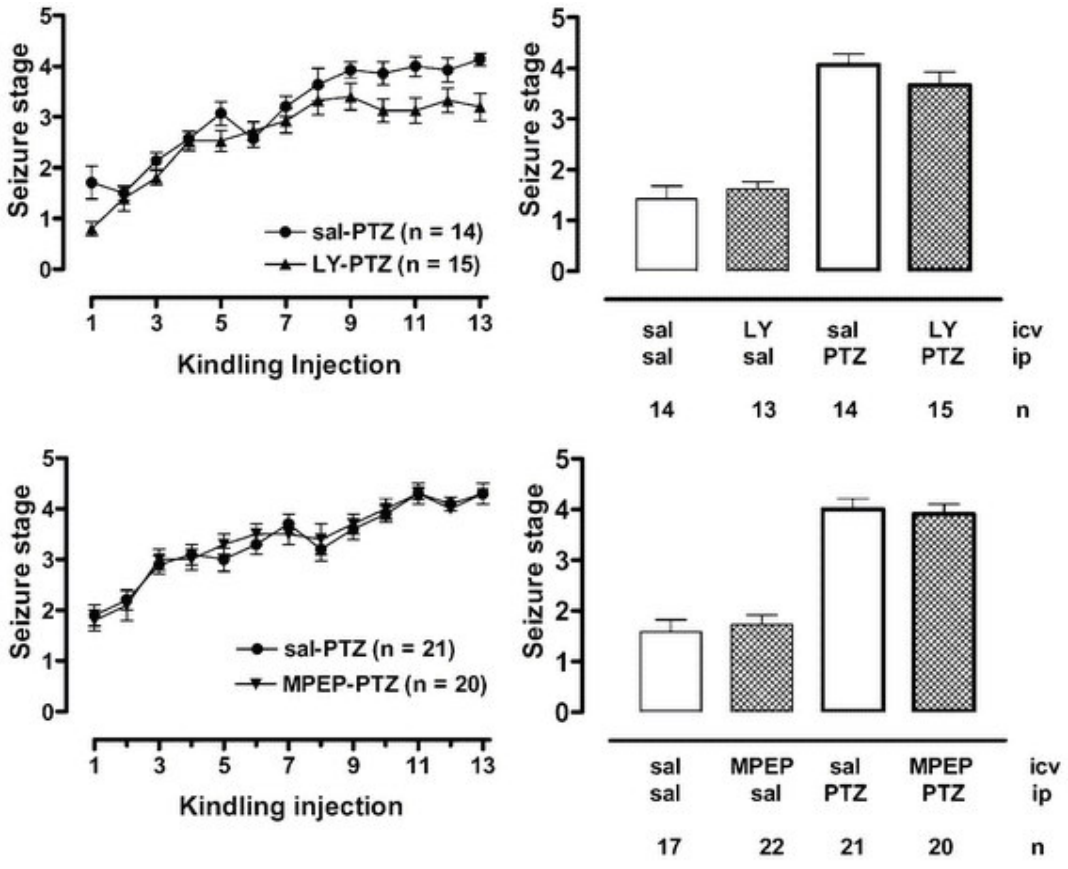

Figure 2.2. The effect of LY and MPEP(0.40 µMol and 0.06 µMol) on severity of seizures in the course of kindling development (*left panels*) and response to challenge (*right panels*). Mean seizure score ± standard error of the mean

Effects of LY and MPEP on kindling development

In animals which were repetitively treated with PTZ, seizure scores constantly increased reflecting an increase in central excitability (Figure 2.2, left panels). Pretreatment with LY did significantly depress the progression of kindled seizure severity ($F_{1,27} = 6.251$, $p = 0.019$) demonstrating anti-kindling efficacy mediated via mGluR1 blockade. In contrast, treatment

with the mGluR5 antagonist had no effect on the development of kindled seizures ($F_{1,39}$ = 0.071, p = 0.79).

Reaction to challenge

One characteristic of the kindled state is enhanced excitability. To test this, the animals received a challenge dose of PTZ 8 days after kindling completion. There was a significant effect of pretreatment (saline vs. PTZ) but not of treatment during kindling development (saline vs. LY or MPEP). As shown in Figure 2.2 (right panels), all the animals from the kindled group had higher scores compared with control animals. Treatment with either LY or MPEP did not modify the reaction to challenge, neither in the control groups nor in the kindled rats. Although the mGluR1 antagonist showed efficacy against acute PTZ seizures and, moreover, it retarded the progression of kindled seizure severity, the kindled state was obtained.

LY
Pretreatment, $F_{1,52} = 96.492$, $p < 0.001$
Treatment, $F_{1,52} = 0.516$, $p = 0.476$
Pretreatment × treatment interaction, $F_{1,52} = 1.319$, $p = 0.206$

MPEP
Pretreatment, $F1,76=112.123$, $p<0.001$
Treatment, $F1,76=0.001$, $p=0.98$
Pretreatment × treatment interaction, $F1,76=0.456$, $p=0.502$

Shuttle-box learning (antagonist given during kindling)

As shown in the Figure 2.3, the number of conditioned reactions in the saline injected control rats constantly increased whereas learning performance in kindled rats was poor in both experiments. Control rats treated with LY and MPEP also showed an increase in the number of conditioned reactions day by day. Kindled rats treated with LY showed a significant improvement in learning to the same level as that of control rats suggesting a protective effect. In contrast, kindled rats treated with MPEP showed the same poor learning performance as that in kindled rats treated with saline.

LY
Day, $F_{1,47} = 177.19$, $p < 0.001$
Day × pretreatment (sal or PTZ), $F_{1,47} = 10.967$, $p = 0.002$
Day × treatment (sal or antagonist), $F_{1,47} = 26.118$, $p = 1.1$
Day × pretreatment × treatment, $F_{1,47} = 16.362$, $p < 0.001$

ANOVA
Day × treatment, $F_{3,47} = 8.41$, $p < 0.001$
Sal/sal vs. LY/sal, $p = 1$
Sal/sal vs. sal/PTZ, $p = 0.006$
Sal/PTZ vs. LY/PTZ, $p = 0.048$
Sal/sal vs. LY/PTZ, $p = 1$

MPEP
Day, $F_{1,76} = 228.228$, $p < 0.001$
Day × pretreatment, $F_{1,76} = 17.68$, $p < 0.001$
Day × treatment, $F_{1,76} = 0.939$, $p = 0.336$
Day × pretreatment × treatment, $F_{1,76} = 6.83$, $p = 0.01$

ANOVA
Day × treatment, $F_{3,76} = 6.509$, $p = 0.001$
Sal/sal vs. MPEP/sal, $p = 1$
Sal/sal vs. sal/PTZ, $p = 0.039$
Sal/PTZ vs. MPEP/PTZ, $p = 0.06$
Sal/sal vs. MPEP/PTZ, $p = 1$

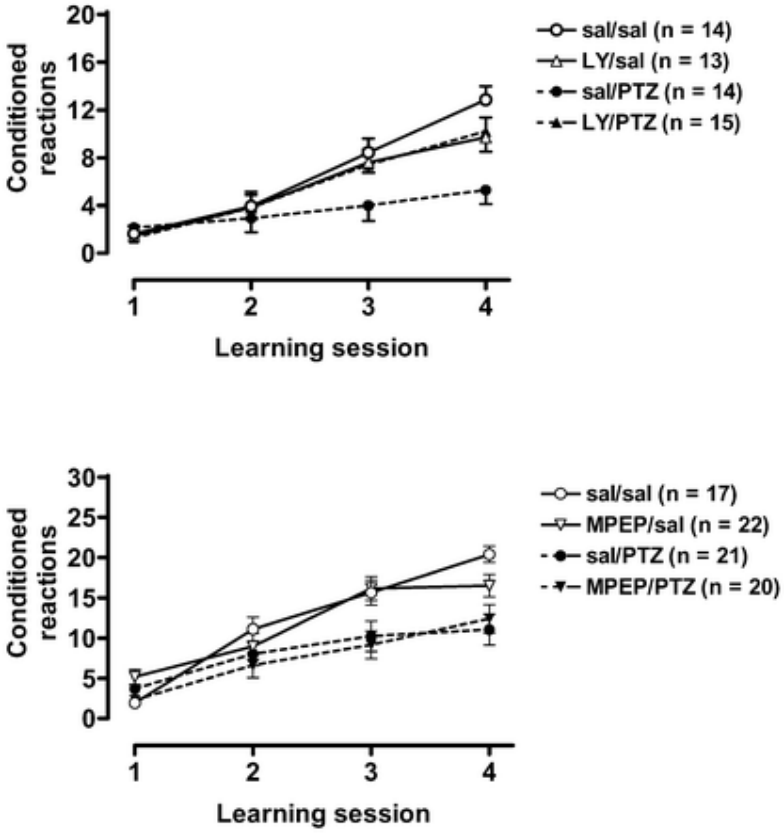

Figure 2.3. Shuttle box performances in LY and MPEP injected animals given during kindling in comparison to controls. Mean ± standard error of the mean

Shuttle box learning (antagonist given prior to the learning session)

As shown in Figure 2.4 the number of conditioned reactions in the saline injected control rats constantly increased whereas learning performance in kindled rats was poor in both experiments. Control rats treated with LY and MPEP also showed an increase in the number of conditioned reactions day by day. Kindled rats treated with LY and MPEP showed a significant improvement and learned at about the same level as that of control rats suggesting a restorative effect in both experiments:

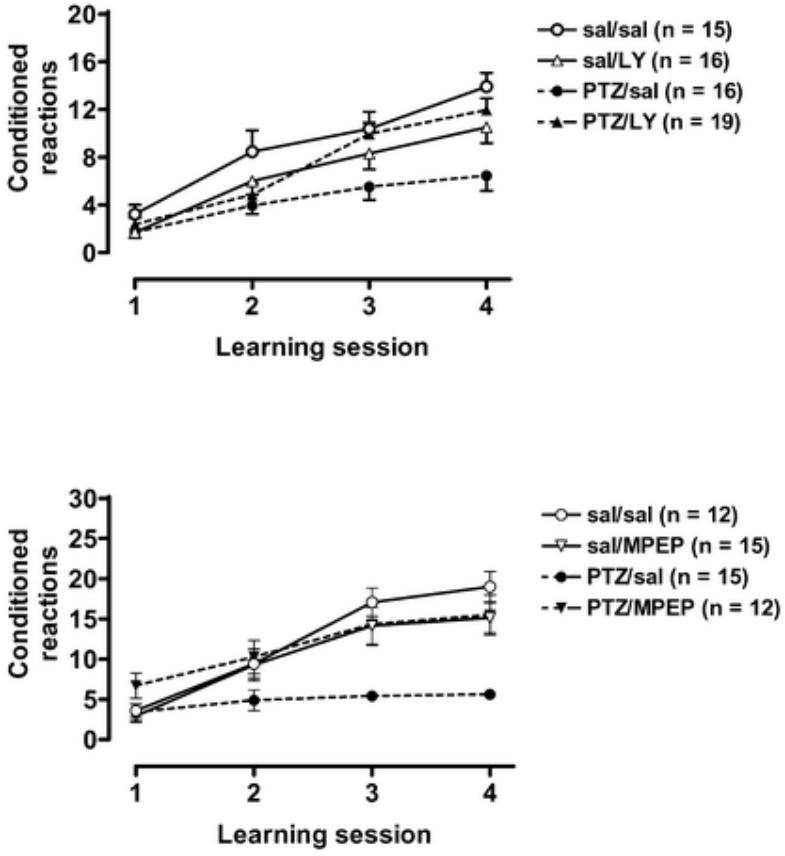

Figure 2.4. Shuttle box performances in LY and MPEP injected animals given prior to the learning session in comparison to controls. Mean ± standard error of the mean

LY
Day, $F_{1,60} = 232.826$, $p < 0.001$
Day × pretreatment, $F_{1,60} = 3.89$, $p = 0.01$
Day × treatment, $F_{1,60} = 3.5$, $p = 0.06$
Day × pretreatment × treatment, $F_{1,60} = 8.69$, $p = 0.005$

ANOVA
Day × treatment, $F_{3,60} = 6.93$, $p < 0.001$
Sal/sal vs. sal/LY, $p = 0.409$
Sal/sal vs. PTZ/sal, $p = 0.001$
PTZ/sal vs. PTZ/LY, $p = 0.05$
Sal/sal vs. PTZ/LY, $p = 0.722$

MPEP
Day, $F_{1,50} = 105.19$, $p < 0.001$
Day × pretreatment, $F_{1,50} = 8.318$, $p < 0.001$
Day × treatment, $F_{1,50} = 0.90$, $p = 0.345$
Day × pretreatment × treatment, $F_{1,50} = 8.172$, $p = 0.006$

ANOVA
Day × treatment, $F_{3,50} = 9.5$, $p < 0.002$
Sal/sal vs. sal/MPEP, $p = 0.007$
Sal/sal vs. PTZ/sal, $p = 0.007$
PTZ/sal vs. PTZ/MPEP, $p = 0.018$
Sal/sal vs. PTZ/MPEP, $p = 1$

Glutamate binding study

PTZ kindling resulted in significantly increased ^{3}H-glutamate binding (Figure 2.5, $U_{12,10}$=25, p<0.05) to hippocampal membranes. The application of both LY and MPEP has no effect on the mGluR1, mGluR I/II, mGluR III (not shown) as well as total glutamate binding sites (Figure 2.5). The ^{3}H-MPEP labeled mGluR 5 binding sites were enhanced in response to PTZ kindling (Figure 2.6, $U_{12,10}$=17, p<0.01). As shown in Figure 2.6, the increase was blocked by LY ($U_{10,6}$=5, p<0.01). The drug itself did not alter the mGluR 5 binding sites in saline controls. MPEP did not significantly influence the glutamate binding sites at all as shown in Figure 2.6.

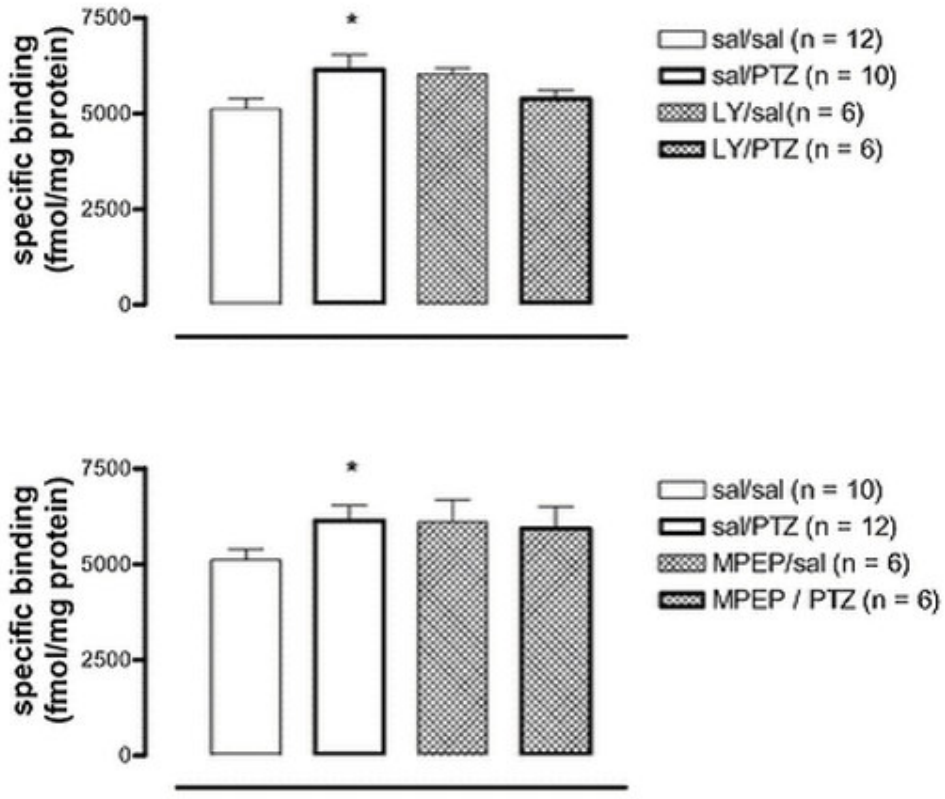

Figure 2.5. Specific ^{3}H-L-glutamate binding to crude synaptic membranes of the hippocampus from control and kindled rats showing total glutamate binding. Mean ± standard error of the mean, *p<0.05

Discussion

There is growing interest in investigating physiological effects mediated by metabotropic glutamate receptors. Group I mGluR are involved in many CNS functions and may participate in a variety of disorders such as pain, epilepsy, ischemia, and chronic neurodegenerative diseases (Bordi and Ugolini 1999; Flor et al., 2002; Folbergrova et al., 2003; Meldrum 1994). These antagonists are considered potential therapeutics in the treatment of these diseases.

In the present study we investigated the effects of mGluR1 and mGluR5 antagonists on acute PTZ convulsion, PTZ kindling and kindling-induced impairments in shuttle-box performance. Blockade of the mGluR1 receptor led to diminished seizures scores in the acute experiment (Figure 2.1). Both substances were effective against convulsions induced by 40 mg/kg PTZ but not when the convulsant was administered in higher doses. This is in agreement with other reports showing similar effects in different epilepsy models (Borowicz et al., 2003; Chapman et al., 2000, 2001). Interaction between group I mGluR and components of the GABAergic neurotransmission seems to be plausible as an explanation of the acute anti-PTZ efficacy (Cozzi et al., 2002; Folbergrova et al., 2003).

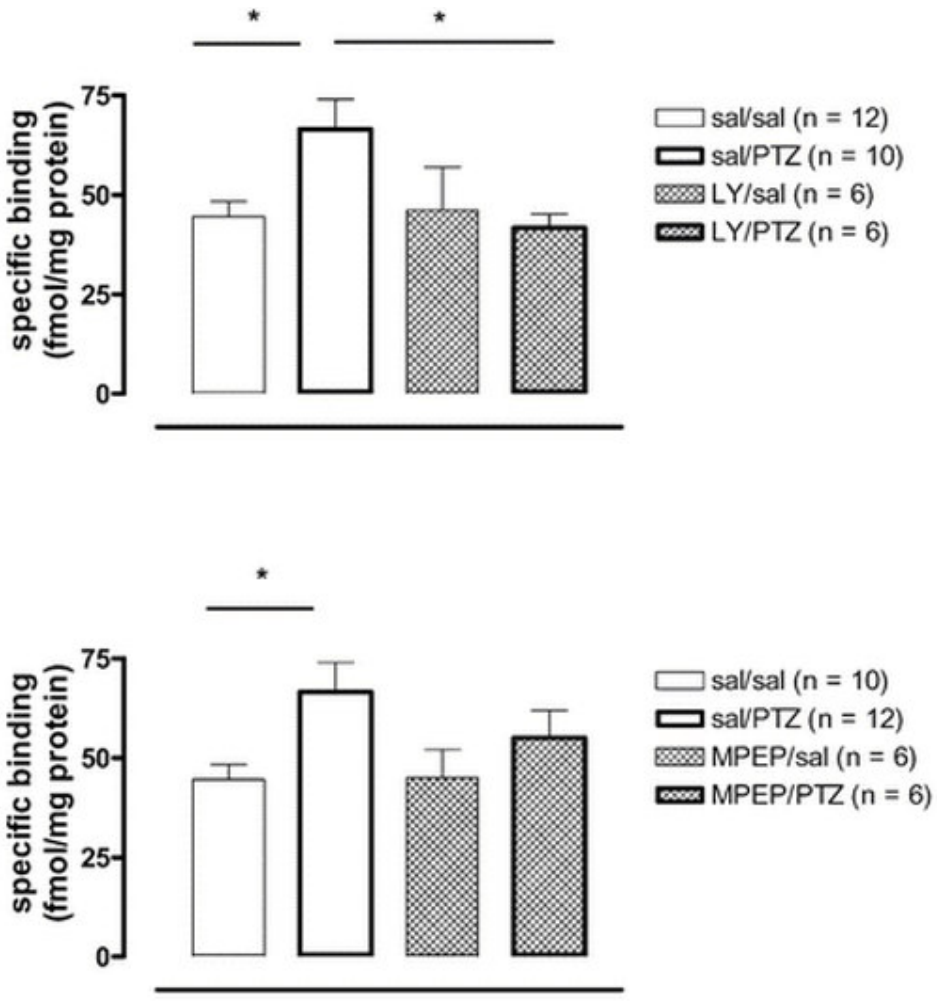

Figure 2.6. Specific ^{3}H-MPEP binding to crude synaptic membranes of the hippocampus from control and kindled rats showing mGluR 5 binding. Mean ± standard error of the mean, *p<0.05

Although both substances showed similar effects in the acute experiment we found striking differences in the kindling experiment. Blockade of mGluR1 depressed the progression of kindled seizure severity. However, in reaction to challenge (Figure 2.2) there was no effect of the treatment with LY suggesting that the developmental component of kindling was not affected. In contrast, blockade of mGluR5 was without effect on kindled

seizures (Figure 2.2). Moreover, in the challenge experiment, the treatment with MPEP did not modify the reaction of either control animals or rats in the kindling group. Obviously, subchronic treatment with MPEP did not change the responsiveness of the glutamatergic system. This is underlined by the data obtained in the binding experiment (Figure 2.5). Kindling resulted in increased glutamate binding which is unchanged by subchronic MPEP application.

A number of studies reported on the anti-kindling effects of ionotropic glutamate receptor antagonists, although their impact on the expression of kindled seizures and kindling progression is under discussion (Borowicz et al., 2001; Doherty and Dingledine 2002; Grecksch et al., 1994; Kodama et al., 1999; Rogawski et al., 2001). Here, we found specific effects of both group I mGluR antagonists. Since both substances were described as effective modulators of GABAergic systems in the CNS (Cozzi et al., 2002; Folbergrova et al., 2003) the mechanisms underlying mGluR I receptors might be mediated via GABA receptors (Chapman et al., 2000; Flor et al., 2002; Lea and Sarvey 2003; Moldrich et al., 2001b, 2001a; Renaud et al., 2002; Tang et al., 2001). Our results are in agreement with others demonstrating anticonvulsant effects of LY and MPEP against acute PTZ-induced seizures (Thomsen and Dalby 1998).

Kindling results in a worsening of shuttle-box learning depending on both the method of kindling induction and the learning model used (Becker et al., 1992, 1997b). Learning impairments were explained in terms of histopathological alterations and kindling-induced modifications in different neurotransmitter systems. Administration of LY in the course of kindling development or, alternatively, prior to each learning session in the shuttle-box improved learning performance in the kindled animals. This is the first report demonstrating protective and restorative efficacy of the substance in an animal model of epilepsy. The protective nature of LY might be mainly based on two specific effects. First, kindling is associated with cell loss in different brain areas (Becker et al., 1997c; Cavazos et al., 1994; Holmes 2002; Kotloski et al., 2002; Osawa et al., 2001; Pohle et al., 1997; Rauca et al., 2000; Spiller and Racine 1994). LY did reduce seizure severity which might result in less severe histopathological damage. Second, the increase in central excitability in kindled animals is also reflected by an increase in glutamate binding sites (Schroeder et al., 1998b) which was found in clinical observations (Blumcke et al., 2000) and in the present study (Figure 2.5). Application of LY prior to kindling significantly reduced the number of mGluR1 binding sites (Figure 2.6). The number of binding sites in sal-injected controls and kindled animals treated with LY is similar. It is speculated that the normalization of the hyperglutamatergic state also contributes to improved learning performance.

Although both substances differed in their effect on kindling development and protection against learning impairment, LY and MPEP treatment prior to the learning test improved shuttle-box performance in the kindled rats. This is the more so interesting since the same dose of MPEP was found to reduce hippocampal long-term potentiation (LTP) and worsened learning of a Y-maze spatial alteration task (Balschun and Wetzel 2002). This phenomenon might help to discuss the restorative effect in the context of reduced/normalized central excitability. Kindling results in enhanced hippocampal LTP (Krug et al., 1998; Ruethrich et al., 1996). A similar effect, i.e. enhancement in hippocampal LTP, was observed in learning experiments (Bergado et al., 1988). According to the plasticity-pathology continuum theory (McEachern and Shaw 1999) we have to differentiate between beneficial alterations and others that may be primarily pathological in nature. Potentiation processes resulting from kindling plus those resulting from learning may led to supramaximal

potentiation which is pathological. Reduction of the supramaximal potentiation by either mGluR1 or mGluR5 blockade prior to the learning test would result in physiological condition, i.e. improvement of learning performance. This hypothesis does not exclude other mechanisms contributing to the effects as found in the study presented. Our findings identify a potential role of mGluR1 and mGluR5 in the amelioration of seizure disorders and, moreover, epilepsy related impairments in learning.

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Chapter 3

Repeated administration of group I mGluR antagonists prevents seizure-induced long-term aberrations in hippocampal synaptic plasticity

Abstract

Kindling induced by repeated application of the convulsant pentylenetetrazole (PTZ) is a validated model of epilepsy and epilepsy-related neuromorphological, neurophysiological and behavioural alterations. In this study, we examined whether kindling-induced long-term aberrations in hippocampal synaptic plasticity can be prevented by application of group I mGluR antagonists.

Kindling resulted in a higher magnitude of long-term potentiation (LTP) induced by a strong high-frequency stimulation in the hippocampal CA1 region in vitro. When the specific mGluR1 antagonist LY 367385 (0.40 μMol) or the specific mGluR5 inhibitor MPEP (0.06 μMol) were given 30 min prior to PTZ, this kindling-induced enhancement of LTP was almost completely prevented. In addition, application of MPEP led to an impaired maintenance of population spike LTP in kindled animals. LY 367385 applied to unkindled control animals caused a reduction of the initial magnitude of population spike LTP. MPEP, in contrast, left the initial magnitude untouched but resulted in a faster decay of potentiation. A single administration of LY 367385 (200 μM) and MPEP (50 μM), respectively, directly into the bath had almost no effect. Our data suggest that the long-lasting aberrations of hippocampal synaptic plasticity induced by the repeated occurrence of generalized epileptic seizures ultimately require a concurrent operation of mGluR1 and mGluR5.

Introduction

Epilepsy is a diverse disorder characterized by the recurrent hypersynchronous discharges of cortical neurons that reside within a localized area of one cerebral hemisphere (focal seizures) or appear throughout the forebrain (generalized seizures) (McCormick and Contreras, 2001 and Morimoto et al., 2004). It is estimated that 0.5–2% of the human population is afflicted with epilepsy (McNamara et al., 1985 and Morimoto et al., 2004). Left untreated, several types of epilepsia invariably result in characteristic behavioural syndromes and cognitive decline (Halgren et al., 1991 and Helmstaedter, 2002). One of the most prominent experimental models of epilepsia is kindling (Goddard, 1967) – a progressive, highly reliable increase of seizure discharge in response to repeated application of electrical stimulation or a chemical epileptogenic compound (McNamara et al., 1985). Kindling permits investigations into the convulsive component of epilepsy, epilepsy-related alterations in behaviour as well as neuromorphological, neurophysiological, and neurochemical determinants of this complex disease.

In previous investigations of rats which acquired the kindling syndrome after repeated injections of the convulsant pentylenetetrazole (PTZ) we found a dramatic impairment in shuttle-box learning which is still persistent 4 weeks after kindling completion (Becker et al., 1992) as well as a number of significant changes in glutamatergic neurotransmission, such as an increase in glutamate binding that was associated with an enhanced binding of metabotropic glutamate receptors (mGluR) (Schroeder et al., 1999). Most prominent was an increase in group I mGluR binding (Schroeder et al., 1999). Stimulation of this group of mGluRs which consists of the mGluR1 and mGluR5 subtypes was reported to elicit long-lasting, ictal-like discharges (Merlin and Wong, 1997 and Camon et al., 1998 see Conn and Pin, 1997 for classification of mGluRs).

In accordance with its effects on learning and memory, kindling has been suggested to interfere with long-term potentiation (LTP), the best investigated model of the cellular mechanisms underlying learning and memory (Racine et al., 1983, McNamara et al., 1985 and Sutula et al., 1994). Thus, while kindling was reported by some groups to result in a long-lasting facilitation of the induction of LTP (Ruethrich et al., 1996, Krug et al., 1997 and Schneiderman 1997) or to produce a potentiation on its own (Racine et al., 1972, Sutula and Steward, 1986, Maru and Goddard, 1987a, Ruethrich et al., 1996 and Omarani et al., 2000), other studies found either no long-term changes of evoked potentials (Giacchino et al., 1984, Piredda et al., 1986, Ruethrich et al., 1996 and Krug et al., 1997), a decrease of potentials (de Jonge and Racine, 1987, Maru and Goddard, 1987b and Gilbert and Mack, 1990) or even a suppression of LTP (Leung and Wu, 2003).

In the present study, we addressed the question of whether inhibition of group I mGluRs either in the course of PTZ-kindling or shortly before evoking an ex vivo LTP might counterbalance putative kindling-induced aberrations in LTP. Our findings suggest a crucial function of group I metabotropic glutamate receptors during the kindling-induced long-term effects on hippocampal synaptic plasticity.

Materials and methods

Ethical approval was sought according to the requirements of the National Act on the Use of Experimental Animals (Germany).

Animals

All experiments were carried out according to the requirements of the National Act on the Use of Experimental Animals (Germany). Male Wistar rats [Shoe:Wist(Shoe), DIMED Schoenwalde GmbH], 8 weeks old, were kept in groups of 5 under controlled laboratory conditions (light regime of 12 h light/12 h dark, light on at 6:00 a.m., temperature 20 ± 2 °C, air humidity 55–60%). They had free access to commercial rat pellets (Altromin 1326) and tap water.

Surgery

For the intracerebroventricular (i.c.v.) injection of substances, a microcannula (outer diameter 0.6 mm) was implanted stereotaxically under Nembutal anaesthesia (40 mg/kg, i.p.) into the right lateral ventricle [coordinates AP −0.25 mm, lat. 1.6 mm (Paxinos and Watson, 1997)] fixed to the skull with two micro-screws and dental cement. Animals were allowed to recover from surgery for at least 1 week.

Application of substances during kindling induction

Pentylenetetrazole (PTZ) (Roth, Germany), LY 367385 [(*S*)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid; Tocris, Germany] and MPEP [2-methyl-6-(phenylethynyl)pyridine hydrochloride; Tocris, Germany] were dissolved in sterile NaCl (sal; 0.9%) up to final concentration. In order to induce kindling, 37.5 mg/kg body weight PTZ (ED_{16} for the

induction of clonic seizures) were applied intraperitoneally (i.p.; injection volume 1 ml/100 g) once every 48 h (altogether 13 injections). Immediately after injection, the behaviour was pursued for 20 min and the seizures stage classified as follows:

Stage 0: no response
Stage 1: ear and facial twitching
Stage 2: myoclonic jerks without rearing
Stage 3: myoclonic jerks, rearing
Stage 4: turn over into side position, clonic-tonic seizures
Stage 5: turn over into back position, generalized clonic-tonic seizures

Only animals that had attained at least three consecutive seizure stages 4 or 5 following 13 PTZ injections, reflecting the complete generalization of convulsive activity were considered to be "kindled" and included in the analysis. Control animals received saline injections according to the same schedule. To test the effect of group I antagonists on kindling development, rats were injected i.c.v. with either 0.40 µMol LY 367385 or 0.06 µMol MPEP (both dissolved in 5 µl saline) 30 min before PTZ-application. The antagonists doses were chosen according to Mannaioni et al., (2001) and Balschun and Wetzel, (2002), respectively. In the case of LY 367385 where the in vivo dose was derived from a published in vitro concentration, the calculation was based on the assumption of an equal distribution of the compound and a brain volume of 2 ml (Riedel et al., 1995). Control animals received the same volume of saline. The chosen antagonists doses were found to effectively reduce the seizure severity (seizure stage) induced by a single injection of 40 mg/kg PTZ while being without any effect when higher PTZ doses of 50 mg/kg and 60 mg/kg, respectively, were applied (Nagaraja et al., 2004).

Hippocampal slice preparation

At least 9 days after completion of kindling, i.e. at a time when PTZ is considered to have been cleared out (Grecksch et al., 1997), rats were stunned by a blow to the neck and hippocampal slices were prepared as described previously (Wilsch et al., 1998). Immediately thereafter, slices were transferred to a submerged-type slice chamber and permanently perfused with 32 °C ACSF.

Electrophysiological long-term recordings

After an incubation of at least 1 h, a glass electrode (filled with ACSF, 1–4 MΩ) was lowered into the *stratum radiatum* or the *stratum pyramidale* of the CA1 region to record field excitatory postsynaptic potentials (fEPSPs) or population spikes that were evoked by a monopolar, lacquer-coated, stainless-steel stimulating electrode positioned in the CA1 dendritic layer. The initial slope of the fEPSP and the amplitude of the population spike (PSA) served as measures of these potentials. After constructing input/output curves, the stimulation strength was adjusted to 40% of the maximum PSA and 30% of the maximum fEPSP slope. This stimulation strength was kept constant throughout the experiment. During baseline recording, three single stimuli (0.1 ms pulse width; 10 s interval) were averaged every 5 min. Once a stable baseline had been established, LTP was induced by the strong tetanization protocol consisting of three stimulus trains of 100 pulses at 100 Hz (0.2 ms pulse width), with a 10 min intertrain interval. LTP was monitored for at least 4 h after applying the

first tetanus. For the recording of LTP of fEPSP and PSA, different groups of animals were used.

Drug perfusion in vitro

In some experiments, slices of PTZ-kindled or control animals were superfused with LY 367385 (200 μM) and MPEP (50 μM), respectively, from 30 min before up to 30 min after tetanization. The compounds were dissolved in ACSF to final concentration.

Statistics

For statistical evaluation of between-group comparisons ANOVA with repeated measures and the Mann–Whitney *U*-test were used whilst the Wilcoxon matched-pairs signed rank test was employed for within-group comparisons.

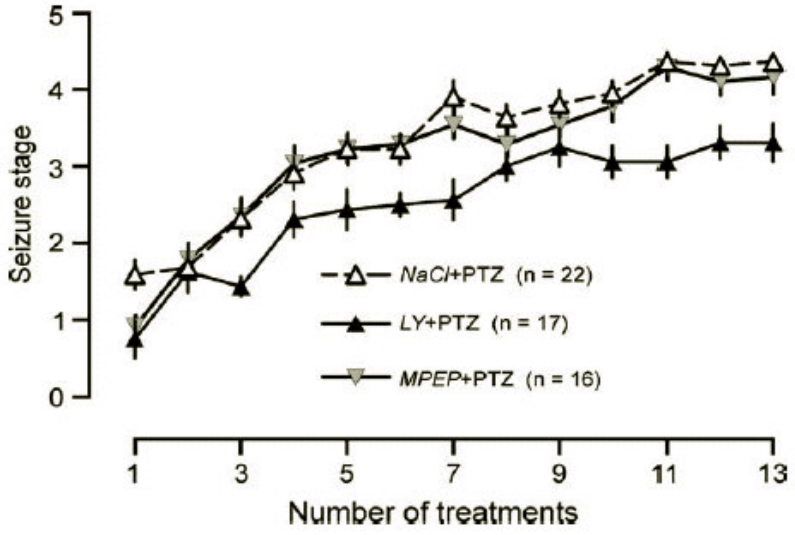

Figure 3.1. The effect of repeated intracerebroventricular (i.c.v.) administration of group I mGluR antagonists on the development of kindling. While LY 367385 (0.40 μMol) significantly depressed the progression of kindled seizure severity ($p < 0.001$, ANOVA) blockade of mGluR5 with the specific antagonist MPEP (0.06 μMol) diminished seizures after the first PTZ-application ($p = 0.022$ Mann–Whitney *U*-test), but failed to have any further influence on kindling development ($p = 0.354$, ANOVA). Only animals that were subsequently used for LTP experiments were included. Substances that were applied i.p. are indicated by normal characters, those given i.c.v. by italics.

Results

Recent studies indicated an important function of group I mGluRs (mGluR1 and mGluR5) in the genesis of long-lasting epileptiform discharges (Merlin and Wong, 1997, Camon et al., 1998, Chapman et al., 1999 and Chapman et al., 2000). Our data point to a differential effect of these mGluR subtypes on PTZ-evoked discharges and kindling development. As depicted in Figure 3.1, inhibition of mGluR1 by the specific antagonist LY 367385 (Clark et al., 1997) had an immediate depressing effect on PTZ-induced seizures which resulted in a retarded

progression of kindled seizure severity ($p < 0.001$, ANOVA). Blockade of mGluR5 with MPEP (Salt et al., 1999), in contrast, depressed seizures after the first PTZ-application ($p = 0.022$ Mann–Whitney *U*-test) but failed to have any further influence on kindling development ($p = 0.354$, ANOVA). Thus, inhibition of mGluR1 but not mGluR5 has an effect on the expression of the kindling 'phenotype'.

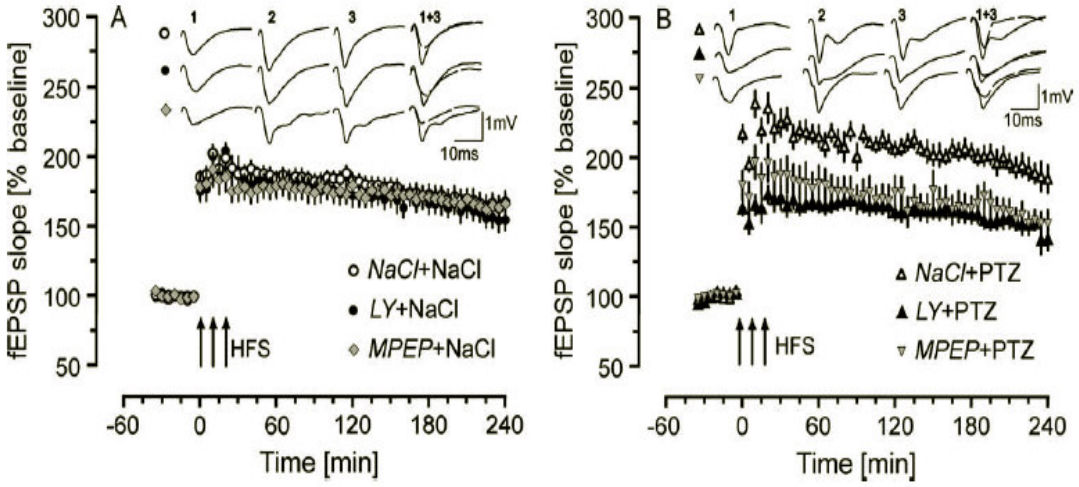

Figure 3.2. The repeated application of group I mGluR antagonists during kindling development counteracts PTZ-induced long-term changes of LTP of the fEPSP in the hippocampal CA1 region in vitro. (A) The intracerebroventricular (i.c.v.) application of the specific mGluR1 antagonist LY 367385 (0.40 µMol) and the specific mGluR5 antagonist MPEP (0.06 µMol) had no effects on LTP of control animals. (B) Rats that had been pretreated during kindling development with LY 367385 (0.40 µMol) and MPEP (0.06 µMol) displayed a clear-cut reduction of the LTP magnitude ($p < 0.001$ and $p = 0.002$, respectively; ANOVA with repeated measures, LY + PTZ $n = 9$; NaCl + PTZ $n = 11$; MPEP + PTZ $n = 8$). Insets show representative recordings taken during baseline recording (1), 10 min after the third 100 Hz train (2), and at the end of recording time (3). Stimulus artefacts have been truncated for clarity. HFS, high-frequency stimulation. Mean ± SEM is given. Substances that were applied i.p. are indicated by normal characters, those given i.c.v. by italics.

Since previous investigations have shown that PTZ-kindling results in long-lasting changes of learning and synaptic plasticity (Becker et al., 1992, Genkova-Papazova and Lazarova-Bakarova, 1995, Ruethrich et al., 1996 and Krug et al., 1997), we addressed the question of whether inhibition of group I mGluRs might counteract long-lasting kindling effects on hippocampal synaptic plasticity. To investigate this topic we prepared hippocampal slices from PTZ-kindled rats 8–12 days after completion of kindling and induced a robust LTP of extracellular recordings in the CA1 region. Animals that had acquired the kindling syndrome displayed a higher magnitude of potentiation of the field EPSP (fEPSP) slope as indicated by the values attained 1 min after tetanization (NaCl + PTZ: 217.3 ± 6.3%, $n = 11$; Figure 3.2B; NaCl + NaCl: 185.8 ± 4.3%, $n = 11$; Figure 3.2A). This difference remained significant until the end of experiment ($p = 0.002$ ANOVA with repeated measures). Interestingly, rats that had been pretreated with the mGluR1 antagonist LY 367385 (0.40 µMol) during kindling development displayed a clear-cut reduction of the LTP magnitude (Figure 3.2B, LY + PTZ 163.4 ± 4.6%, $n = 9$, vs. NaCl + PTZ; $p < 0.001$; ANOVA with repeated measures) i.e. to values that were even below those of saline controls (Figure

3.2A NaCl + NaCl). Likewise, in animals that received the specific mGluR5 antagonist MPEP (0.06 µMol) (Salt et al., 1999) prior to PTZ-application the kindling-induced increase of LTP magnitude was completely prevented (Figure 3.2B, MPEP + PTZ 177.8 ± 10.7%, $n = 8$, $p = 0.002$ ANOVA with repeated measures). In contrast, the application of these mGluR antagonists had no effects on LTP when applied to control animals (Figure 3.2A). Thus, repeated application of group I mGluR antagonists does not seem to exert long-term effects on synaptic transmission of control animals.

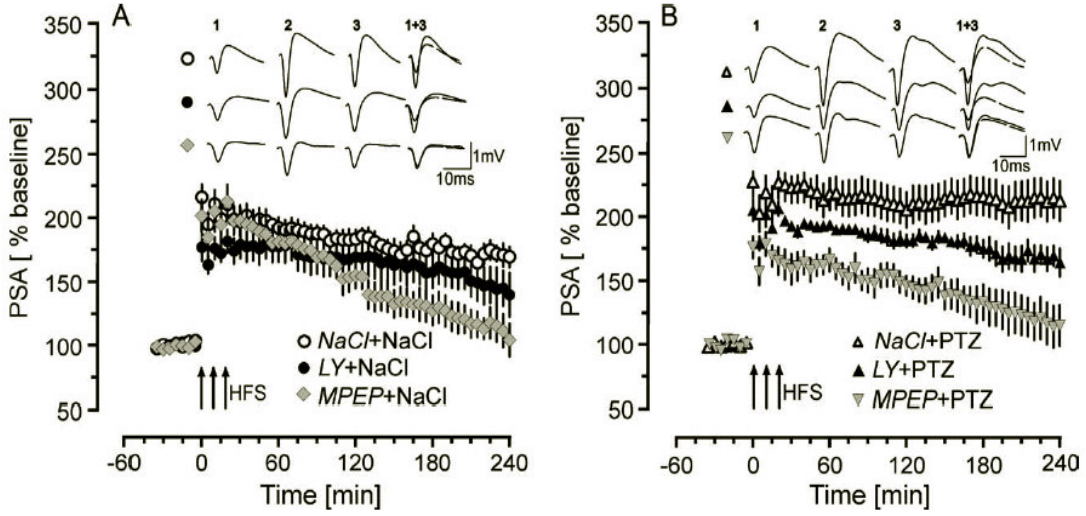

Figure 3.3. The repeated application of the specific mGluR5 antagonist MPEP during kindling development has stronger effects on PTZ-induced long-term changes of LTP of the PSA in the hippocampal CA1 region in vitro than the application of LY 367385. (A) While the intracerebroventricular (i.c.v.) application of the specific mGluR1 antagonist LY 367385 (0.40 µMol) had no significant effects on LTP of control animals, the specific mGluR5 antagonist MPEP (0.06 µMol) led to a clear-cut impairment of LTP maintenance without reducing the initial magnitude of potentiation (4 h: $p = 0.001$ Mann–Whitney *U*-test, NaCl + NaCl $n = 11$; MPEP + NaCl $n = 8$). (B) Pretreatment with LY 367385 before kindling reduced the magnitude of LTP (LY + PTZ $n = 8$; NaCl + PTZ $n = 11$). However, this change reaches the level of statistical significance only at some time points (60 min: $p = 0.020$, 65 min: $p = 0.015$, 70 min: $p = 0.015$, 150 min: $p = 0.044$, Mann–Whitney *U*-test). In contrast, application of MPEP to PTZ-kindled animals caused a marked reduction of the initial LTP magnitude ($p = 0.009$ Mann–Whitney *U*-test, MPEP + PTZ $n = 8$; NaCl + PTZ $n = 11$) together with a marked impairment of LTP maintenance leading to values that were indistinguishable from baseline after about 190 min (Wilcoxon matched-pairs signed rank test). See Figure 3.2 for further explanation.

Earlier studies indicated that kindling predominantly enhances potentiation of the population spike amplitude (PSA) (Ruethrich et al., 1996 and Becker et al., 1997). Therefore, we explored in a separate series of experiments whether similar changes as found in LTP of fEPSP could be also observed in LTP of PSA. Our data, compiled in Figure 3.3, show a moderately increased initial magnitude of LTP in PTZ-kindled animals (228.7 ± 11.9, $n = 11$; Figure 3.3B NaCl + PTZ) as compared with unkindled controls (216.0 ± 10.0, $n = 11$; Figure 3.3A NaCl + NaCl, n.s. Mann–Whitney *U*-test) and a more robust maintenance resulting overall in a significant difference across the recording period of 4 h ($p = 0.039$ ANOVA with repeated measures). Pretreatment with LY 367385 before kindling caused a reduction of the initial magnitude (Figure 3.3B, LY + PTZ 206.3 ± 8.4, $n = 8$) to values that closely resemble

those of untreated controls. Application of LY 367385 to control rats further decreased the potentiation as shown in Figure 3.3A (LY + NaCl: 176.5 ± 11.0, $n = 8$).

Strikingly, injection of MPEP prior to kindling had even a stronger effect on PSA LTP as compared with LY 367385. Thus, a clear reduction of the initial LTP magnitude (Figure 3.3B, MPEP + PTZ 176.9 ± 8.4, $n = 8$, vs. NaCl + PTZ, $p = 0.009$ Mann–Whitney *U*-test) was accompanied by a marked impairment of LTP maintenance leading to values that were indistinguishable from baseline after about 190 min (Wilcoxon matched-pairs signed rank test). The impaired maintenance of potentiation after repeated MPEP application was not confined to kindled animals but was also observed in control animals (Figure 3.3A, 4 h: NaCl + NaCl 168.9 ± 7.9%, $n = 11$; MPEP + NaCl 105.0 ± 12.9%, $n = 8$, $p = 0.001$ Mann–Whitney *U*-test). Therefore, mGluR5 inhibition resulted in two distinguishable effects on kindling-induced changes in PSA LTP: (i) a reduction of the initial magnitude of potentiation which was unique to kindled animals and (ii) an impairment of LTP maintenance which could be also observed in control animals.

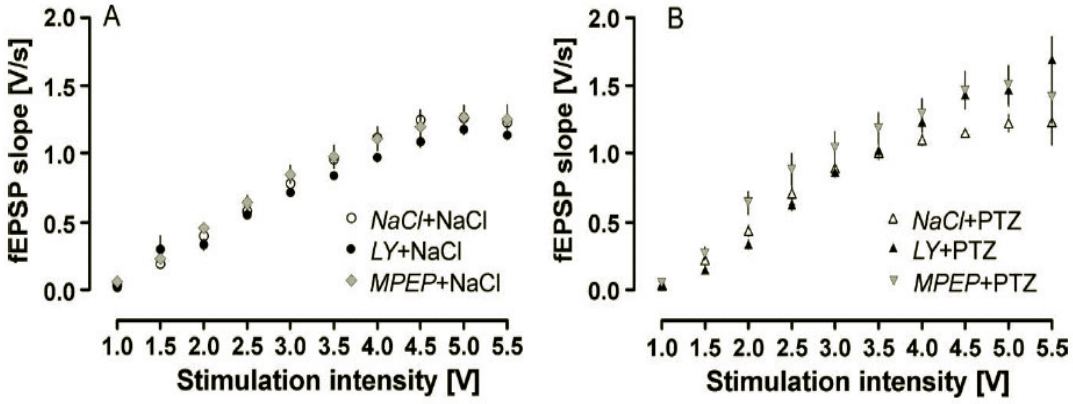

Figure 3.4. Input/output (I/O) curves of the fEPSP reveal only minor effects of the i.c.v. application of group I antagonists during kindling development. (A) Pretreatment with LY caused smaller fEPSP slopes at higher intensities as compared with untreated controls (4 V $p = 0.040$ Mann–Whitney *U*-test). In contrast, application of MPEP was without any effect on I/O curves of control animals. (B) The application of LY 367385 and MPEP, respectively, tended to increase fEPSP slopes of kindled animals after strong stimulation (4.5 V: LY $p = 0.028$; MPEP $p = 0.026$, Mann–Whitney *U*-test). Mean ± SEM is given.

The differences found in LTP of kindled animals after pretreatment with group I antagonist might be due to specific changes of mechanisms underlying LTP or an alteration of basic synaptic transmission and excitability. To examine the latter possibility we compared the I/O curves of experimental groups. As compiled in Figure 3.4A and B there was no discernible difference between PTZ-treated ($n = 16$) and control animals ($n = 15$) when the fEPSP was plotted vs. stimulation intensity. However, the application of LY 367385 ($n = 11$) and MPEP ($n = 9$) resulted in a tendency of greater fEPSP slopes at higher stimulation intensities indicating an increased capacity of synaptic transmission (Figure 3.4B, 4.5 V: $p = 0.028$ and 0.026, respectively). In addition, pretreatment with LY caused smaller fEPSP slopes at higher intensities as compared with untreated controls (Figure 3.4A, 4 V $p = 0.040$

Mann–Whitney *U*-test; *LY* + NaCl $n = 9$). In contrast, there was no difference in the I/O curves of PSA of all groups (data not shown).

While these experiments demonstrated the long-term efficacy of group I antagonists when given during kindling development, we checked in another set of experiments whether application of the same antagonists into the bath immediately before LTP induction has any functional consequences on the kindling-induced enhancement of potentiation. Because the experiments with the application of group I antagonists in vivo demonstrated a high sensitivity of PSA LTP we used this parameter for the following experiments.

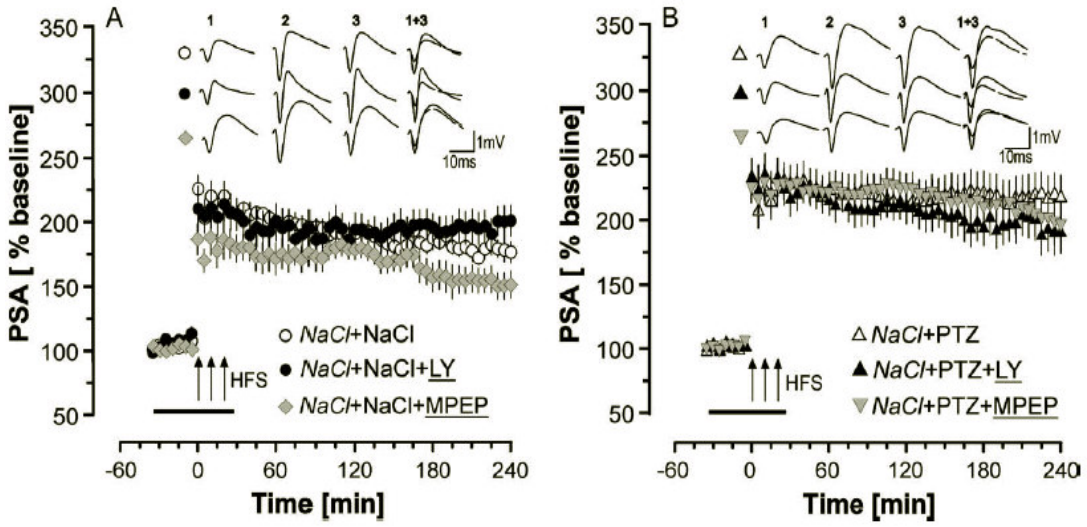

Figure 3.5. A single bath-application of group I antagonists is unable to counterbalance PTZ-induced long-term changes of LTP. (A) LY 367385 (200 µM) applied via the perfusion line from 30 min before up to 30 min after tetanization did not have any effect on LTP when applied to slices of control rats. In contrast, MPEP (50 µM) reduced the initial magnitude of LTP (0 min: $p = 0.047$, 5 min: $p = 0.038$ Mann–Whitney *U*-test, NaCl + MPEP $n = 8$; NaCl + NaCl $n = 11$). (B) Neither LY 367385 nor MPEP altered the PTZ-induced enhancement of potentiation when given to slices of PTZ-kindled animals. Horizontal bar indicates the time of drug application. Substances that were given i.p. are indicated by normal characters, those that were injected i.c.v. by italics and compounds which were applied via the perfusion line are underlined. See Figure 3.2 for further explanation.

As shown in Figure 3.5A, LY 367385 (200 µM) applied via the perfusion line from 30 min before up to 30 min after tetanization had neither an effect on LTP when applied to slices of control rats (*NaCl* + NaCl + LY 201.3 ± 16.7, $n = 9$) nor when given to slices of PTZ-kindled animals (*NaCl* + PTZ + LY 228.3 ± 13.7, $n = 7$; Figure 3.5B). In contrast, MPEP (50 µM) did not alter the PTZ-induced enhancement of potentiation (Figure 3.5B) but reduced the initial magnitude of LTP if applied to slices of unkindled controls (Figure 3.5A, *NaCl* + NaCl + MPEP 182.4 ± 11.6, $n = 8$, vs. *NaCl* + NaCl; 0 min: $p = 0.047$, 5 min: $p = 0.038$, Mann–Whitney *U*-test). Thus, an 'acute' administration of group I antagonists is apparently unable to prevent the kindling-induced long-term enhancement of potentiation. In agreement with the findings obtained with kindled rats, inhibition of mGluR5 seems to be more potent in affecting LTP even in unkindled control animals.

Discussion

Kindling and LTP have been suggested to exploit similar mechanisms (Racine et al., 1983, McNamara et al., 1985 and Sutula et al., 1994). Here we used the repeated injection of the $GABA_A$-antagonist PTZ to generate kindling, which was described to trigger long-lasting changes in the induction of LTP (Ruethrich et al., 1996 and Krug et al., 1997) or a potentiation on its own (Ruethrich et al., 1996, Omrani et al., 2000 and Palizvan et al., 2001). Since electrical kindling was reported to result in a potentiation of evoked responses as well (Racine et al., 1972, Racine et al., 1995 and Maru and Goddard, 1987b), kindling-induced potentiation has been suggested to contribute to the developing epileptogenesis. Other groups, however, failed to see such effects, i.e. they observed either no long-term changes of transmission after different types of kindling (Giacchino et al., 1984, Piredda et al., 1986, Ruethrich et al., 1996 and Krug et al., 1997), a decrease of evoked potentials (de Jonge and Racine, 1987, Maru and Goddard, 1987b and Gilbert and Mack, 1990) or even a suppression of LTP (Leung and Wu, 2003).

In the present study, we do not find significant differences between the I/O curves of PTZ-kindled and control animals, widely excluding any kindling-induced potentiation of normal synaptic transmission and excitability. This is corroborated by a recent paper of Maru et al., (2002) who described a kindling protocol that resulted in behavioural convulsions and a prolonged duration of after-discharges without any signs of synaptic potentiation. Our data, however, uncover the tendency that the repeated application of LY 367385 or MPEP during kindling induction led to greater fEPSP slopes at higher stimulation intensities. Apparently, the repeated administration of group I antagonists induces moderate long-term changes of basal synaptic transmission. However, these slight changes at higher stimulation intensities are unlikely to be of major physiological relevance.

In contrast to the missing effect of PTZ-kindling on basal synaptic function and excitability, we found a clear increase in an LTP of PSA that had been induced by a strong tetanization protocol. This is in accordance with earlier data obtained with weak tetanization paradigms in the CA1 region in vitro (Krug et al., 1997) or in the dentate gyrus in vivo (Ruethrich et al., 1996). Most notably, however, we found a marked increase in LTP of the field EPSP slope demonstrating that the kindling-induced long-term changes in synaptic plasticity are not confined to neuronal excitability but might include synaptic changes as well. The higher magnitude of potentiation in slices of kindled rats obtained after both a weak single tetanization (Ruethrich et al., 1996) or a triple strong tetanization (this study) is clearly different from a saturation of potentiation that would be expected if tetanization and the kindling-induced surplus-potentiation would exploit the same mechanisms. Therefore, the available experimental evidence indicates that the enhanced LTP of fEPSP and PSA in kindled rat is not simply due to a strengthening of the normal induction mechanism but caused by the additional involvement of as yet unknown processes.

Here we describe that a repeated ‘chronic’ inhibition of group I mGluRs during kindling development prevents an enhancement of LTP, whilst a single, acute application of group I antagonists just before LTP recording has no effect. This is indicative of a consolidation of kindling-induced changes of synaptic plasticity which is dependent on functionally active group I mGluRs. Activation of this group of mGluRs has been reported recently to be required for the generation of ictal discharges (Merlin and Wong, 1997, Camon et al., 1998, Sayin and Ruteki, 2003 and Smolders et al., 2004).

In contrast to the short-time effects of group I mGluR activation and inhibition that are mostly described, the changes in LTP found by us are long-lasting and suggest mGluR-mediated functional alterations elapse with a certain time course. A distinct time course of kindling-induced long-term changes is also supported by a recent study of Palizvan et al., (2001). A mechanism that could underlie such lasting alterations is a change in the expression level as particularly described for mGluR1 following electrical kindling in rats and TLE in patients (Akbar et al., 1996 and Blumcke et al., 2000). Importantly, our data clearly demonstrate that the long-term effects of group I mGluR antagonists on kindling-induced enhancement of LTP are not directly related to the kindling 'phenotype' itself. Thus, while LY 367385 but not MPEP had a significant effect on the development of kindling, MPEP was more effective than LY 367385 in counteracting the kindling-induced changes in LTP.

A central question is whether the long-term effects of PTZ-kindling on hippocampal LTP need both group I mGluR subtypes (mGluR1 and mGluR5) to occur, or whether activation of either subtype alone is sufficient to bring the changes about. In accordance with a recent study which used mGluR1 and mGluR5 knock-out mice (Stoop et al., 2003), our data demonstrate that inhibition of only one receptor subtype abolishes the surplus-potentiation almost completely. Apparently, the kindling-induced enhancement of LTP ultimately requires a concurrent operation of mGluR1 and mGluR5. Each subtype can be suggested to be linked to specific molecular steps, but only the functioning of these steps ensures that the long-term changes in LTP will be initiated and maintained. A cooperative action of synaptically activated mGluR1 and mGluR5 was also inferred from in vitro studies (Lee et al., 2002 and Thuault et al., 2002), with mGluR5 acting as the principal mGluR subtype in the initiation of bursting activity and mGluR1 in its maintenance (Merlin, 2002, Lee et al., 2002, Thuault et al., 2002 and Lanneau et al., 2002).

Beyond epilepsia and kindling, group I mGluRs have been implicated in different forms of synaptic plasticity such as long-term potentiation (LTP) as well as in learning and memory formation (Nakanishi, 1994, Conn and Pin, 1997 and Balschun et al., 1999). In our study, only MPEP but not LY 367385 caused a long-term effect on LTP when repeatedly applied i.c.v. to control animals. Likewise, an impairment of late LTP was observed in previous experiments when MPEP was applied i.c.v. prior to induction of LTP in the dentate gyrus of freely-moving rats (Balschun and Wetzel, 2002). Corresponding data were recently obtained in the dentate gyrus in vivo (Naie and Manahan-Vaughan, 2004) and in the CA1 region in vitro (Francesconi et al., 2004). The latter is in apparent contradiction to the almost normal potentiation seen in the present study after bath-application of 50 μM MPEP but is in accordance with the data of Doherty et al., (2000) who did not see any effect on CA1-LTP in vitro using LY344545, another mGluR antagonist which is selective towards mGluR5 at the used concentration. The variable efficacy of mGluR5 inhibition on LTP might be due to differences in the relative strength of tetanization and the resulting degree of saturation as well as to another excitation/inhibition ratio under the particular experimental conditions. According to earlier data of our group (Wilsch et al., 1998), the weaker a potentiation and the less saturated an LTP, the higher is the susceptibility towards an inhibition of group I mGluRs. In agreement with this rule and the only faint distribution of mGluR1 in the neuropil of the CA1 region (Blumcke et al., 2000) we did not see any effect of specific mGluR1 antagonist LY 367385 on a saturated LTP induced by a strong tetanization. This result was gained irrespective of whether LY 367385 was repeatedly applied i.c.v. in vivo or given directly into the bath during recording. Interestingly, both LY 367385 and MPEP could alleviate the worsening of shuttle-box learning in kindled animals when given prior to the

learning experiment, but only LY 367385 was found to prevent the detrimental effects of PTZ-kindling on learning when given during kindling development (Nagaraja et al., 2004).

Evaluated together, our data lead us to the following conclusions:

(i) Kindling results in long-lasting changes in hippocampal LTP that are mediated via activation of mGluR1 and mGluR5 and consolidate with a certain time course.

(ii) These changes ultimately require a concurrent operation of mGluR1 and mGluR5 but they do not seem to be directly related to the kindling-phenotype.

(iii) Repeated inhibition of mGluR5 resets the level of hippocampal synaptic plasticity not only during kindling but also under normal conditions.

Our findings suggest a pivotal role of the concurrent activation of mGluR1 and mGluR5 in epilepsia-induced long-term aberrations in hippocampal network functions.

Acknowledgements

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Chapter 4

The use of patch-clamp recordings of Purkinje cells in rat cerebellar slices as a tool to assess synaptic plasticity

Introduction

The patch-clamp technique is a powerful and versatile method for studying electrophysiological properties of neurons. It allows for the recording and manipulation of currents flowing through ion channels, enabling one to monitor physiological membrane potential changes such as action potentials (Sakmann and Stuart, 1995 for review). This chapter describes the techniques we used to study long-term synaptic gain changes in excitatory synapses onto Purkinje cells in rat cerebellar slices.

Studying long-term synaptic plasticity in Purkinje cells in rat cerebellar slices by use of patch-clamp recordings

The patch-clamp setup

In our experiments we used a standard patch-clamp setup consisting of a microscope (Zeiss Axioskop FS) on a vibration isolation table (Newport) within a Faraday cage (Figure 4.1), a patch-clamp amplifier (HEKA EPC-9), a peristaltic pump (Rainin Dynamax) to supply the slice chamber with artificial cerebrospinal fluid (ACSF), stimulus isolation units (Cygnus) for the stimulation electrodes, an oscilloscope (Hameg) and a computer running PULSE + PULSEFIT 8.53 (HEKA). Rat cerebellar slices were placed in a slice chamber, which was connected to a fixed stage that also carried the micromanipulator holding the amplifier probe and the attached patch pipette, micromanipulators for positioning the stimulation electrode(s) (Luigs and Neumann), a reference electrode, and two tubes supplying and retrieving artificial cerebrospinal fluid (ACSF) to and from the slice bath (Figure 4.2). The slices were observed with a 100 W HBO lamp (Zeiss).

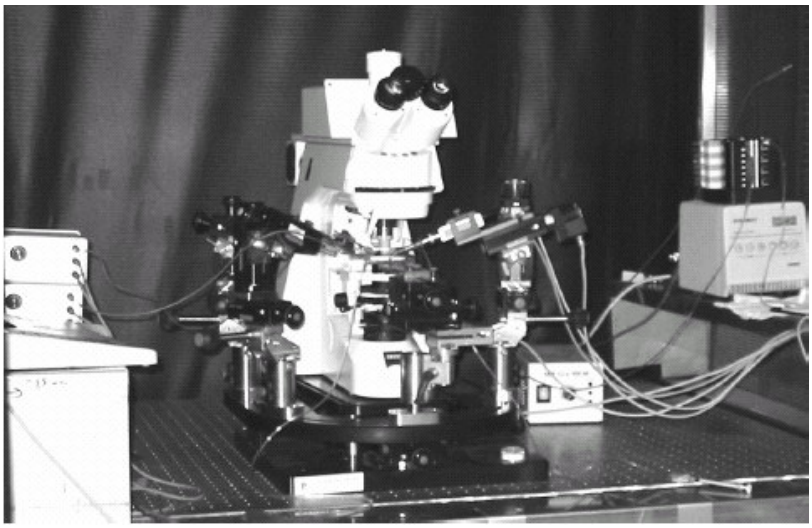

Figure 4.1. The patch-clamp setup used in our experiments

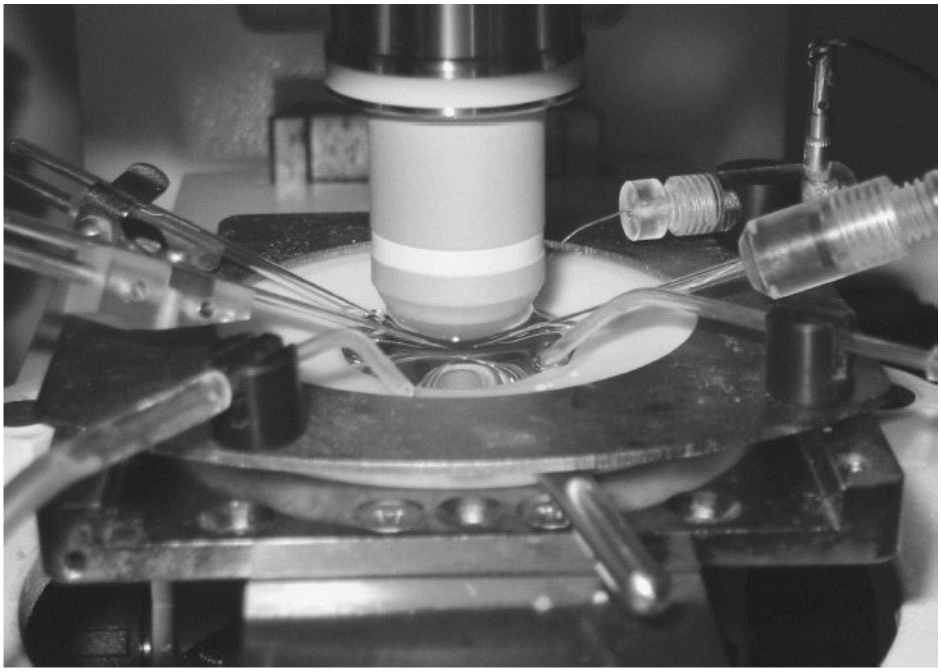

Figure 4.2. A close-up of the slice bath recording chamber, with brain slice, reference electrode, patch pipette and cannula for ACSF supply (right), stimulation pipettes and cannula for ACSF discharge (left), as seen during recording.

Borosilicate glass single barrel capillaries (World Precision Instruments PG52165-4) were used to pull patch-clamp recording and stimulation pipettes, on a Flaming/Brown pipette puller (Sutter). After fire polishing using a microforge (Narishige), the recording pipette tips were typically 1-2 µm in diameter. When filled with the recording saline their resistance was typically 2-5 MΩ.

Patch clamp recording from the soma of Purkinje cells in rat cerebellar slices

Sagittal slices of the cerebellar vermis (200-250 µm) were prepared from postnatal-day 18-25 Sprague-Dawley rats by using a vibratome (Leica) and ice-cold standard ACSF containing 124 mM NaCl, 5 mM KCl, 1.25 mM Na_2HPO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose, bubbled with 95% O_2/5% CO_2. After slicing, the slices were placed in an incubation chamber filled with ACSF, and bubbled with 95% O_2/5% CO_2 at room temperature. After a recovery period of at least 1 h, the slices were placed in the slice chamber that was perfused at a flow rate of 3 ml/min with roomtemperature ACSF supplemented with 20 µM bicuculline methiodide to block γ-aminobutyric acid type A receptors.

Under visual control, stimulation electrodes filled with ACSF were carefully placed in the molecular and/or granular cell layer around the Purkinje cell that was selected for recording (Figure 4.3). For standard EPSC recordings, the recording electrode was filled with a K+-based solution containing 9 mM KCl, 10 mM KOH, 120 mM K gluconate, 3.48 mM $MgCl_2$, 10 mM HEPES, 4 mM NaCl, 4 mM Na_2ATP, 0.4 mM Na_3GTP, and 17.5 mM sucrose

(300 ± 5 mM ; pH 7.25 ± 0.3). In all experiments, the calcium chelator BAPTA (5 mM (K_4^+ salt)) was added to the patch pipette saline. All drugs used for ACSF or recording pipette solutions were purchased from Sigma, except for BAPTA (Molecular Probes).

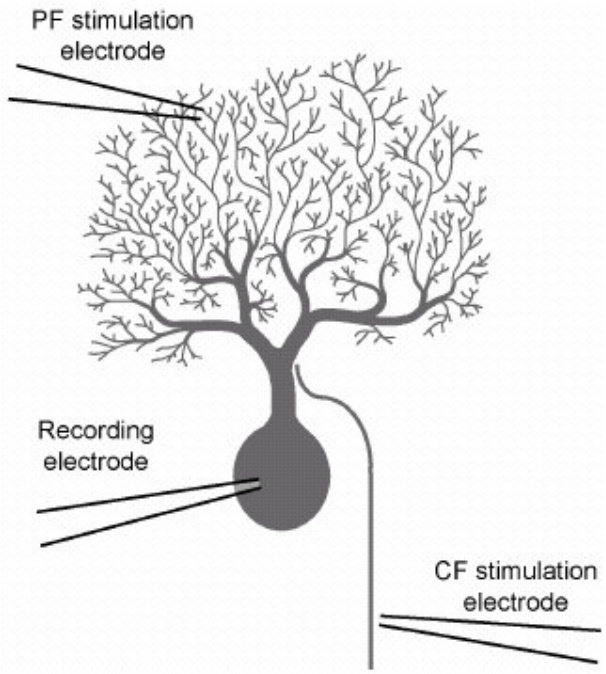

Figure 4.3. A schematic drawing of a Purkinje cell and the recording and stimulation electrodes. The recording electrode is attached to the Purkinje cell soma. The stimulation electrodes are placed in the granule cell layer, through which the climbing fiber ascends, and in the molecular layer, in which the parallel fibers are located.

When entering the bath, positive pressure was applied to the patch pipette, generating a pressure wave of pipette solution in front of the tip, in order to keep the tip clean and open and to clean the Purkinje cell membrane. Once the patch pipette tip touched the membrane (as seen by the dimpling of the membrane), the positive pressure was released, and slight negative pressure was applied until the membrane was tightly sealed to the electrode tip. Hyperpolarization of the patch pipette to -65 mV was used to aid seal formation. The forming of the seal could be registered as a large increase in resistance, typically reaching values over 10^9 Ω (1 GΩ: 'gigaseal'). Subsequently the patch membrane was ruptured by brief pulses of suction to the patch pipette, thereby creating a hole in the plasma membrane and gaining access to the Purkinje cell interior. This rupture leaves the seal between the pipette and plasma membrane intact, thus preventing leak currents flowing between the pipette and the reference electrode, and separating the Purkinje cell's constituents from the ACSF in the bath. This configuration is characterized by a low-resistance access to the cell interior through the pipette tip (typically 2-5 MΩ), allowing a voltage-clamp of the whole cell ('whole-cell' configuration). In whole-cell 'voltage-clamp' mode the membrane potential of the neuron is controlled by the potential applied to the pipette electrode, enabling the recording of membrane currents. 'Current clamp' mode enables one to monitor the changes in membrane potential, which constitute the physiological response of a cell, such as action potentials. Currents were filtered at 2.9 kHz, digitized at 8 kHz, and acquired by using PULSE 8.53 software (HEKA). During voltage-clamp recordings, holding potentials were chosen in the range of -70 to -60 mV.

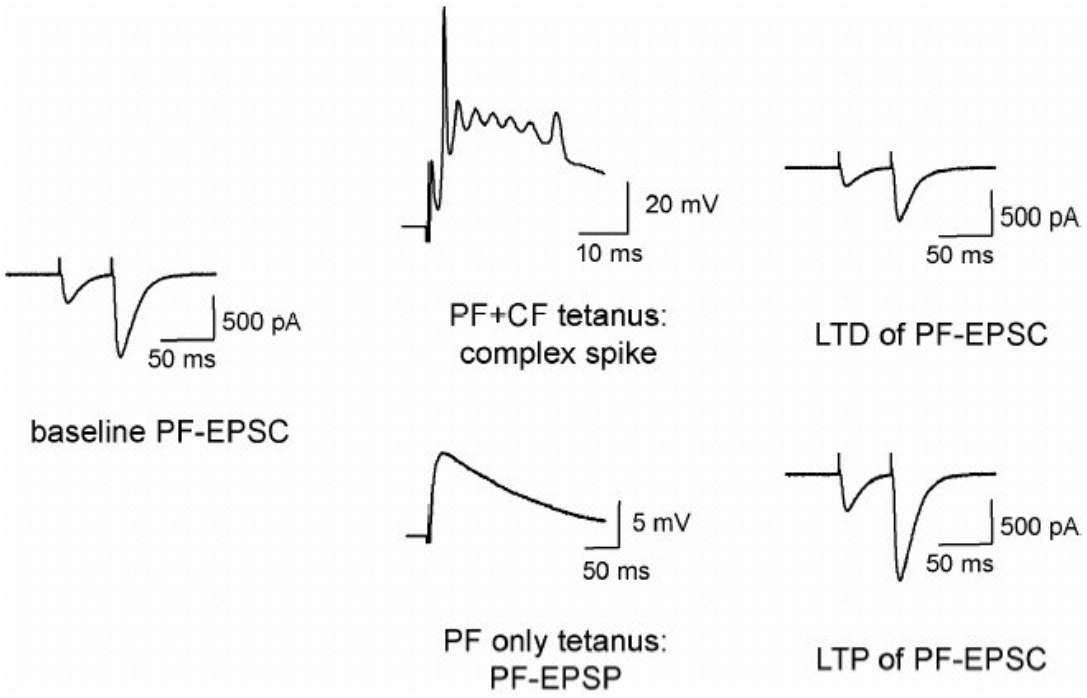

Figure 4.4. Induction of long-term PF-PC synaptic plasticity. A representative trace of the PF-evoked EPSC (left panel). Climbing fibre stimulation elicits a complex spike after PF+CF coativation and parallel PF-only stimulation elicits PF-EPSP, respectively (mid panel). A reduction of PF-PC synaptic strength, which is expressed as decreased PF-EPSC amplitude (top right panel). PF-only stimulation induces an increase of PF-PC synaptic strength, expressed as an increase of PF-EPSC amplitude (bottom right panel).

Synaptic gain changes

In the majority of our experiments we investigated long-term changes in the strength of the PF-PC synapse (Figure 4.4). In cerebellar slices, PF-LTD can be induced by coactivation of PF and CF for 5 min at a rate of 1 Hz (Karachot et al., 1994), while PF-LTP can be induced by activation of the PF alone for 5 min at 1 Hz (Lev-Ram et al., 2002). After breaking the gigaseal, EPSCs in response to PF stimulation were recorded at 0.05 Hz in voltage-clamp mode. We recorded these PF-EPSCs, until a stable baseline with only small variations in amplitude was recorded for 5-10 min. Test responses were evoked at a frequency of 0.05Hz using 0.5-2μA pulses. To evoke EPSCs, paired pulses (100ms interpulse interval) were applied to the PF input. In all experiments, cells were switched to current-clamp mode during tetanization, in order to allow the PC to fire complex spikes. The protocol for inducing presynaptic LTP consisted of 120 pulses at 8Hz to the PFs alone. For PF-CF co-stimulation, the CF input was activated with every second PF stimulus, resulting in 4Hz and 8Hz activation, respectively. The LTP protocol used has been described to induce a presynaptically expressed form of PF-LTP (Salin et al., 1996). Induction of presynaptic PF-LTP depends on presynaptic calcium influx, activation of calcium/calmodulin-sensitive adenylyl cyclase I, and the subsequent activation of cAMP-dependent kinase (PKA; Salin et al., 1996; Chen and Regehr, 1997; Linden, 1997; Storm et al., 1998). After the tetanus we

resumed the 0.05 Hz PF-stimulation in voltage-clamp mode, and, if possible, we recorded the EPSCs for 20-30 more minutes. When PF-LTP was successfully induced, PF EPSCs were larger in size after successful PF-LTP induction by PF-only stimulation. In some experiments, bath-application of the CB receptor agonist WIN55,212-2 (2μM) caused a pronounced reduction in EPSC amplitudes on its own showing LTD of PF-EPSC.

In order to establish the localization of the synaptic gain changes, we used a paired-pulse facilitation (PPF) paradigm (Figure 4.4). PPF is a very short-term enhancement in synaptic efficacy attributed to residual presynaptic Ca^{2+} facilitating more transmitter release (Atluri and Regehr, 1996; Salin et al., 1996). Changes in PPF during PF-LTP would argue that PF-LTP is expressed presynaptically, whereas unchanged PPF would suggest a postsynaptic expression site.

PF-LTP can be induced by 8Hz PF tetanization, but is blocked when the CF is co-stimulated. CF activity can be substituted for by bath-application of the CB receptor agonist WIN55,212-2. In the presence of the CB1 receptor antagonist AM251, CF activity no longer suppresses PF-LTP. The results of the experiments mentioned in this paragraph will be described in detail in chapter 5.

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Chapter 5

Climbing Fiber-Evoked Endocannabinoid Signaling Heterosynaptically Suppresses Presynaptic Cerebellar Long-Term Potentiation

Abstract

Endocannabinoid signaling has been demonstrated to mediate depolarization-induced suppression of excitation at climbing fiber (CF) and parallel fiber (PF) synapses onto cerebellar Purkinje cells. Here, we show that CF-evoked release of cannabinoids (CBs) additionally suppresses a presynaptic form of long-term potentiation (LTP) at PF synapses. PF-LTP can be induced by 8 Hz PF tetanization but is blocked when the PF tetanization is paired with 4 or 1 Hz CF coactivation. CF activity can be substituted for by bath application of the CB receptor agonist WIN55,212-2 [***R***(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone]. In the presence of the CB1 receptor antagonist AM251 [***N***-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1***H***-pyrazole-3-carboxamide], CF activity no longer suppresses PF-LTP. Presynaptic potentiation can also be obtained by the adenylyl cyclase activator forskolin. WIN55,212-2 blocked this forskolin-mediated enhancement, showing that CB1 receptor activation interferes with the adenylyl cyclase–protein kinase A cascade, which participates in LTP induction. CF activity has been described to promote the induction of postsynaptic PF-long-term depression (LTD) and to impair postsynaptic PF-LTP. Our observation that CF activity blocks the induction of presynaptic LTP suggests that the CF input controls all forms of presynaptic and postsynaptic PF plasticity and that CF activity provides a "safety lock" to prevent an enhancement of transmitter release while postsynaptic AMPA receptor function is downregulated during LTD.

Introduction

Long-term depression (LTD) at cerebellar parallel fiber (PF)–Purkinje cell (PC) synapses is considered a cellular correlate of cerebellar motor learning (Hansel et al., 2001; Ito, 2001). Whereas PF-LTD induction requires the simultaneous activity of the PF and the climbing fiber (CF) inputs onto PCs at low frequencies (e.g., 1 Hz for 5 min), PF stimulation alone leads to the induction of long-term potentiation (LTP) (Lev-Ram et al., 2002; Coesmans et al., 2004). The CF acts as a heterosynaptic control switch for postynaptic PF plasticity: CF-evoked complex spikes evoke large dendritic calcium transients that trigger LTD induction, whereas LTP results from smaller calcium transients in the absence of complex spikes (Coesmans et al., 2004). PF stimulation alone can also elicit a presynaptically expressed form of PF-LTP when applied for short durations at high frequencies (e.g., 8 Hz for 15 s) (Salin et al., 1996). Induction of presynaptic PF-LTP depends on the activation of calcium/calmodulin-sensitive adenylyl cyclase I and the subsequent activation of cAMP-dependent kinase [protein kinase A (PKA)] (Salin et al., 1996; Chen and Regehr, 1997; Linden, 1997; Storm et al., 1998; Jacoby et al., 2001).

In postsynaptic PF plasticity, CF activity determines whether LTD or LTP is induced (Lev-Ram et al., 2002; Coesmans et al., 2004). So far, it has not been examined whether CF activity can suppress presynaptic LTP. We reasoned that such a "safety lock" might exist to prevent the induction of presynaptic LTP while LTD is expressed postsynaptically, because the postsynaptic downregulation of response amplitudes might otherwise be accompanied by an increase in transmitter release. Retrograde endocannabinoid signaling might provide a possible link between CF activity and the suppression of presynaptic PF-LTP. At cerebellar and hippocampal synapses, dendritically released endocannabinoids can bind to presynaptically located CB1 receptors (Diana et al., 2002) and cause depolarization-induced suppression of inhibition (Llano et al., 1991; Pitler and Alger, 1992; Wilson et al., 2001), or its

equivalent at excitatory synapses, depolarization-induced suppression of excitation (DSE) (Kreitzer and Regehr; 2001). The release of endocannaboids involved in DSE at PF–PC synapses can be activated by CF activity (Brenowitz and Regehr, 2005), suggesting that complex spike-associated calcium transients in PC dendrites are sufficient to initiate the release process.

There have been contradictory reports on the effects of retrograde endocannabinoid signaling on synaptic plasticity in the cerebellum. It has been demonstrated that cannabinoids impair PF-LTD induction (Levenes et al., 1998), but also that cannabinoid signaling is required for PF-LTD induction (Safo and Regehr, 2005). The suppressive effect was explained by a reduction in transmitter release during tetanization, and the permissive effect was explained by a CB1 receptor-mediated release of nitric oxide (NO) from PF terminals. Here, we demonstrate that endocannabinoid signaling can suppress presynaptic PF-LTP. We show that this retrograde signaling mechanism is recruited by CF activity to suppress presynaptic PF-LTP and that this effect can be attributed to the CB1 receptor-mediated blockade of adenylyl cyclase. This CB1 receptor-mediated suppression of presynaptic LTP provides an alternative explanation for the permissive effect of retrograde endocannabinoid signaling in PF-LTD induction.

Materials and methods

Slice preparation:

Sagittal slices of the cerebellar vermis (200–250 μm thick) were prepared from postnatal day 18–25 Sprague Dawley rats in ice-cold artificial CSF (ACSF). The slices were kept in ACSF containing the following (in mM): 124 NaCl, 5 KCl, 1.25 Na_2HPO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose bubbled with 95% O_2 and 5% CO_2. The ACSF used for perfusion was supplemented with 20 μM bicuculline methiodide to block $GABA_A$ receptors. Whole-cell patch-clamp recordings were performed at room temperature using an EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Recording electrodes were filled with a solution containing the following (in mM): 9 KCl, 10 KOH, 108 K-gluconate, 3.48 $MgCl_2$, 10 HEPES, 4 NaCl, 4 Na_2ATP, 0.4 Na_3GTP, 5 BAPTA (K_4^+ salt), and 17.5 sucrose, pH 7.25. When the BAPTA concentration was raised to 30 mM (see Figure 5.1 *B*,*D*), 15 mM $CaCl_2$ was added to maintain the resting calcium concentration. The K-gluconate concentration was lowered to maintain the desired osmolarity and ionic strength. All drugs were purchased from Sigma (St. Louis, MO), except for BAPTA (Invitrogen, Eugene, OR), AM251 [*N*-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; Tocris, Bristol, UK], and PKC [19-36] (Calbiochem, La Jolla, CA). AM251, WIN55,212-2 [*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone], and forskolin were dissolved in DMSO and kept as stock solution at –20°C.

Electrophysiology:

Currents were filtered at 3 kHz, digitized at 8 kHz, and acquired using PULSE software (HEKA Elektronik). During voltage-clamp experiments, PCs were held at potentials in the range of –60 to –70 mV. For extracellular stimulation, standard patch pipettes were filled with external saline. The CF input was activated in the granule cell layer, and the PF input was activated in the molecular layer. Test responses (typically ~200 pA) were evoked at a frequency of 0.05 Hz using 0.5–2 μA pulses. To evoke EPSCs, paired pulses (100 ms

interpulse interval) were applied to the PF input. In all experiments, cells were switched to current-clamp mode during tetanization. The protocol for inducing presynaptic LTP consisted of 120 pulses at 8 Hz to the PFs. In experiments in which the CF was coactivated, the presence of a CF response was confirmed in the beginning of the recording, but CF stimulation was subsequently only resumed for tetanization. Recordings were excluded from the study if the series or the input resistance varied by >15% over the course of the experiments. All values are shown as percentage of baseline ± SEM. For statistical analysis, we used the paired Student's *t* test and the Mann–Whitney *U* test where appropriate.

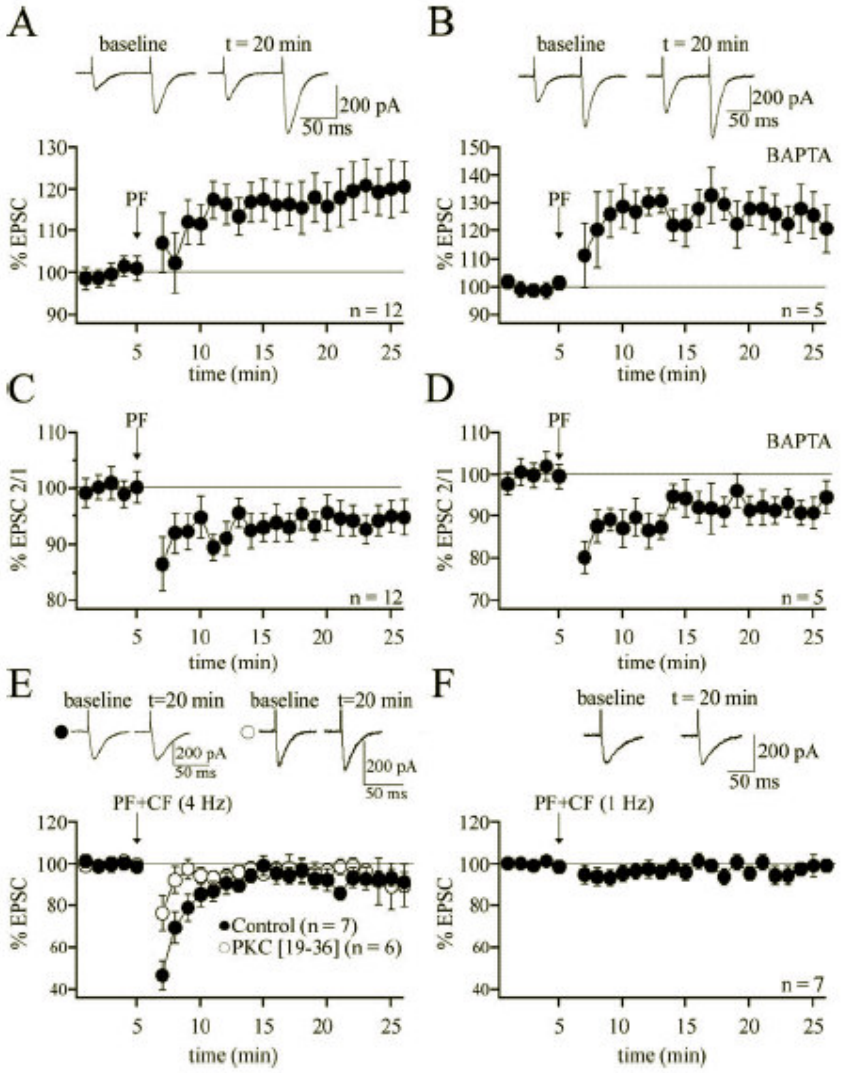

Figure 5.1. CF activity suppresses the induction of presynaptic PF-LTP. ***A***, PF-LTP can be induced by PF stimulation at 8 Hz for 15 s (n = 12). Each data point represents the average of three successive test responses evoked at 0.05 Hz. The traces on top show EPSCs before and after LTP induction. The arrow indicates the onset of tetanization. ***B***, PF-LTP can be induced when BAPTA (30 mM) is added to the internal saline (n = 5). ***C***, PPF ratio (EPSC 2/EPSC 1) from the LTP group shown in ***A***. ***D***, PPF ratio from the BAPTA group shown in ***B***. ***E***, PF-LTP is abolished when 8 Hz PF stimulation is paired with 4 Hz CF stimulation (n = 7). LTP suppression using paired CF stimulation can also be observed when PKC [19-36] (100 µM) is added to the internal saline (n = 6). ***F***, PF-LTP is blocked when the 8 Hz PF stimulation is paired with CF stimulation at 1 Hz (n = 7). Error bars indicate SEM.

Results

To characterize the effect of CF-evoked cannabinoid signaling on presynaptic PF-LTP, we performed whole-cell patch-clamp recordings from PCs in rat cerebellar slices. During the test periods before and after tetanization, EPSCs were monitored in voltage-clamp mode. For tetanization, recordings were switched to current-clamp mode. LTP was observed after 8 Hz PF tetanization for 15 s. This manipulation resulted in a potentiation of EPSC amplitudes (120.2 ± 6.3% of baseline; n = 12; last 5 min) (Figure 5.1 *A*) that reached statistical significance ($p < 0.01$; paired Student's t test). The LTP protocol used has been described to induce a presynaptically expressed form of PF-LTP (Salin et al., 1996). The presynaptic origin of the potentiation was confirmed by activating PF–EPSC pairs at an interpulse interval of 100 ms and monitoring the paired-pulse facilitation (PPF) ratio. The LTP observed after tetanization was associated with a modest reduction in the PPF ratio (94.3 ± 2.9% of baseline; n = 12; last 5 min) (Figure 5.1 *C*), which reached statistical significance ($p < 0.01$; paired Student's t test), indicating a significant presynaptic component of the potentiation. In subsequent experimental groups (see below), we restricted measurements of the PPF ratio to test for the efficacies of drugs known to act presynaptically. The reason is that the PPF ratio is a simple, but rather unreliable, indicator of the expression site of synaptic gain changes. To obtain independent proof of the presynaptic origin of this form of LTP, we applied the 8 Hz PF tetanization protocol when the calcium chelator BAPTA (30 mM) was added to the internal saline. At this concentration, BAPTA blocks the induction of postsynaptic LTP (Coesmans et al., 2004□). In the presence of BAPTA, EPSC amplitudes were still potentiated (124.7 ± 7.9% of baseline; n = 5; last 5 min; $p < 0.05$) (Figure 5.1 *B*), and the PPF ratio was reduced (92.2 ± 3.4%; n = 5; last 5 min; $p < 0.05$) (Figure 5.1 *D*). Neither the changes in the EPSC amplitudes nor the PPF alterations differed between the control group and the BAPTA group ($p > 0.05$; Mann–Whitney U test). These results suggest that the LTP obtained with 8 Hz PF tetanization is indeed presynaptically expressed.

To examine whether paired CF activity impairs the probability to induce LTP, we costimulated the PF and CF inputs. The same PF tetanization protocol was applied as described above, but now the CF input was coactivated with every second PF stimulation pulse, effectively resulting in a 4 Hz stimulation for 15 s. Although the PF activation pattern was the same, application of this pairing protocol did not result in PF-LTP induction (92.9 ± 4.6% of baseline; n = 7; last 5 min; $p > 0.05$) (Figure 5.1 *E*). This difference in LTP observed in the absence of paired CF stimulation was statistically significant ($p < 0.01$, Mann–Whitney U test). To be able to exclude the possibility that paired PF and CF stimulation led to the induction of postsynaptic LTD, which might simply mask presynaptic LTP, we applied the same stimulus protocol when LTD induction was blocked by adding the PKC inhibitory peptide PKC [19-36] (100 μM) to the internal saline (Hansel et al., 2001). In the presence of PKC [19-36], paired stimulation still did not elicit LTP (93.4 ± 7.9% of baseline; n = 6; last 5 min; $p > 0.05$) (Figure 5.1 *E*), indicating that LTD did not mask presynaptic LTP.

Paired PF and CF stimulation also blocks the induction of presynaptic LTP when the frequency of CF stimulation is reduced to 1 Hz, which is in the range of spontaneous complex spike activity observed *in vivo* (98.4 ± 3.8% of baseline; n = 7; last 5 min) (Figure 5.1 *F*). However, the transient EPSC depression seen after pairing with CF stimulation at 4 Hz was not observed after 1 Hz CF stimulation. Thus, it seems that this short-term depression depends on the level of CF activity, but it might not transfer into LTD because of the short duration of tetanization applied here.

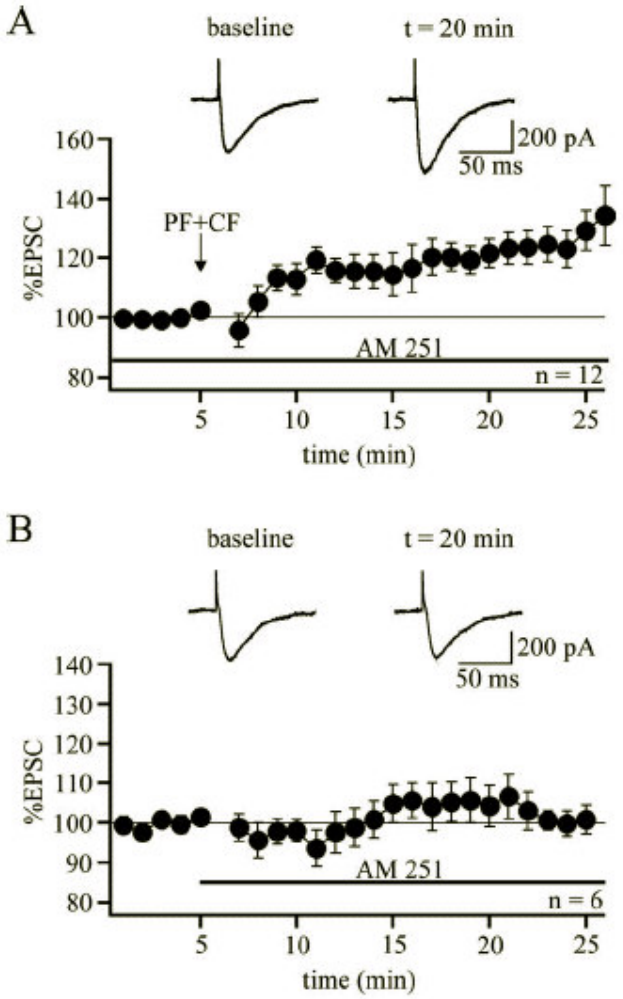

Figure 5.2. The CB1 receptor antagonist AM251 rescues PF-LTP. ***A***, PF-LTP is observed when PF and CF synapses are coactivated in the presence of AM251 (5 µM; $n = 12$). ***B***, Bath application of AM251 does not alter EPSC amplitudes ($n = 6$). The horizontal bar indicates the period in which AM251 was bath applied. Error bars indicate SEM.

Retrograde endocannabinoid signaling can provide a link between CF activity and inhibition of transmitter release at PF terminals (Brenowitz and Regehr, 2005). To test whether the CF-mediated suppression of PF-LTP involves the activation of CB1 receptors, we applied paired 8 Hz PF and 4 Hz CF tetanization, while the CB1 receptor antagonist AM251 was present in the bath (5 µM). In the presence of AM251, PF-LTP was rescued (127.0 ± 6.8% of baseline; $n = 12$; last 5 min; $p < 0.01$) (Figure 5.2 *A*). This potentiation was significantly different from the EPSC changes resulting from PF and CF stimulation in the absence of the drug ($p < 0.01$, Mann–Whitney *U* test). In the absence of tetanization, AM251 did not alter EPSC amplitudes (100.9 ± 3.3%; $n = 6$; last 5 min; $p > 0.05$) (Figure 5.2 *B*). The opposite strategy is to test whether PF tetanization alone at 8 Hz for 15 s can still elicit LTP when CB receptors are tonically activated. Bath application of the CB receptor agonist WIN55,212-2 (2 µM) caused a pronounced reduction in EPSC amplitudes (37.2 ± 5.1% of baseline; $n = 7$; last 5 min; $p < 0.01$) (Figure 5.3 *A*), which was associated with a significant increase in the PPF ratio (123.8 ± 8.5%; $n = 7$; last 5 min; $p < 0.01$) (Figure 5.3 *B*), indicating that the drug acts on presynaptic CB receptors to reduce transmitter release. When WIN55,212-2 was present in the bath, application of the otherwise LTP-inducing PF tetanization protocol did not cause a potentiation (96.1 ± 3.5%; $n = 17$; last 5 min; $p > 0.05$) (Figure 5.3 *C*). This blockade was significantly different from the LTP seen under control

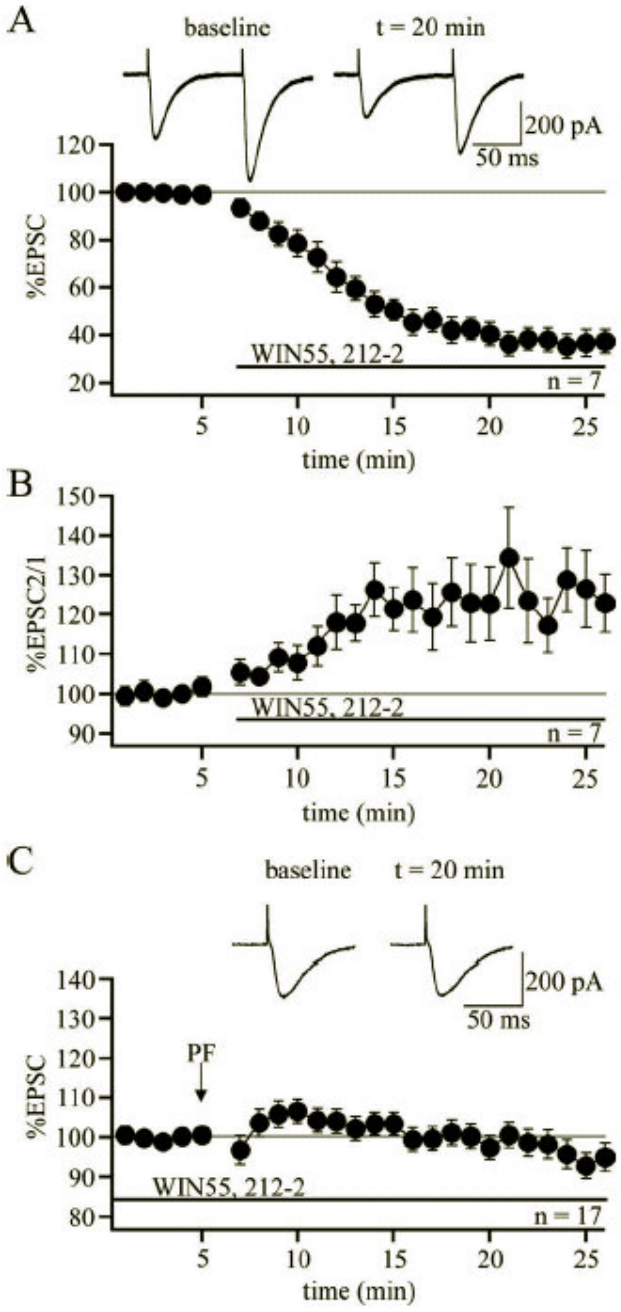

Figure 5.3. The CB receptor agonist WIN55,212-2 blocks PF-LTP. ***A***, Bath application of WIN55,212-2 (2 μM) depresses PF-EPSCs (n = 7). ***B***, PPF ratio (EPSC 2/EPSC 1) from the group shown in ***A***. The horizontal bar indicates the presence of WIN55,212-2 in the bath. ***C***, PF-LTP induced by 8 Hz PF stimulation for 15 s is abolished in the presence of WIN55,212-2 (n = 17). Error bars indicate SEM.

conditions ($p < 0.01$, Mann–Whitney U test). Together, the results obtained from the CB1 receptor antagonist (AM251) and the CB receptor agonist (WIN55,212-2) experiments suggest that activation of presynaptic CB1 receptors blocks the induction of presynaptic PF-LTP and that this pathway can be recruited by CF activity.

CB1 receptor activation can inhibit adenylyl cyclase activity and can modify K- and Ca-selective channels (Ameri, 1999). It has been shown that the induction of presynaptic PF-LTP involves the activation of adenylyl cyclase (Salin et al., 1996; Storm et al., 1998). Therefore, we next examined whether, under our experimental conditions, receptor activation

would interfere with the adenylyl cyclase pathway. To do so, we bath applied the adenylyl cyclase activator forskolin (50 µM). Forskolin application resulted in a strong potentiation of EPSCs (178.4 ± 17.7% of baseline; $n = 6$; last 5 min; $p < 0.01$) (Figure 5.4 *A*), which was associated with a significant reduction in the PPF ratio (89.2 ± 4.7%; $n = 6$; last 5 min; $p < 0.01$) (Figure 5.4 *B*). In contrast, when forskolin was added to the bath in the presence of WIN55,212-2, no subsequent enhancement of EPSC amplitudes could be observed (99.9 ± 3.8%; $n = 6$; last 5 min) (Figure 5.4 *C*). This effect was significantly different from the potentiation seen in the absence of WIN55,212-2 ($p < 0.01$; Mann–Whitney *U* test). As forskolin activates adenylyl cyclase and thus acts downstream of K- or Ca-selective ion channels, the blockade of the forskolin-mediated EPSC potentiation by WIN55,212-2 suggests that the CB receptor agonist indeed interferes at the level of adenylyl cyclase activation.

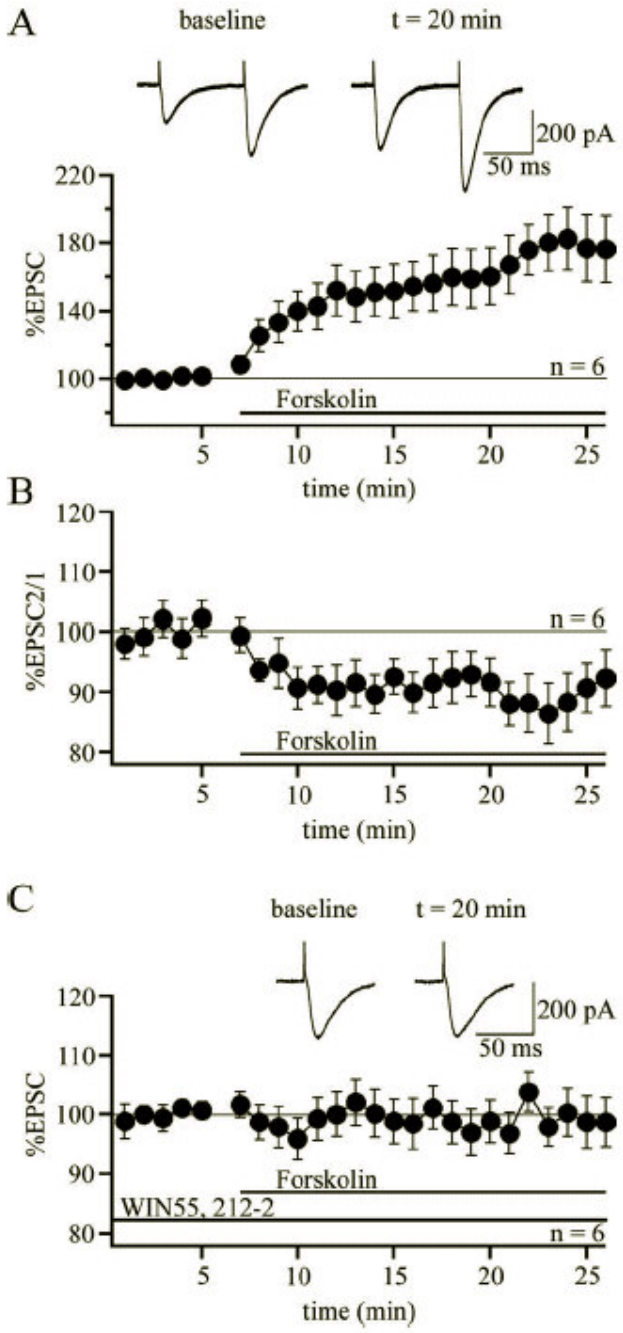

Figure 5.4. WIN55,212-2 prevents the activation of adenylyl cyclases by forskolin. ***A***, Bath application of the adenylyl cyclase activator forskolin (50 µM) enhances PF-EPSCs ($n = 6$). ***B***, PPF ratio (EPSC 2/EPSC 1) from the group shown in ***A***. ***C***, WIN55,212-2 (2 µM) abolishes the forskolin-mediated enhancement of PF-EPSCs ($n = 6$). The horizontal bars indicate the presence of WIN55,212-2 and forskolin, respectively, in the bath. Error bars indicate SEM.

Discussion

The main finding of this study is that activity of the heterosynaptic CF input suppresses the induction of a presynaptically expressed form of PF-LTP through retrograde cannabinoid signaling. Derivatives of the cannabinoid Δ^9-tetrahydrocannabinol, the primary psychoactive component of the cannabis plant, are powerful modulators of synaptic transmission. In the brain, the endocannabinoids anandamide and 2-arachidonylglycerol function as modulators of transmitter release by binding to G-protein-coupled CB1 receptors, which are located on synaptic terminals. CB1 receptor activation can both inhibit adenylyl cyclases and modify K- and Ca-selective channels (Ameri, 1999). The latter mode of action has been shown to be involved in the acute effects of CB1 receptor activation on PF-EPSPs (Daniel et al., 2004) and DSEs (Brown et al., 2004). The resulting downregulation of transmitter release has consequences for LTP/LTD induction: cannabinoids impair LTP as well as LTD induction at glutamatergic synapses (Levenes et al., 1998; Misner and Sullivan, 1999) but have also been reported to facilitate the induction of LTD (Gerdeman et al., 2002). Moreover, cannabinoids have been described to reduce GABA release (Chevaleyre and Castillo, 2003). This effect likely explains the observation that endocannabinoids can, through heterosynaptic interaction, also facilitate LTP induction (Carlson et al., 2002; Chevaleyre and Castillo, 2004). The effects of CB1 receptor activation on K- and Ca-selective ion channels and adenylyl cyclases might interfere with presynaptic forms of long-term plasticity as well. For example, it has been shown that presynaptic LTD at neocortical synapses requires CB1 receptor activation (Sjöström et al., 2003).

Here, we report that endocannabinoid signaling can be triggered by CF activity and that the subsequent activation of CB1 receptors impairs presynaptic PF-LTP. Endocannabinoid signaling is well suited to mediate this CF-evoked suppression because it can be triggered in a calcium-dependent way (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll; 2001; Brenowitz and Regehr, 2003), and because CB1 receptors are located at PF terminals. Our data show that cannabinoids indeed act as mediators of the CF effect. When the PF and CF inputs were coactivated, bath application of the CB1 receptor antagonist AM251 could rescue PF-LTP. Moreover, effects of CF stimulation could be mimicked by tonically activating CB1 receptors with bath application of WIN55,212-2, which impaired LTP induction. Agonists of CB1 receptors have been reported previously to reduce transmitter release (Levenes et al., 1998; Takahashi and Linden, 2000; Daniel et al., 2004). Activation of CB1 receptors has several effects, which all result in a reduction of transmitter release: (1) it can lead to a downregulation of voltage-dependent calcium channels; (2) it can cause an upregulation of K channels; and (3) it can interfere with the activation of adenylyl cyclases and thus with the PF-LTP induction cascade. All three mechanisms could account for the observed effects. By showing that in the presence of the CB receptor agonist WIN55,212-2 the adenylyl cyclase activator forskolin no longer potentiates PF-EPSCs, we confirm that CB receptor activation indeed suppresses the activity of adenylyl cyclase. This finding does not exclude the possibility that Ca- or K-selective channels are also modified, but it suggests that during tetanization, the activation of CB1 receptors blocks the adenylyl cyclase–PKA signaling cascade that otherwise leads to the induction of presynaptic LTP through phosphorylation of the PKA substrate RIM1α (Castillo et al., 2002; Lonart et al., 2003).

The observed endocannabinoid-mediated suppression of presynaptic PF-LTP occurs under conditions that also favor the induction of postsynaptically expressed LTD (paired PF and CF activity). However, when LTD induction was blocked by adding the PKC inhibitory peptide PKC [19-36], CF coactivation was still able to suppress presynaptic LTP, suggesting that LTD

does not simply mask LTP. Rather, the CF-evoked suppression of presynaptic LTP might provide a safety lock, which prevents that LTP is expressed presynaptically, while LTD is expressed postsynaptically. The signaling cascades involved partially overlap with those described for DSE; CF activity causes complex spike-associated calcium transients that trigger the release of endocannabinoids (Brenowitz and Regehr, 2005). It is remarkable that the CF apparently controls all forms of PF plasticity. Simultaneous CF activity postsynaptically promotes the induction of PF-LTD by providing a large calcium transient, whereas postsynaptically expressed PF-LTP is triggered by smaller calcium transients in the absence of CF activity (Coesmans et al., 2004). Postsynaptic PF-LTP depends on the activation of protein phosphatases PP1, PP2A, and PP2B (Belmeguenai and Hansel, 2005), whereas PF-LTD depends on the activation of PKC (for review, see Hansel et al., 2001). Thus, it can be argued that CF activity, by promoting the PKC cascade, has a tight grip on the polarity of postsynaptic PF plasticity. Our observation that CF activity additionally suppresses the induction of presynaptic PF-LTP supports the notion that the CF input heterosynaptically controls all forms of PF synaptic plasticity.

The suppressive effect of CF coactivation was not only observed with CF stimulation at 4 Hz, but also at 1 Hz, suggesting the possibility that presynaptic LTP is permanently suppressed under resting conditions. In our recordings, spike activity is prevented in PCs by constant current injection. CF stimulation at 1 Hz thus provides a transient increase in PC activity that might trigger endocannabinoid release only under these *in vitro* conditions. Even if endocannabinoids were released under resting conditions *in vivo*, there are physiological activity patterns favoring presynaptic LTP because complex spikes are not constantly fired at 1 Hz. Transient inhibition of CFs by elevated cerebellar activity has been shown to facilitate the extinction of conditioned eyelid responses (Medina et al., 2002) and might create time windows without complex spike activity during which LTP can be induced.

Previous reports have suggested that cannabinoids interfere with the induction of PF-LTD. A previous study has shown that cannabinoid signaling impairs PF-LTD induction, which was explained by reduced transmitter release during tetanization (Levenes et al., 1998). The opposite effect was reported recently, showing that cannabinoid signaling is permissive for PF-LTD induction (Safo and Regehr, 2005). The resulting paradox that a retrograde messenger modifies the induction probability of postsynaptic LTD was addressed by suggesting that CB1 receptor activation might trigger NO release from PF terminals. Because NO has been suggested to be required for LTD induction, CB1 receptor activation could promote LTD induction, despite an accompanying transient reduction in transmitter release. The study by Safo and Regehr (2005) and ours complement each other and, in fact, provide two sides of the same coin. Whereas these authors demonstrated that cannabinoid signaling is required for the induction of PF-LTD (which is postsynaptically expressed), we demonstrate here that cannabinoid signaling suppresses a presynaptic form of PF-LTP. This LTP suppression might well provide a better explanation for the endocannabinoid-mediated promotion of PF-LTD than NO signaling as suggested by Safo and Regehr (2005). First, it has recently been shown that NO might indeed be involved in LTD induction; however, it is not released from PF terminals, but rather from interneurons (Shin and Linden, 2005). Second, simultaneous induction of presynaptic LTP would counteract postsynaptic LTD. It is likely that the CF-evoked calcium transients in PC dendrites can postsynaptically promote LTD and, through the release of endocannabinoids, presynaptically suppress LTP at the same time. The latter effect would facilitate LTD induction and enhance the observed LTD magnitude.

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Chapter 6

General Discussion

In the preceding chapters I have described several aspects of hippocampal and cerebellar function. There is growing interest in investigating physiological effects mediated by mGluRs as they are distributed throughout the CNS, and play an important role in the modulation of synaptic transmission causing long-term changes in synaptic transmission in the hippocampus and the cerebellum. The relation between synaptic transmission, synaptic plasticity, and learning behaviour was studied using various models and techniques. Glutamate receptors mediate most excitatory neurotransmission in the brain and play an important role in neural plasticity, neural development and neurodegeneration. Group I mGluRs are involved in many CNS functions and may participate in a variety of disorders such as pain, epilepsy, ischemia, and chronic neurodegenerative diseases (Bordi and Ugolini 1999; Flor et al. 2002; Folbergrova et al. 2003; Meldrum 1994). The mGluR antagonists are considered potential therapeutics in the treatment of these diseases. In the present study we investigated the effects of mGluR1 and mGluR5 antagonists on PTZ kindling. Beyond epilepsy and kindling, group I mGluRs have been implicated to play a role in different forms of synaptic plasticity such as LTP/LTD as well as in learning and memory formation. In this chapter the results, their correlation, and some functional implications of mGluRs in hippocampus and cerebellar plasticity will be discussed.

6.1. mGluRs in epileptogenesis

Excessive activation of glutamate receptors is thought to play a role in the pathophysiology of many neurological diseases including epilepsy. While it is well documented that the overactivation of ionotropic glutamate receptors leads to seizures and excitotoxic injury, little is known about the role of mGluRs in epileptogenesis and neuronal injury. It is well documented that the overactivation of iGluRs with their specific subtype agonists NMDA, kainate, and AMPA leads to seizures and excitotoxic injury throughout the CNS and, in particular, in the hippocampus, one of the most vulnerable cerebral regions to injury after seizures (Meldrum and Garthwaite, 1990; Choi, 1994).

mGluRs have been implicated in different forms of synaptic plasticity, such as LTP and LTD, memory formation (Conn and Pin, 1997; Nakanishi, 1994; Riedel, 1996; Riedel et al., 1996b) and changes associated with epilepsy (Chapman, 1998). An interesting feature of mGluR-mediated effects is that they are often long-lasting. Changes produced by mGluR stimulation have long-lasting effects on cortical epileptiform activities. Changes in the expression of mGluRs (Blümcke et al., 2000) and in their function (Dietrich et al., 1999; Nagerl et al., 2000) have been found in the human hippocampus of temporal lobe epilepsy (TLE).

Because mGluR function is so intimately linked with regulation of glutamatergic and GABAergic neurotransmission, it is not surprising that this family of receptors strongly influences the induction, propagation, and termination of epileptic activity in the CNS (Chapman, 1998). For this reason, mGluRs have the potential to be effective new targets for the treatment of epilepsy. Indeed, numerous mGluR ligands have been demonstrated to induce, modulate, or inhibit seizure activity in a variety of *in vivo* experimental models of epilepsy. Evidence from these models of epilepsy indicates that mGluR expression and function are rapidly altered during epileptogenesis. However, which mGluRs should be targeted for therapeutic intervention for epilepsy or epileptogenesis? Should activation or antagonism of mGluR functions be the goal of new therapeutic agents? In order to be able to answer these questions we require better understanding of the roles of individual mGluRs in triggering and maintaining seizure activity.

There have been many data with a focus on the mechanisms of actions of mGluRs and how they may be functionally related to the hyperexcitability of seizure activity. It was reported that group I mGluRs sustain or promote seizures (Camon et al., 1998; Chapman et al., 1999; 2000), whereas group II and group III mGluRs suppress seizures via presynaptic inhibition of glutamate release (Attwell et al., 1998; Gasparini et al., 1999). In contrast to group I mGluR, which seems to be more implicated in pathways leading to enhanced neurotoxicity, group II and III mGluRs have been proposed as potential targets for neuroprotective drugs in various disease.

The identification of subtype-selective agonists and antagonists has permitted evaluation of mGluRs as potential targets in the treatment of epilepsy. The non-selective mGluR agonist (1S, 3R)-1- aminocyclopentane-1, 3-dicarboxylic acid (ACPD) has been demonstrated to be convulsive in a number of studies (Sacaan and Schoepp, 1992; Tizzano et al., 1993, 1995). Subsequent studies have demonstrated similar convulsive effects for the group I-selective agonist, 3,5- dihydroxyphenylglycine (3,5-DHPG) in both rats (Camon et al., 1998) and mice (Tizzano et al., 1993), strongly implicating a role for activation of group I mGluRs in the convulsant effects of ACPD. Furthermore, selective antagonists of group I mGluRs, such as (S)-4- carboxy-3-hydroxyphenylglycine (CHPG), are anticonvulsive in a variety of seizure models. More recently, the characterization of mGluR1 and mGluR5 selective antagonists has made it possible to address the contribution of each group I subtype to epileptic activity. The mGluR1 selective antagonists, (+)-2-methyl-4-carboxyphenylglycine (LY367385), and (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) were effective anticonvulsants in models of both generalized convulsive and absence seizures (Chapman et al., 1999). Two mGluR5 selective noncompetitive antagonists, MPEP and SIB 1893, also have been reported to have anticonvulsant properties (Chapman et al., 2000). Unlike the convulsant properties of group I mGluR agonists, activation of group II and III mGluRs typically results in an anticonvulsant profile.

6.2. mGluRs in kindling and kindling induced learning performance

Group I mGluRs are involved in many CNS functions and may participate in a variety of disorders such as pain, epilepsy, ischemia, and chronic neurodegenerative diseases (Flor et al., 2002; Folbergrova et al., 2003). The Group I mGluR antagonists are considered potential therapeutics in the treatment of these diseases. In the present study the effects of mGluR1 and mGluR5 antagonists on PTZ kindling were investigated. Kindling is associated with transsynaptic changes, synaptic re-organization, LTP, changes in synaptic morphology, protein synthesis and axonal transport (McNamara et al., 1980). As an epilepsy experimental model associating neuronal plasticity and seizures, kindling is unique in providing opportunities to study the ability of drugs to modify these progressive changes. Such ability may be due to the underlying mechanisms of drug action on seizure development (Albertson et al., 1981). The kindling-induced enhanced seizure activity is understood to indicate a long-lasting alteration of the neuronal excitability, which can be regarded as suggestive of neuronal plasticity (Cain, 1989).

PTZ kindling is an acknowledged animal model for epilepsy and refers to a phenomenon in which repeated injection of a convulsant causes gradual seizure development culminating in generalized tonic–clonic seizures associated with a deficit in shuttle-box learning when tested after the end of the kindling procedure (Becker et al., 1992). Impairment of GABAergic inhibition is thought to play a crucial role in the processes underlying epileptogenesis in the PTZ-kindling model (Getova et al., 1998; Corda et al., 1991). Examination of PTZ-kindled rat brains revealed a significant neuronal cell loss priority in

vulnerable hippocampal CA1 and CA3 structures as well as in the hilus and it is possibly the cause of cognitive deficits (Pohle et al., 1997). PTZ kindling has been associated with a variety of alterations on the behavioral, neurophysiological, and neurochemical level resulting in long-lasting changes in hippocampal and cortical glutamate receptor density, potentiation of monosynaptic field potential in the dentate gyrus upon perforant path stimulation and worsening shuttle box learning (Becker et al., 1994; Schroeder et al., 1993, 1994; Ruethrich et al., 1996; Schroeder et al., 1998).

In *Chapter 2,* the interference of mGluRs in seizure-induced learning defecits and long-term plasticity were outlined and described in detail. Blockade of the mGluR1 receptor led to diminished seizure scores in the acute experiment. Both substances were effective against convulsions induced by 40 mg/kg PTZ but not when the convulsant was administered in higher doses. This is in agreement with other reports showing similar effects in different epilepsy models (Chapman et al., 2000; Borowicz et al., 2003). An explanation of the acute anti-PTZ efficacy by mGluR1 antagonists may be induced via functional changes in GABAergic neurotransmission (Cozzi et al., 2002; Folbergrova et al., 2003). It is possible that blocking of mGluR1 receptors may enhance inhibitory synaptic transmission and this potentiation of GABAergic neurotransmission prevents the development of seizure.

In previous investigations of rats which acquired the kindling syndrome a dramatic impairment in shuttle-box learning was found, and it was still persistent 4 weeks after kindling completion (Becker et al., 1992). In the present study the effects of mGluR1 and mGluR5 antagonists on kindling-induced impairments in shuttle-box performance were investigated. Kindling results in a worsening of shuttle-box learning depending on both the method of kindling induction and the learning model used (Becker et al., 1992, 1997b). Learning impairments were explained in terms of histopathological alterations and kindling-induced modifications in different neurotransmitter systems. Administration of LY 367385 in the course of kindling development or, alternatively, prior to each learning session in the shuttle-box improved learning performance in the kindled animals. Although both substances differed in their effect on kindling development and protection against learning impairment, LY 367385 and MPEP treatment prior to the learning test improved shuttle-box performance in the kindled rats.

As demonstrated in behavioral investigations, mGluRs apparently play an important part in hippocampus-dependent learning paradigms. Evidence obtained using general and specific antagonists of group I mGluRs suggests that these receptors are critically important for both hippocampal synaptic plasticity in vivo (Manahan-Vaughan et al., 1999; Naie and Manahan-Vaughan, 2004) and hippocampus-based learning (Balschun et al., 1999; Naie and Manahan-Vaughan, 2004). It was found that retention in the Y-maze spatial alternation was impaired by MCPG and 4-CPG (Balschun and Wetzel, 1998). It was also reported that water-maze learning was lessened by MCPG (Richter-Levin et al., 1994; Bordi et al., 1996). Mutant mice, deficient for mGluR 5, showed deterioration in two hippocampus-dependent tasks, the Morris water maze and contextual fear conditioning (Jia et al., 1998; Lu et al., 1997). Experiments using agonists of group I mGluRs, ACPD and tADA resulted in an improvement of memory (Bianchin et al., 1994; Riedel et al., 1995b). Daily application of the mGluR5 antagonist MPEP has also been shown to result in an impairment of spatial memory in an eight-arm radial maze (Naie and Manahan-Vaughan, 2004). In our experiments with kindled rats, a group I mGluR subtype specific altered performance is seen in the shuttle-box after the application of the mGluR1 antagonists LY 367385 and MPEP.

Neuroanatomical, behavioral, and electrophysiological experiments have shown that the mGluR5 subtype, in particular, is critical for the associative strengthening of neural

connections during learning (Lu et al., 1997; Jia et al., 1998; Riedel et al., 2000). Little is known about the precise contribution of mGluR1 to hippocampal plasticity and learning. Previous studies using transgenic mice lacking mGluR1 have shown normal hippocampal LTD expression but impaired LTP (Aiba et al., 1994). In contrast, it was also reported that hippocampal LTP was normal in mGluR1-deficient mice (Conquet et al., 1994). The contribution of mGluR5 to synaptic plasticity and associative learning may be related to the fact that it has a mutual potentiative relationship with NMDA receptors (NMDARs) (Doherty et al., 1997; Alagarsamy et al., 1999, 2001). Transgenic mice lacking mGluR5 display a complete loss of the NMDAR-mediated component of long-term potentiation (LTP) in the CA1 region of the hippocampus (Lu et al., 1997; Jia et al., 1998) and show impairments in memory tasks, including the acquisition and use of spatial information in the Morris water maze (Lu et al., 1997), that depend on NMDAR-mediated plasticity in CA1 (Morris et al., 1990; Tsien et al., 1996). Therefore, group I mGluRs may be involved in the fine tuning of hippocampal synaptic plasticity and learning.

6.3. mGluRs in kindling induced long-term plasticity

The involvement of iGluRs and mGluRs has been shown in LTP mechanisms. Although many studies support a role of mGluRs in synaptic plasticity and memory formation (Nakanishi, 1994; Riedel et al., 1996a; Conn and Pin 1997), the involvement of different mGluR groups and subtypes in particular physiological circuits and functions such as hippocampal synaptic plasticity and learning is still under debate. For instance, some authors described an inhibition of hippocampal LTP by the group I/II mGluR specific antagonist *S*-α-methyl-4-carboxyphenylglycine (MCPG) (Bashir et al., 1993; Bortolotto et al., 1994; Brown et al., 1994; Richter-Levin et al., 1994; Little et al., 1995; Riedel et al., 1995a), whereas in other studies the MCPG actions could not be confirmed (Chinestra et al., 1993; Izumi and Zorumski, 1994; Manzoni et al., 1994; Selig et al., 1995; Thomas and O'Dell, 1995; Martin and Morris, 1997). An alternative explanation for the ambiguous actions of MCPG was presented by Bortolotto et al., (1994) in the "molecular switch" hypothesis. It was postulated that activation of mGluRs before LTP sets an input-specific molecular switch that then negates the necessity of further mGluR-activation during LTP-induction. Other groups, however, failed to find experimental evidence for the existence of this molecular switch (Selig et al., 1995; Thomas and O'Dell, 1995; Martin and Morris, 1997). Studies using mGluR1 knockout mice confused the issue further. A significantly lower magnitude of LTP in the CA1 area of the hippocampus was found in these mutants (Aiba et al., 1994). In contrast to this, Conquet et al., (1994) did not observe a change of CA1 and dentate LTP in these mutants. Another possibility is that mGluR activation is required only when LTP is induced by a weak tetanus (Thomas and O'Dell, 1995; Wilsch et al., 1998). It has been shown previously that mGluR agonists can facilitate the induction of LTP both *in vivo* (Manahan-Vaughan and Reymann, 1995) and *in vitro* (Behnisch and Reymann, 1993; McGuinness, 1991a). The broad-spectrum mGluR agonist 1S, 3R-ACPD facilitates instantaneous (McGuinness, 1991b; Otani et al., 1993) and subsequent induction of LTP with a tetanus (Cohen et al., 1998), and can also cause a slow-onset potentiation on its own (Manahan-Vaughan and Reymann, 1995; Breakwell et al., 1996).

In vivo, metabotropic receptor antagonists have been reported to block the induction and maintenance of LTP, mainly at perforant path/dentate granule cell synapses. Metabotropic receptor agonists have been reported to facilitate the induction and maintenance of LTP at the same synapses. Recently, it was reported that mGluR5, but not mGluR1, is required for the induction of L-LTP. It was reported that the mGluR5 inhibitor MPEP inhibits

L-LTP, a protein synthesis-dependent form of synaptic plasticity (Francesconi et al., 2004). This is in agreement with the work of Rodriguez et al., who has reported that MPEP impairs NMDAR-dependent LTP at thalamic input synapses to the lateral amygdala in brain slices (Rodriguez et al., 2002). Furthermore, the induction and expression of group I mGluR-dependent discharges have been found to be independent of NMDA receptors (Galoyan and Merlin, 2000) and to involve molecular steps, such as a tyrosine-kinase dependent activation of extracellular signal-regulated kinases 1 and 2 (Zhao et al., 2004) and activation of phospholipase D coupled receptors (Rico and Merlin, 2004). Previous findings suggest that in kindled animals, and perhaps in patients with epilepsy, there is an upregulation of mGluR1 function which could contribute to the underlying seizure susceptibility, and potentially the epileptogenesis, of kindled animals and patients with epilepsy. These findings provide further support for the hypothesis that mGlu1 receptors may play an important role in seizures and seizure-related disorders. Finally, previous studies have shown that mGluR5 facilitate hippocampal bursting and are involved in the induction of persistent bursting activity in epileptiform conditions (Merlin, 2002; Thuault et al., 2002; Stoop et al., 2003), suggesting that the detrimental effects of MPEP on cortical bursting activity observed may also be linked to its potent anticonvulsant effects (Chapman *et al.*, 2000).

In *Chapter 3,* the interference of mGluRs in seizure-induced long-term plasticity was outlined and described in detail. Kindling and LTP have been suggested to exploit similar mechanisms (Racine et al., 1983; McNamara et al., 1985; Sutula and Steward, 1987; Sutula et al., 1994). Here, in our studies repeated injection of the $GABA_A$-antagonist PTZ was used to generate kindling, which was described to trigger long-lasting changes in the induction of LTP (Ruethrich et al., 1996; Krug et al., 1997; Schneiderman, 1997) or a potentiation on its own (Ruethrich et al., 1996; Omrani et al., 2000; Palizvan et al., 2001). A central question is whether the long-term effects of PTZ-kindling on hippocampal LTP requires the activation of both group I mGluR subtypes (mGluR1 and mGluR5), or whether activation of either subtype alone is sufficient to bring the changes about. In the present study, it is described that a repeated 'chronic' inhibition of group I mGluRs during kindling development prevents an enhancement of LTP, while a single, acute application of group I antagonists just before LTP recording has no effect. This is indicative of a consolidation of kindling-induced changes of synaptic plasticity, which is dependent on functionally active group I mGluRs. Importantly, the data clearly demonstrate that the long-term effects of group I mGluR antagonists on the kindling-induced enhancement of LTP are not directly related to the kindling 'phenotype' itself. Thus, while LY 367385 but not MPEP had a significant effect on the development of kindling, MPEP was more effective than LY 367385 in counteracting the kindling-induced changes in LTP. Our electrophysiological findings suggest a pivotal role of the concurrent activation of mGluR1 and mGluR5 in epilepsia-induced long-term aberrations in hippocampal network functions.

The data supports that the mGluRs may regulate both physiological and pathophysiological synaptic alterations. These receptors appear to play a very important role in maintaining synaptic function in healthy dynamic ranges, and furthermore, play a critical role in the induction of both stable plasticity and persistent information storage. Evaluated together, the data leads to the following conclusions:

* Both mGluR1 and mGluR5 play a specific role in the convulsive component of kindling.

* There is a potential role of mGluR1 and mGluR5 in the amelioration of seizure disorders and also in epilepsy related impairments during avoidance learning.

* Kindling results in long-lasting changes in hippocampal LTP that are mediated via activation of mGluR1 and mGluR5 and consolidate with a certain time course.

* These changes ultimately require a concurrent operation of mGluR1 and mGluR5 but they do not seem to be directly related to the kindling-phenotype.

* Repeated inhibition of mGluR5 resets the level of hippocampal synaptic plasticity not only during kindling but also under normal conditions.

Taken together, group I mGluRs appears to play an important role in a wide variety of neural functions such as learning, memory and plasticity.

6.4. mGluR mediated plasticity of the PF-PC synapse

Cerebellar Purkinje cells abundantly express mGluR1 (Masu et al., 1991), a group I mGluR subtype that couples to the $G\alpha_{q/11}$ protein and phospholipase C (Abe et al., 1992; Masu et al., 1991). The *mGluR1* null mutant (*mGluR1*$^{-/-}$) mice or rats treated with antiserum to mGluR1 suffer from impairment of motor coordination, indicating that mGluR1 is essential for cerebellar functions (Aiba et al., 1994; Conquet et al., 1994; Sillevis Smitt et al., 2000). Activation of mGluR1 causes various postsynaptic effects in PCs, including IP3-mediated local Ca^{2+} release (Finch and Augustine 1998; Takechi et al., 1998), slow EPSCs (Batshelor and Garthwaite 1997; Tempia et al., 1998), and induction of long-term synaptic depression (Aiba et al., 1994; Conquet et al., 1994; Hansel and Linden, 2000; Ichise et al., 2000; Kano and Kato, 1987; Linden et al., 1991; Shigemoto et al., 1994).

Besides these postsynaptic effects, the group I mGluRs (including mGluR1 and mGluR5) are also reported to influence presynaptic functions. They modulate neurotransmitter release in various regions of the CNS (Anwyl, 1999). For example, activation of mGluR1 in PCs (Conquet et al., 1994;) and mGluR1 or mGluR5 in hippocampal neurons (Gereau and Conn, 1995) causes a reversible suppression of excitatory transmission that is presumably of presynaptic origin. However, detailed immunohistochemical studies indicate that the group I mGluRs are hardly found at presynaptic fibers but are densely concentrated at the perisynaptic region of postsynaptic membrane (Baude et al., 1993; Lujan et al., 1996; Lujan et al., 1997; Nusser et al., 1994). It is, therefore, conceivable that some retrograde signal exists from postsynaptic group I mGluRs to the presynaptic terminals. Another example of the presynaptic actions exerted by postsynaptic group I mGluRs is that mGluR1 in the postsynaptic PC is essential for the elimination of redundant CF synapses during postnatal cerebellar development. This process is impaired in *mGluR1*$^{-/-}$ mice (Kano et al., 1997; Levenes et al., 1997), and the defect is restored by introducing *mGluR1α* transgene into *mGluR1*$^{-/-}$ PCs under the control of a PC-specific promoter (Ichise et al., 2000). Because the CF synapse elimination eventually results in retraction of surplus CFs, these results also suggest the existence of a mechanism that enables communication from postsynaptic mGluR1 to CF terminals. It was found that activation of postsynaptic mGluR1 in PCs causes presynaptic inhibition that is mediated by cannabinoid receptors (Maejima et al., 2001).

6.5. CF-evoked endocannabinoid signaling suppresses PF-LTP

Recent studies have demonstrated that endogenous cannabinoids (endocannabinoids) are released from depolarized postsynaptic neurons in a Ca^{2+}-dependent manner, act retrogradely onto presynaptic CB1 receptors, and suppress neurotransmitter release. This type of modulation has been found in the inhibitory synapse of the hippocampus (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) and CF and PF synapses in the cerebellum (Kreitzer and

Regehr, 2001). It was shown that the mGluR1-dependent mechanism works in a synergistic manner with the depolarization-induced suppression to efficiently modulate excitatory synaptic transmission in PCs (Maejima et al., 2001). They also suggested a link via short-lived endocannabinoids between the postsynaptic group I mGluRs and presynaptic cannabinoid receptor.

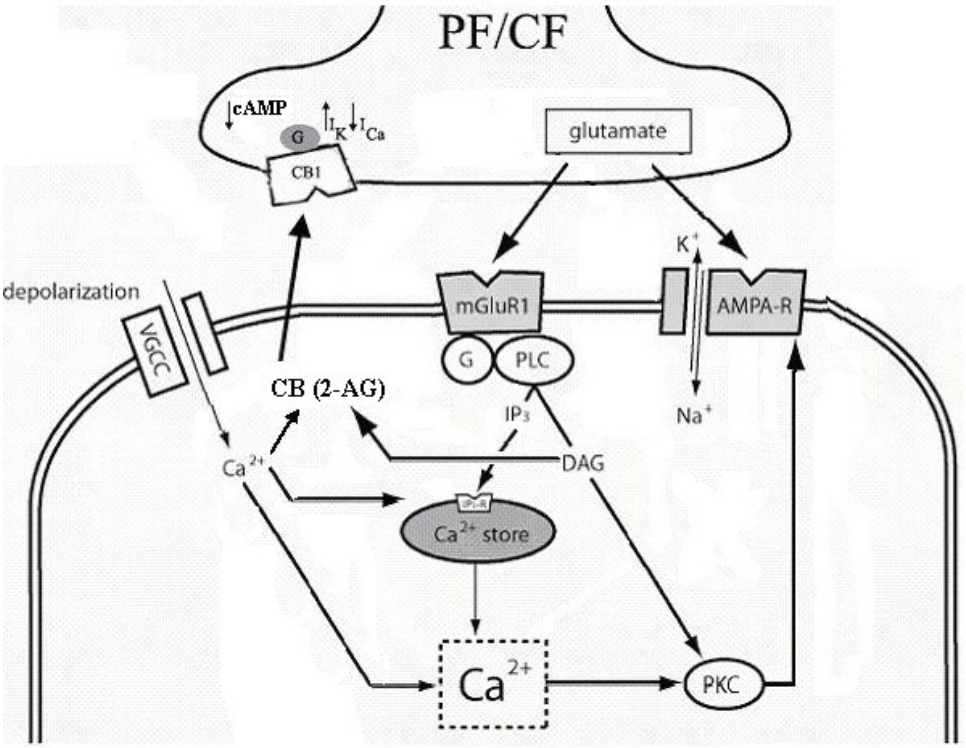

Figure 6.1: Model of the endocannabinoid signaling at the parallel and climbing fiber synapse to Purkinje cell. Cannabinoids are produced in response to stimulation of excitatory fibers and resultant elevation of intracellular calcium. DAG is generated from Phosphoinositides by phospholipase C and is converted to 2-AG by DAG lipase. CB: Cannabinoid, 2-AG : 2- arachidonylglycerol, CB1: Cannabinoid receptor type 1, VGCC: voltage-gated sodium channels, mGluR1: metabotropic glutamate receptor type 1, AMPA-R: AMPA receptor, G: G protein, PLC: phospholipase C, IP_3: inositol 1,4,5-triphosphate, DAG: 1,2-diacylglycerol, PKC: protein kinase C, c-AMP: cyclic adenosine monophosphate, CF: climbing fiber, PF: parallel fiber. *(Adapted from Coesmans, Cerebellar Plasticity in Health and Disease, PhD thesis, Rotterdam 2004).*

In *Chapter 5,* the suppression of PF-LTP by CF-evoked endocannabinoid signaling is outlined and described in detail. In the brain, the endocannabinoids anandamide and 2-arachidonylglycerol (2-AG) function as modulators of transmitter release by binding to G-protein-coupled CB1 receptors, which are located on presynaptic terminals. CB1 receptor activation can both inhibit adenylyl cyclases and modify K- and Ca-selective channels (Figure 6.1). Endocannabinoid signaling has been demonstrated to mediate depolarization-induced suppression of excitation at CF and PF synapses onto cerebellar PCs. Here, we show that CF-evoked release of CBs additionally suppresses a presynaptic form of LTP at PF synapses. CF activity can be substituted for by bath-application of the CB receptor agonist WIN55,212-2. In the presence of the CB1 receptor antagonist AM251, CF activity no longer suppresses PF-LTP. Presynaptic potentiation can also be obtained by the adenylyl cyclase activator

forskolin. WIN55,212-2 blocked this forskolin-mediated enhancement, showing that CB1 receptor activation interferes with the adenylyl cyclase-protein kinase A cascade, which participates in LTP induction. In postsynaptic PF plasticity, CF activity determines whether LTD or LTP is induced (Lev-Ram et al., 2002; Coesmans et al., 2004). It has not been examined so far, whether CF activity can suppress presynaptic LTP. We reasoned that such a 'safety lock' might exist in order to prevent the induction of presynaptic LTP while LTD is expressed postsynaptically, since the postsynaptic downregulation of response amplitudes might otherwise be accompanied by an increase in transmitter release. A possible link between CF activity and the suppression of presynaptic PF-LTP might be provided by retrograde endocannabinoid signaling. The release of endocannaboids involved in DSE at PF-PC synapses can be activated by CF activity (Brenowitz and Regehr, 2005), suggesting that complex spike-associated calcium transients in PC dendrites are sufficient to initiate the release process. We suggest that simultaneous induction of presynaptic LTP would counteract postsynaptic LTD. It is likely that the CF-evoked calcium transients in PC dendrites can postsynaptically promote LTD and, through the release of endocannabinoids, suppress presynaptic LTP at the same time. The latter effect would facilitate LTD induction as well as enhance the observed LTD amplitude.

The CB1 receptor shows a widespread distribution at presynaptic terminals of various regions, including the cerebellum and hippocampus (Egertova and Elphick, 2000; Matsuda et al., 1993). Activation of CB1 receptor depresses synaptic transmission in various regions of the brain, including the cerebellum (Levenes et al., 1998; Takahashi and Linden, 2000), hippocampus (Misner and Sullivan, 1999), and cerebral cortex (Auclair et al., 2000). Thus, presynaptic inhibition mediated by the CB1 receptor is ubiquitous in the brain (Di Marzo et al., 1998). On the other hand, most neurons in the brain express either mGluR1 or mGluR5 on the postsynaptic site. Because mGluR1 and mGluR5 activate the same intracellular signaling cascade involving phospholipase Cβ (Abe et al., 1992; Masu et al., 1991), most neurons in the brain may have the capability to produce endocannabinoids in response to glutamate. Therefore, the presynaptic inhibition involving a retrograde signal from postsynaptic group I mGluRs to presynaptic CB1 receptors may be of general importance in neural functions.

Recent studies have revealed another important pathway for endocannabinoid production in neurons. In hippocampal neurons and cerebellar PCs, depolarization-induced transient elevation of $[Ca^{2+}]_i$ has been shown to produce endocannabinoids that suppress neurotransmitter release from presynaptic terminals (Kreitzer and Regehr, 2001; Maejima et al., 2001; Montgomery and Madison, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). The group I mGluR-induced Ca^{2+}-independent pathway and the depolarization-induced Ca^{2+}-dependent pathway may function in a synergistic manner in vivo. Furthermore, activation of mGluR1/mGluR5 leads to Ca^{2+} mobilization from the internal store and may facilitate the Ca^{2+}-dependent release of endocannabinoids. Taken together, it is highly likely that the endocannabinoid-mediated presynaptic inhibition can be an important and widespread mechanism in the brain through which the activity in postsynaptic neurons can influence the presynaptic functions.

Perspectives

Changes in synaptic efficacy are thought to be crucial to experience-dependent modifications of neural function. The diversity of mechanisms underlying these changes is far greater than previously expected. In the last few years, a new class of use-dependent synaptic plasticity that requires retrograde signaling by endocannabinoids and presynaptic CB1 receptor

activation has been identified in several brain structures. Endocannabinoid-mediated plasticity encompasses many forms of transient and long-lasting synaptic depression and is found at both excitatory and inhibitory synapses. In addition, endocannabinoids can modify the inducibility of non-endocannabinoid-mediated forms of plasticity. Thus, the endocannabinoid system is emerging as a major player in synaptic plasticity. Given the wide distribution of CB1 receptors in the CNS, the list of brain structures and synapses expressing endocannabinoids-mediated plasticity is likely to expand. Recent developments, such as the generation of CB1 and CB2 knock-out mice and synthesis of selective antagonists of the CB1 and CB2 receptors, have provided insight into the (patho) physiological roles of the endocannabinoid system. However, some important fundamental aspects of the endogenous cannabinoid system remain to be discovered. For example, the proteins responsible for the biosynthesis of endocannabinoids and their transport into cells have not been identified yet. It is likely that novel CB receptor subtypes as well as novel endogenous ligands will be found. The understanding of the complex interplay of the endocannabinoid system with other neurotransmitters in the CNS and their function as retrograde messengers will greatly enhance our knowledge about the physiological roles of the endocannabinoid system. This may provide useful information to exploit the cannabinoid system for therapeutic intervention in various diseases.

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List of abbrevations

2-AG, 2-arachidonylglycerol;

AC, adenyl cyclase

ACPD, *trans*-(1S,3R)-1-amino-1,3-cyclopentanedicarboxylic acid;

ACSF, artificial cerebrospinal fluid

AD, afterdischarge;

AIDA, (RS)-1-aminoindan-1,5-dicarboxylic acid;

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid;

cAMP, cyclic adenosine monophosphate;

4C3HPG, (*S*)-4-carboxy-3-hydroxyphenylglycine;

4CPG, (*S*)-4-carboxyphenylglycine;

CA1, cornu ammonis region 1;

CA3, cornu ammonis region 3;

CB, cannabinoid;

CB1, cannabinoid receptor subtype 1;

CB2, cannabinoid receptor subtype 2;

CCG1, (2*S*,3*S*,4*S*)- (carboxycyclopropyl)glycine-1;

CNS, central nervous system;

CPP, 3-((±)-2-carboxypiperazine-4-yl)propenyl-1-phosphonate;

DAG diacylglycerol;

DG, dentage gyrus;

DHPG, (*R*,*S*)-3,5-dihydroxyphenylglycine;

EAA, excitatory amino acid;

EPSC, excitatory postsynaptic current;

EPSP, excitatory postsynaptic potential;

fEPSP, field excitatory postsynaptic potential;

ER, endoplasmic reticulum;

GABA, γ-aminobutyric acid;

i.c.v., intra cerebroventricular injection;

iGluR, ionotropic glutamate receptor;

i.p., intra peritoneal injection;

I/O, input/output;

IP_3, inositol trisphosphate;

KA, kainate;

LTP, long-term potentiation;

LTD, long-term depression;

LY 367385, (S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid;

MCPG, S-*α*-methyl-4-carboxyphenylglycine;

mGluR, metabotropic glutamate receptor;

mGluR1, metabotropic glutamate receptor type 1;

mGluR5, metabotropic glutamate receptor type 5;

MPEP, 2-Methyl-6- (phenylethynyl) pyridine hydrochloride;

NBQX, 2, 3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulphonamide;

NMDA, N-methyl-D-aspartic acid;

NMDAR, NMDA receptor;

PC, purkinje cell;

PF, parallel fibre;

PI, phosphatidylinositol;

PIP_2, phosphatidylinositol-bi-phosphate;

PKA, cAMP-dependent protein kinase;

PKC, protein kinase C;

PLC, phospholipase C;

PPF, paired pulse facilitation;

PSA, population spike amplitude;

PTZ, pentylenetetrazol;

tADA, trans -azetidine-2,4-dicarboxylic acid;

TLE, temporal lobe epilepsy;

VGCC, voltage gated calcium channel;

Summary

Metabotropic glutamate receptors (mGluRs) are distributed throughout the central nervous system and play important roles in the modulation of synaptic transmission. The mGluRs regulate long-term potentiation (LTP)/long-term depression (LTD) and learning and memory in hippocampus. This group of glutamate receptors are also reported to exert anticonvulsive and neuroprotective efficacy, especially group I mGluRs. Kindling induced by repeated application of the convulsant pentylenetetrazole (PTZ) is a validated model of epilepsy and epilepsy-related neuromorphological, neurophysiological and behavioural alterations. In this thesis, the role of group I metabotropic glutamate receptors (mGluRs) in PTZ induced seizures, kindling, kindling-related learning deficits and LTP were examined.

LY 367385 (mGluR1) and MPEP (mGluR5), which are group I mGluR antagonists, were used in rats to investigate their effects on PTZ seizures, kindling, and kindling-related learning deficits. Both substances showed anticonvulsant efficacy against seizures induced by lower doses of PTZ (40 mg/kg), but they were ineffective in counteracting seizures evoked by higher PTZ doses (50 and 60 mg/kg). For the kindling experiment a dose of 37.5 mg/kg body weight PTZ was injected once every 48 h. When these substances were given in the course of kindling induction, LY significantly depressed the progression of kindled seizure severity. In contrast, MPEP was ineffective in this experiment. One characteristic of the kindled state is enhanced excitability. To test this, the animals received a challenge dose of PTZ (32.5 mg/kg) 8 days after kindling completion. Treatment with either LY or MPEP did not modify the reaction to challenge dose of PTZ. Kindling results in a worsening of shuttle-box learning. LY improved shuttle-box learning when administered in the course of kindling development or when given prior to the learning experiment. This suggests protective and restorative effectiveness. In contrast, MPEP was only effective on the learning performance of kindled rats when given prior to the shuttle-box experiment, which demonstrates restorative effectiveness. Kindling is associated with an increase in glutamate binding. LY counteracted this increase whereas MPEP was ineffective. It was concluded that mGluR1 and mGluR5 play a specific role in the convulsive component of kindling. The beneficial action of the antagonists on kindling-induced impairments in shuttle-box learning may be associated with their effect on glutamatergic synaptic activity.

In the later part of the investigations, it was examined whether kindling-induced long-term aberrations in hippocampal synaptic plasticity can be prevented by application of group I mGluR antagonists. Kindling resulted in a higher magnitude of LTP induced by a strong high-frequency stimulation in the hippocampal CA1-region *in vitro*. When the specific mGluR1 antagonist LY 367385 (0.40 µMol) or the specific mGluR5 inhibitor MPEP (0.06 µMol) were given 30 min prior to PTZ, this kindling-induced enhancement of LTP was almost completely prevented. In addition, application of MPEP led to an impaired maintenance of population spike LTP in kindled animals. LY 367385 applied to unkindled control animals caused a reduction of the initial magnitude of population spike LTP. MPEP, in contrast, left the initial magnitude untouched but resulted in a faster decay of potentiation. A single administration of LY 367385 (200µM) and MPEP (50µM), respectively, directly into the bath had almost no effect on LTP. Our data suggest that the long-lasting aberrations of hippocampal synaptic plasticity induced by the repeated occurrence of generalized epileptic seizures ultimately require a concurrent operation of mGluR1 and mGluR5. Together, the data document a specific role of mGluR1 and mGluR5 in the pathophysiological mechanisms induced by PTZ kindling and the effect of kindling on learning and LTP.

In addition to its role in hippocampal plasticity, mGluR1 has been shown to play a role in long-term changes in synaptic transmission of cerebellum and motor learning. Connections in the mammalian cerebellar cortex are highly plastic. Plastic changes have been described at the parallel fiber-Purkinje cell synapse, where presynaptic LTP and postsynaptic LTP and LTD have been described, and at the climbing fiber-Purkinje cell synapse, where postsynaptic LTD has been noted. LTD at cerebellar parallel fibre (PF)-Purkinje cell (PC) synapses require activation of group I mGluRs. Previous studies indicated the essential role of mGluR1 activation on PF-PC plasticity. The current thesis studied the cause of this effect and linked it to the other forms of known types of plasticity at the excitatory afferent synapses of the PCs.

Group I mGluRs are reported to influence both presynaptic and postsynaptic functions. For example, activation of mGluR1 in Purkinje cells and hippocampal neurons causes a reversible suppression of excitatory transmission that is presumably of presynaptic origin. Activation of postsynaptic mGluR1 in Purkinje cells causes presynaptic inhibition that is mediated by cannabinoid (CB) receptor activation that are located on the presynaptic terminals. Endocannabinoid signaling has been demonstrated to mediate depolarization-induced suppression of excitation (DSE) at climbing fiber (CF) – Purkinje cell and parallel fiber (PF) – Purkinje cell synapses. We studied whether CF-evoked cannabinoid signaling additionally suppresses a presynaptic form of LTP at PF synapses.

Presynaptic PF-LTP can be induced by 8Hz PF tetanization, but is blocked when the CF is co-stimulated. CF activity can be substituted for by bath-application of the CB receptor agonist WIN55,212-2 (2 μM). In the presence of the CB1 receptor antagonist AM251 (5 μM), CF activity no longer suppresses PF-LTP. Presynaptic potentiation can also be obtained by the adenylyl cyclase activator forskolin (50 μM). WIN55, 212-2 blocked this forskolin-mediated enhancement, showing that CB1 receptor activation interferes with the adenylyl cyclase-protein kinase A cascade, which participates in LTP induction. CF activity has been described to promote the induction of postsynaptic PF-LTD and to impair postsynaptic PF-LTP. Our observation that CF activity blocks the induction of presynaptic LTP suggests that the CF input controls all forms of pre- and postsynaptic PF plasticity and that CF activity provides a safety lock to prevent an enhancement of transmitter release while postsynaptic AMPA receptor function is downregulated during LTD. The main finding of this study is that activity of the heterosynaptic CF input suppresses the induction of a presynaptically expressed form of PF-LTP through retrograde cannabinoid signaling. This investigation can lead to better understanding of the role of cannabinoids in the modulation of synaptic transmission. The discovery of the endocannabinoids and the availability of new pharmacological tools have revitalized the field of cannabis research in the past few years. It is possible that the pharmacological manipulation of the endocannabinoid system may offer a safer approach to the cannabis-based medicines in the future.

Populaire samenvatting

Metabotrope glutamaatreceptoren (mGluRs) komen overal in het centrale zenuwstelsel voor en spelen een belangrijke rol in de modulatie van de synaptische transmissie. De mGluRs reguleren zowel lange termijn potentiatie (LTP) als lange termijn depressie (LTD) en zijn gelinkt aan hippocampaal gemedieerd geheugen. Deze groep glutamaatreceptoren, en met name de type I mGluRs hebben een anticonvulsieve en zenuwcel-beschermende werking. Kindling, geïnduceerd door herhaalde toediening van het convulsieve middel pentylenetetrazole (PTZ), is een veel gebruikt model voor epilepsie, de daaraan gerelateerde veranderingen in de morfologie en fysiologie van hersencellen en veranderingen in gedrag. In dit proefschrift is de rol van type I mGluRs in PTZ-geïnduceerde epileptische aanvallen (convulsies), kindling, de aan kindling gerelateerde leerdefecten en LTP onderzocht.

Het effect van het blokkeren van mGluRs op PTZ-geïnduceerde convulsies, kindling en aan kindling gerelateerde leerproblemen werd onderzocht in ratten, met behulp van de type I mGluR-antagonisten LY 367385 (mGluR1) en MPEP (mGluR5). Beide substanties bleken te beschermen tegen convulsies bij toediening van lage doses PTZ (40 mg/kg) maar bleken ineffectief bij hogere doses PTZ (50 en 60 mg/kg). Voor het kindling- experiment werd een dosis van 37,5 mg per kg lichaamsgewicht per 48 uur toegediend. Wanneer tijdens kindling-inductie LY werd toegediend, dan bleek dat de door kindling geïnduceerde convulsie minder toenam in ernst, terwijl MPEP hierop geen effect had. Na toediening van hogere dosis PTZ hadden zowel LY als MPEP geen effect. Verder bleek dat kindling een verslechtering in shuttle-box leren veroorzaakt. LY verbeterde het shuttle-box leren wanneer het werd toegediend tijdens de kindling ontwikkeling of voor het leerexperiment. Dit suggereert een beschermend en herstellend effect van LY. MPEP had alleen effect op de leerprestaties van ratten waarin kindling was geïnduceerd, als we MPEP toedienden vóór het shuttle-box experiment. Dit suggereert dat MPEP herstellend werkt. Kindling wordt ook geassocieerd met een versterking van de binding van glutamaat. LY ging deze versterking tegen, terwijl MPEP er geen effect op had. We concluderen dat mGluR1 en mGluR5 een specifieke rol spelen in de convulsieve component van kindling. De positieve uitwerking van type I mGluR-antagonisten op de door kindling geïnduceerde verslechtering van shuttle-box leren zou gerelateerd kunnen zijn aan hun effect op de activiteit van glutamaterge synapsen in de hippocampus.

Hierna werd gekeken of de door kindling geïnduceerde langdurige afwijkingen in hippocampale synaptische plasticiteit kunnen worden voorkomen door middel van toediening van type I mGluR-antagonisten. *In vitro* experimenten lieten zien dat LTP-inductie door sterke, hoogfrequente stimulatie van het CA1 gebied in de hippocampus resulteerde in een sterkere potentiatie wanneer er eerder kindling was geïnduceerd. Toediening van de specifieke mGluR1-antagonist LY 367385 (0.40 µMol) of de specifieke mGluR5 remmer MPEP (0.06 µMol), 30 minuten voor PTZ toediening, voorkwam deze door kindling geïnduceerde versterking van LTP vrijwel volledig. Verder veroorzaakte de toediening van MPEP ook een verminderde duur van de 'population spike LTP' in ratten waarin kindling was geïnduceerd. LY veroorzaakte een reductie in de amplitude van de populatie spike LTP in de hippocampus van ratten waarin niet eerst kindling was geïnduceerd. MPEP daarentegen had geen effect op de amplitude, maar leidde tot een sneller verval van de LTP. Directe toediening van LY (200 µM) en MPEP (50 µM) had geen significant effect op de LTP. Deze data suggereren dat mGluR1 en mGluR5 samen actief zijn bij de langdurige verstoring van hippocampale synaptische plasticiteit na het herhaaldelijk optreden van gegeneraliseerde epileptische aanvallen. Onze data tonen aan dat mGluR1 en mGluR5 een specifieke rol spelen

in de pathofysiologiche mechanismen gerelateerd aan PTZ-kindling en in het effect van kindling op LTP en leren.

Naast de centrale rol van type I mGluR in hippocampale synaptische plasticiteit is het aangetoond dat mGluR1 een rol speelt bij langdurige synaptische veranderingen in het cerebellum, welke vermoedelijk ten grondslag liggen aan motorisch leergedrag. Verbindingen in de hersenschors van zoogdieren zijn sterk plastisch, wat wil zeggen dat de sterkte ervan kan veranderen. Zulke plastische veranderingen zijn uitgebreid onderzocht voor de parallelvezel-Purkinjecel synaps, waar presynaptisch LTP en postsynaptisch LTD zijn beschreven. Ook op het niveau van de klimvezel-Purkinjecel synaps is postynaptische LTD beschreven. Het is aangetoond dat voor LTD van de parallelvezel-Purkinjecelsynaps in het cerebellum activatie van mGluR1 nodig is. Dit proefschrift onderzoekt de onderliggende mechanismen hiervan en ook het verband met andere vormen van plasticiteit van de excitatoire synapsen van de Purkinjecellen.

Groep I mGluRs kunnen zowel pre- als postsynaptische functies beïnvloeden. Bijvoorbeeld, activatie van mGluR1 in Purkinjecellen veroorzaakt een omkeerbare onderdrukking van excitatoire transmissie die waarschijnlijk presynaptisch van aard is. Activatie van postsynaptische mGluR1 in Purkinjecellen veroorzaakt presynaptische inhibitie die wordt gemedieerd door cannabinoid-receptoren (CB). In cerebellaire synapsen kunnen endocannabinoiden binden aan presynaptisch gelokaliseerde CB1 receptoren. Endocannabinoiden spelen een rol in depolarisatie-geïnduceerde onderdrukking van excitatie (DSE) van zowel de parallelvezel-Purkinjecel en klimvezel-Purkinjecel synaps. We hebben onderzocht of de endocannabinoiden die vrijkomen bij klimvezel-activatie een presynaptische vorm van LTP in de parallelvezel-Purkinjecel synaps kunnen onderdrukken.

Presynaptische PF-LTP kan worden geïnduceerd door de parallelvezel met 8Hz te tetaniseren, maar wordt geblokkeerd wanneer de klimvezel ook actief is. Klimvezel-activiteit kan *in vitro* worden vervangen door toediening van de CB receptor agonist WIN55,212-2 (2 μM). In de aanwezigheid van de CB1-receptor-antagonist AM251 (2μM) blokkeert klimvezel-activiteit niet langer de PF-LTP. Presynaptische potentiatie kan ook worden verkregen door de adenylylcyclase-activator forskolin (50 μM). WIN55 blokkeert deze forskolin-gemedieerde potentiatie. Dit toont aan dat CB1 receptor activatie invloed heeft op de adenylylcyclase – proteine-kinase A cascade die nodig is voor LTP-inductie. Klimvezel-activiteit vergemakkelijkt de inductie van postsynaptische PF-LTD en verkleint de kans op postsynaptische PF-LTP. Onze observatie dat klimvezel- activiteit de inductie van presynaptische LTP blokkeert, suggereert dat de klimvezel-input alle vormen van pre- en postsynaptische PF plasticiteit bestuurt. Verder suggereert onze data ook dat klimvezel-activiteit een veiligheid inbouwt die voorkomt dat er een te hoge neurotransmitter-uitstoot plaatsvindt, terwijl de postsynaptische AMPA-receptor- functie is verminderd tijdens LTD. De belangrijkste vinding van onze studie is dat klimvezel-activiteit de inductie van presynaptische PF-LTP onderdrukt door middel van retrograde cannabinoide signalen. Dit onderzoek kan leiden tot een beter begrip van de rol die cannabinoiden spelen in de modulatie van synaptische transmissie. De ontdekking van de endocannabinoiden en de beschikbaarheid van nieuwe farmacologische gereedschappen hebben het cannabis-onderzoek nieuw leven ingeblazen. Voor de toekomst kan dit betekenen dat farmacologische manipulatie van het endocannabinoid-systeem cannabis-achtige medicijnen veiliger in het gebruik kan maken.

List of publications

1. **Nagaraja, R.Y.,** Grecksch, G., Reymann, K.G., Schroeder, H., Becker, A., 2004. Group I metabotropic glutamate receptors interfere in different ways with pentylenetetrazole seizures, kindling, and kindling-related learning deficits. *Naunyn Schmiedebergs Arch.Pharmacol. 370, 26-34.*

2. **Nagaraja, R.Y.,** Becker, A., Reymann, K.G., Balschun, D., 2005. Repeated administration of group I mGluR antagonists prevents seizure-induced long-term aberrations in hippocampal synaptic plasticity. *Neuropharmacology. 2005; 49: 179-87.*

3. van Beugen*, B., **Nagaraja*, R.Y.,** Hansel, C., 2006. Climbing fiber-evoked endocannabinoid signaling heterosynaptically suppresses presynaptic cerebellar LTP. *The Journal of Neuroscience, 26(32):8289–8294*

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CURRICULUM VITAE

The author of this work was born in Yelahanka, Bangalore on October 24 1976. Bangalore is the capital city of Karnataka, a southern state of India. He moved on to Mysore with his parents in 1979. Mysore is the "cultural" capital of Karnataka and is 140 kms away from Bangalore. He started his early school in Mysore and was in Mysore throughout for his education. After passing out of the secondary school he attended the pre-university course at the Sarada Vilas College in Mysore from 1992-1994. He gained entry to the University of Mysore and studied for his bachelor's degree in Science at St. Philomena's College in Mysore. He studied chemistry, botany and zoology in St. Philomena's College from 1995-1998, to obtain his bachelor's degree. In August 1998 he started his master's studies at the Mysore University for two years. In October 2000 he obtained his master's degree in Biosciences. After his master's degree he worked as temporary faculty in Government science college, Bangalore. In January 2002 he joined as an assistant for scientific work in the Department for Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore (CFTRI), India. His younger brother drew his interest toward doing research in Europe. In May 2002 he began his work as a Research Assistant in the Institute for Pharmacology and Toxicology, under PD Dr. Axel Becker, Magdeburg, Germany. He spent two and half years in Magdeburg working in a collaborative project of Dr. Axel Becker and Prof. Klaus .G. Reymann (Department of Neuropharmacology of the Leibniz Institute for Neurobiology, Magdeburg). In January 2005 he joined the Department of Neuroscience in the Medical Faculty of the Erasmus University, Rotterdam, under the supervision of Dr. Christian Hansel to continue his research. He is presently working on the cellular mechanisms of cerebellar synaptic plasticity and is looking forward to persue his career further in neuroscience research.

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