# Investigation of Cellular Mechanisms of Hippocampal LTP and LTD

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Sheeja Navakkode Gangadharan

## Abstract

Processes of functional plasticity, i.e. long-lasting changes of the strength of synaptic connectivity in response to relative short-lasting afferent stimulation, are the most likely mechanisms underlying memory storage in the adult brain. The best studied models of functional plasticity are long-term potentiation (LTP) and long-term depression (LTD).

It is well known that the 3'-5'-cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway is essential for the prolonged mainentance of LTP as well as LTD. Therefore, it was interesting to investigate, how substances with a direct action on cellular cAMP-regulation would affect LTP/LTD. Rolipram, a specific type IV-specific cAMP phosphodiesterase (PDE) inhibitor, was therefore used in my initial studies to investigate its effect on late plastic events during functional CA1 plasticity in rat hippocampal slices in vitro. My studies showed that, an early form of LTP which normally decays to the baseline within 2-3 h (early-LTP) can be converted to a long-lasting LTP (late-LTP) lasting up to 6 h, if rolipram was applied during a weak tetanization. This rolipram-reinforced LTP (RLTP) was NMDA-receptor- and protein synthesis-dependent. The formation of cAMP during late-LTP in region CA1 requires dopaminergic receptor activity (Frey et al., 1989;Frey et al., 1990), thus we have studied whether RLTP was influenced by inhibitors of the D1/D5-receptor. Application of the specific D1/D5 antagonist SCH23390 did not prevent RLTP, suggesting that the phosphodiesterase inhibitor acts downstream of the D1/D5-receptors. Further studies were conducted to investigate whether rolipram can interact with processes of synaptic tagging. Synaptic tagging provides a conceptual basis for characterizing the mechanisms by which newly synthesized proteins that prolong functional changes in synaptic strength may act at

specific, recently activated synapses (Frey and Morris, 1997;Frey and Morris, 1998a). Inhibition of PDE and subsequent induction of RLTP in one synaptic population S1 was able to transform early- into late-LTP in a second, independent synaptic population S2 of the same neurons. This supports our hypothesis that cAMP-dependent processes are directly involved in the synthesis of plasticity-related proteins (PRPs).

It has been reported recently that, an atypical PKC isotype PKMzeta (PKMζ) is a first LTP specific PRP which is both necessary and sufficient for long-lasting LTP maintenance, but not for LTD (Sajikumar et al., 2005b). Thus, our assumption was that rolipram may specifically activate the synthesis of PKMζ only during LTP or it is involved in a more general regulation of the synthesis of PRPs necessary for both LTP and LTD. Thus, if inhibition of PDEs can reinforce an early form of LTP, the next question was whether rolipram could reinforce an early form of LTD into a late one.

In addition to the action of rolipram on LTP, I show here, in the CA1 region of hippocampal slices from male adult rats in vitro that rolipram also converts an early form of LTD (early-LTD) that normally decays within 2-3 h, to a long-lasting LTD (late-LTD) if rolipram was applied during LTD-induction. Rolipram-reinforced LTD (RLTD) was NMDA-receptor- and protein synthesis-dependent. Furthermore, it was dependent on the synergistic co-activation of dopaminergic D1/D5- and glutamate receptors. The question arose whether synaptic tagging occurs during RLTD. I found that early-LTD in a synaptic input S1 was transformed into late-LTD, if early-LTD was induced in a second independent synaptic pathway S2 during the inhibition of PDE by rolipram, supporting the interaction of processes of synaptic tagging during RLTD.

Although the mechanism of action of different forms of LTP is well understood, signalling cascades for LTD still remain poorly understood. I therefore delineated the

pathway for the possible mechanism of action of rolipram during the reinforcement of early-LTD. I could show that extracellular signal-regulated kinase (ERK1/ERK2) cascade is recruited during RLTD. Inhibition of the ERK signaling cascade with specific inhibitors of mitogen-activated protein kinases (MAPK), U0126 or PD98059 prevented the maintenance of RLTD. I further investigated the specific pathways by which ERK1/ERK2 is activated during RLTD. Thus MAPK-activation was triggered during RLTD by the synergistic interaction of NMDA-receptor- and D1/D5-receptor-mediated Rap/B-Raf pathways but not by the Ras/Raf-1 pathway in adult hippocampal CA1 neurons, which was revealed by the use of pathway-specific inhibitors, manumycin for Ras/Raf-1 pathway and lethal toxin-82 (LT-82) for Rap/B-Raf pathway. Thus for the first time I report that PDE4B3 could represent a process-non-specific PRP which regulates the synthesis of either LTP- and/or LTD- plasticity-related proteins (PRPs).

Next, I was interested to investigate the question of what exactly is the putative nature of the synaptic tag? Are there specific ´tags´ for LTP and LTD? I studied the role of two promising candidates: Calcium/calmodulin-dependent protein kinase II (CaMKII) and mitogen- activated protein kinases (MAPK) on the setting of a synaptic tag during LTP and LTD. First I could confirm the results obtained from other laboratories that CaMKII or MAPK inhibition during the induction blocks the maintenance of LTP/LTD. However, I found that CaMKII or MAPK inhibition after the induction of LTP/LTD had no effect on the maintenance of the processes.

In a next series of experiments I have investigated whether CaMKII can mediate the setting of the synaptic tags in LTP or LTD. Induction of late-LTP in S1 followed by early-LTP in S2 and in presence of CaMKII inhibitor, KN-62 prevented processes of synaptic tagging during LTP while application of KN-62 did not affect synaptic tagging

during LTD. It means setting of tags in LTP is CaMKII mediated while in LTD it is independent of CaMKII.

If CaMKII mediates the setting of synaptic tags in LTP, but not during LTD, the question was which kinase mediates the setting of the LTD-specific tags? By using two mitogen-activated protein (MAP) kinase kinase 1 (MKK1 or MEK) inhibitors, U0126 and PD98059, I could find that setting of LTD tag is mediated by MAPK. Thus LTP tagging is specifically mediated by CaMKII and LTD tagging by MAPK.

Having determined the specifity of LTP- and LTD-specific tags I was now interested to find out the implication of tag-specificity for processes of cross-tagging. I could confirm the findings obtained in LTP/ LTD tagging, that CaMKII and MAPK mediates the setting of LTP /LTD-specific tags respectively in cross-tagging.

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# 1. Introduction

## 1.1. Learning and memory

One of the most important features of the mammalian central nervous system is its capacity for processing and storing information. Learning involves the acquisition of new information; memory is the retention of, and ability to recall information, personal experiences, and procedures (skills and habits) (Squire, 2004). The neural basis of memory is usually studied indirectly by monitoring the effects of brain damage on subsequent cognitive abilities or by measuring neural activity in terms of the hemodynamic, magnetic, or electrical field changes. At the beginning of 20th century Cajal (Jones, 1994b;Jones, 1994a) proposed that neuronal networks are not cytoplasmically continuous, but communicate with each other at distinct junctions, which Sherrington named ´synapses´ (Sherrington CS, 1906). External events are represented in the brain as spatio-temporal patterns of activity within preexisting neuronal circuits. Processes involved in learning and memory formation must therefore occur within preexisting neuronal circuits. The physical representation of a memory is referred to as the engram or memory trace (Dudai, 1996;Dudai, 2004).

Although there is considerable information regarding the properties of memory formation and decay, studying the physical manifestation of memory remains difficult, beginning with a determination of where do memories reside. One of the most intensive searches to localize memory traces-or engrams-within the brain was initiated by Karl Spencer Lashley in the 1920's (Bruce, 2001). Lashley set out to determine the effect of various brain lesions on learning in rats. At the time, Lashley framed his work on the generally accepted belief that, the engram could be located in specific areas of the

neocortex based on the Broadman's cytoarchitectural maps. However, the memory deficits were not localized to specific brain regions, suggesting that the memory traces were distributed throughout the cortex (Thompson, 1991;Thompson and Kim, 1996).

### 1.2. Classification of memory

In subsequent research, psychologists have distinguished several types of memory and have determined that there is considerable localization of function that was missed in Lashley's work. We now know that different types of information require the engagement of different neural systems. Two major subdivisions of memory are declarative (explicit) and nondeclarative (implicit). Declarative memory, memory for facts and events, is associated with awareness and intention to recall. It is generally rapidly acquired, flexible, and prone to distortion (Cohen and Squire, 1980;Squire, 2004;Squire et al., 1993). Nondeclarative memory includes priming, motor skill and emotional memory. It is nonconscious, slowly acquired (except for priming), and inflexible (Squire, 2004).

Declarative memories rely on structures in the medial temporal lobe, including the hippocampus and the entorhinal, parahippocampal, and perirhinal cortices (Squire et al., 1993). Lesions to these structures produce deficits in declarative memory tasks (Scoville and Milner, 2000;Scoville and Milner, 1957;Zola-Morgan et al., 1986;Squire et al., 1993;Squire and Alvarez, 1995). Declarative memory can be further subdivided into episodic memory, involving recollections associated with a time and place, and semantic memory, which is the recollection of facts without the environmental and temporal context. Patients with bilateral medial temporal lobe lesions show both anterograde and retrograde amnesias (Scoville and Milner, 2000;Scoville and Milner, 1957). They cannot

acquire new episodic memories nor retrieve episodic memories stored shortly prior to the time of lesion. They can, however, retrieve declarative memories learned in the more distant past, suggesting that the storage of such information may depend, at least temporarily, on intact and functional medial temporal lobes. A significant role for the hippocampus in declarative memory was identified following neuropsychological research involving a human patient that had undergone bilateral lesions of both the hippocampus and surrounding cortical structures (Scoville and Milner, 1957). The removal of large sections of his temporal lobes including hippocampus left "H.M." unable to form any new personal memories, but his tragic loss revolutionized the field of neurobiology and made "H.M." the most-studied individual in the history of brain research.

Another type of memory associated with awareness involves the short-term retention of a perceptual representation and is termed working memory. Working and declarative memory are dissociable because amnesic patients experience severe explicit memory deficits but normal working memory, and patients with parietal or frontal lobe lesions show poor working memory but normal explicit memory (Warrington and Weiskrantz, 1971).

### 1.3. The Hippocampus

The hippocampus is widely considered to be critical for the initial storage of declarative memories. It receives extensive input from neocortical systems and feeds information back to those same systems (McClelland et al., 1995). It has been suggested that the hippocampus provides a compressed trace for the temporary linking of component neocortical traces that must be activated together to read out the memory

in its entirety. The hippocampus plays a fundamental role in episodic memory, the kind that will let us remember a pleasant dinner party years later. Unlike our memory of facts and events, however, our spatial memory appears to be confined to the hippocampus. This structure seems to be able to create a mental map of space with the help of “place cells” (Nakazawa et al., 2004).

The hippocampus, named for its resemblance to the sea horse (*hippo*= horse, kampos= sea monster; Greek) is formed by two interlocking sheets of cortex and in cross-section has a well defined laminar structure with layers visible, where rows of pyramidal cells are arranged. The different cell layers and sections are defined by the series of connections made. The information from the visual, auditory, and somatic associative cortices arrives first at the parahippocampal region of the cortex, and then passes through the entohorinal cortex and then on to the hippocampus proper.

### 1.3.1. Trisynaptic loop of the hippocampus

Within the hippocampus, the information passes through three distinct regions in succession. The hippocampus proper is composed of regions with tightly packed pyramidal neurons, mainly areas CA1, CA2, and CA3. (“CA” stands for Cornu Ammonis, or Horn of Ammon. The reference is to the ram’s horns of the Egyptian God Ammon, whose shape these three areas together roughly resemble). This makes what is called the trisynaptic circuit or trisynaptic loop of the hippocampus. Information enters this one-way loop via the axons of the entorhinal cortex, known as perforant fibres (or the perforant path, because it perforates or penetrates through the subiculum and the space that separates it from the dentate gyrus). These axons make the loop’s first connection, with the granule cells of the dentate gyrus. From these cells, the mossy fibers in turn

project to make the loop's second connection with the dendrites of the pyramidal cells in area CA3. The axons of these cells divide into two branches. One branch forms the commissural fibers that project to the contralateral hippocampus via the corpus callosum. The other branch forms the Schaffer collateral pathways that make the third connection in the loop, with the cells in area CA1 (Fig.1).

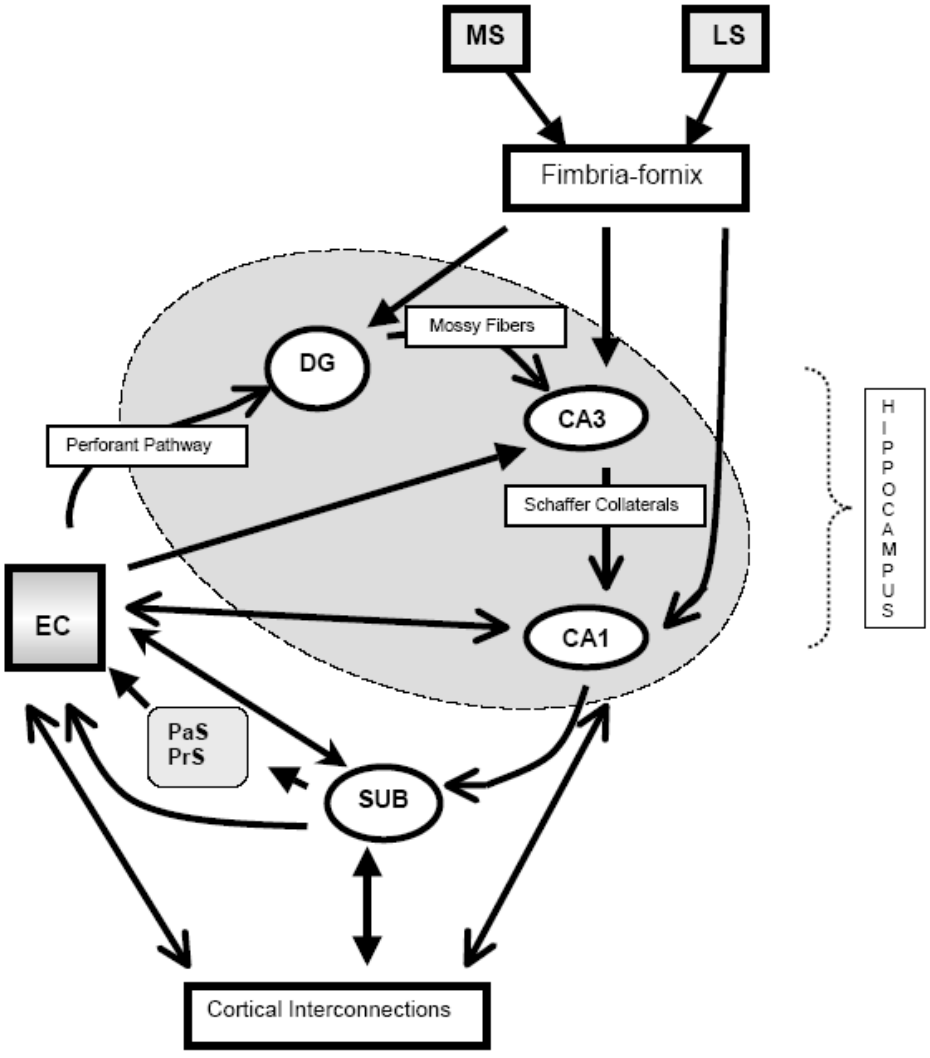



**Fig. 1. Schematic representation of major intrinsic connections of the mammalian hippocampal formation** (adapted from Amaral and Witter, 1995). EC, entorhinal cortex; DG, dentate gyrus; MS, medial septum; LS, lateral septum; CA1 and CA3, fields of Ammon's horn; SUB, subiculum; PaS, parasubiculum; PrS, presubiculum.

### 1.3.2. Hippocampus, an ideal structure for investigating synaptic plasticity

Hippocampus is one of the useful structures for brain slice preparation and for investigating synaptic plasticity. The main reason is because of its structure, that allows a slice to be cut whilst preserving a large number of neurons and their interconnecting axons (Andersen et al., 1969;Amaral and Witter, 1989). The dendritic structure of the three main hippocampal cell types and their interconnecting axons lay in a single plane. This plane is oriented normal to the ventricular surface and to the longitudinal axis of the hippocampus. The lamellar structure allows slices to be taken without destroying the neurons together with their dendrites and axons. The highly organized and laminar arrangement of synaptic pathways with its extensive connections makes the hippocampus (Fig.1, adapted from (Amaral and Witter, 1989)) a convenient model for studying synaptic function in vitro and in vivo (Andersen et al., 1969;Amaral and Witter, 1989).

Brain slices offer a variety of novel opportunities, the most obvious being visual inspection. Depending upon the brain region, histological landmarks can be seen with an ordinary dissecting microscope. In many ways the tissue can be seen in a gross microscopic slide. This allows visual control of electrode placement. It is also possible to direct electrodes to known parts of a given cell. For example, in the hippocampus, an electrode may be placed in the apical or basal dendritic tree of pyramidal cells at known distances from the soma to record the activity of a small group of synapses.

Hippocampal slices in vitro also allow a comparison of the effectiveness of proximal and distal synapses to the same cell to be made. A great advantage is the lack of anaesthesis. This is of obvious importance for many studies on neuronal excitability,

but is also invaluable for many pharmacological studies. Furthermore, in the slice preparation, the influence of the blood brain barrier is removed. The ability to change the tissue concentration of interesting molecules at will provides good experimental control of the preparation. In addition to the temperature and oxygen concentration, the pH, ionic concentration and hormonal levels can be changed at will. The slice neurons are consequently under less synaptic bombardment than cells in the intact brain. Other modulating influences (neuromodulators, biological clocks, hormones) are also absent. The acute hippocampal slice preparation has been widely used to study the cellular mechanisms underlying activity-dependent forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD).

### 1.4. Hebbian learning

Learning and memory involve ongoing adaptations of brain circuitry throughout the life time in response to the environment and are generally thought to result from alterations in synaptic connectivity within the central nervous system (HEBB, 1959;Iriki et al., 1989). The synaptic connectivity changes create new networks or circuits that are believed to represent newly acquired memories. Hebb (1949) increased our understanding of how networks of neurons might store information with the provocative theory that memories are represented by reverberating assemblies of neurons. Hebb recognized that a memory so represented cannot reverberate forever and that some alteration in the network must occur to provide integrity both to make the assembly a permanent trace and to make it more likely that the trace could be reconstructed as a remembrance. Neurons communicate with each other only at synapses, the activity of the assembly or network is most easily altered by changes in synaptic function. Hebb

(1949) formalized this idea in his famous book ´The Organisation of Behaviour´ in what is known as Hebb's Postulate:

*"When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased."*

Hebb's Postulate is very close to a modern-day definition of long-term potentiation (LTP). Bliss and Lomo first reported that brief tetanic stimulation of the perforant path in anesthetized rabbits increased the efficacy of synaptic transmission measured as changes of the population excitatory post-synaptic potential (EPSP) recorded extracellularly in the dentate gyrus. This prolonged increase in synaptic efficacy after a brief high-frequency stimulation of afferent fibers was named long-term potentiation (Bliss and Lomo, 1973). LTP is considered as a cellular correlate of learning and memory (Bliss and Collingridge, 1993).

The effectiveness of LTP as a mechanism for information storage would be severely limited if processes that decrease synaptic strength did not also exist. In area CA1 of the rat hippocampus, prolonged periods of low-frequency afferent stimulation elicit a long-term depression (LTD) that is specific to the stimulated input. Dunwiddie and Lynch discovered long-term depression (LTD) that was found to occur at the synapses between the Schaffer collaterals and the CA1 pyramidal cells in the hippocampus (Dunwiddie and Lynch, 1978). LTD is defined as ‘persistent decrease in synaptic efficacy after a relatively short episode of low-frequency stimulation’ (LFS) (Bear and Malenka, 1994;Braunewell and Manahan-Vaughan, 2001). Since the work of Hebb, 1949 and the discovery of LTP and LTD (Bliss and Lomo, 1973;Dunwiddie and Lynch, 1978), these theoretical connections among neurons that strengthen as a result of

activity are referred to as Hebb synapses. The contention that LTP and LTD might serve as a memory storage device stemmed, at least in part, from its discovery in the hippocampus, a structure that is critical to the formation of certain types of memories.

### 1.5. Properties of LTP and LTD

Basic properties of LTP and LTD include input-specificity, associativity, cooperativity, and late-associativity. LTP and LTD are input-specific in the sense that it is restricted to activated synapses rather than to all of the synapses on a given cell. This feature of LTP is consistent with its involvement in memory formation. If activation of one set of synapses led to activation of all other synapses-even inactive ones-being potentiated, it would be difficult to selectively enhance particular sets of inputs, as is presumably required for learning and memory. Another important property of LTP and LTD are associativity. As noted, weak stimulation of a pathway will not by itself trigger LTP or LTD. However, if one pathway is weakly activated at the same time that a neighbouring pathway onto the same cell is strongly activated, both synaptic pathways undergo LTP or LTD. This selective enhancement of conjointly activated sets of synaptic inputs is often considered a cellular analog of associative or classical conditioning. More generally, associativity is expected in any network of neurons that links one set of information with another. The third basic property of LTP is synaptic cooperativity, i.e. LTP can be induced either by strong tetanic stimulation of a single pathway, or cooperatively via the weaker stimulation of many. It is explained by the presence of a stimulus threshold that must be reached in order to induce LTP. Late-associativity is a novel property of LTP and LTD. It describes intersynaptic interventions within a time frame of a few minutes to a few hours (Frey and Morris, 1997;Frey and Morris,

1998b;Frey and Morris, 1998a). More clearly, a weak protein synthesis-independent early-LTP/-LTD in one synaptic input can be transformed into a late, protein synthesis-dependent form, if a protein synthesis-dependent late-LTP/-LTD is induced in the second synaptic input preceded by the weak events in the first synaptic input (weak before strong) within a specific time frame (Frey and Morris, 1998a;Frey and Morris, 1998b;Kauderer and Kandel, 2000;Sajikumar and Frey, 2004a).

### 1.5.1. Multiple Phases of LTP and LTD

Brief high-frequency stimulation of the CA3-CA1 synapses can result in LTP, which can be divided into several temporal phases characterized by different underlying mechanisms. In general, it is divided into induction, expression and maintenance. The initial induction phase of LTP i.e. so named 'posttetanic potentiation' (PTP) with a duration of several seconds to minutes is characterized by presynaptic mechanisms, i.e. transient increase in transmitter release. PTP is followed by a 'short-term potentiation' (STP) with a duration up to one hour. Postsynaptic events like activation of transmitter receptors by local protein kinases (e.g. CaMKII, tyrosine kinase) (Dobrunz et al., 1997;Huang, 1998) are responsible for the maintenance of that phase. STP can be followed by at least two further phases: early-and late-LTP (Matthies et al., 1990;Huang, 1998). Early-LTP is a transient form of LTP which lasts 2-3 h in vitro and 7-8 h in vivo, while late-LTP lasts for 8-10 h in vitro and days or even months in intact animals (Abraham and Bear, 1996;Abraham, 2003) (Fig. 2).

The different forms of LTP can be specifically induced by distinct stimulus protocols in acute slices in vitro (Frey et al., 1993;Huang and Kandel, 1994). A single high-frequency stimulus train of distinct stimulation strength can induce early-LTP, but

such a protocol is normally not sufficient to induce late-LTP. The induction of late-LTP, on the other hand, requires repeated or stronger trains of high-frequency stimulation. Processes specifically involved in early- and late- phases of LTP require different cellular signaling pathways.

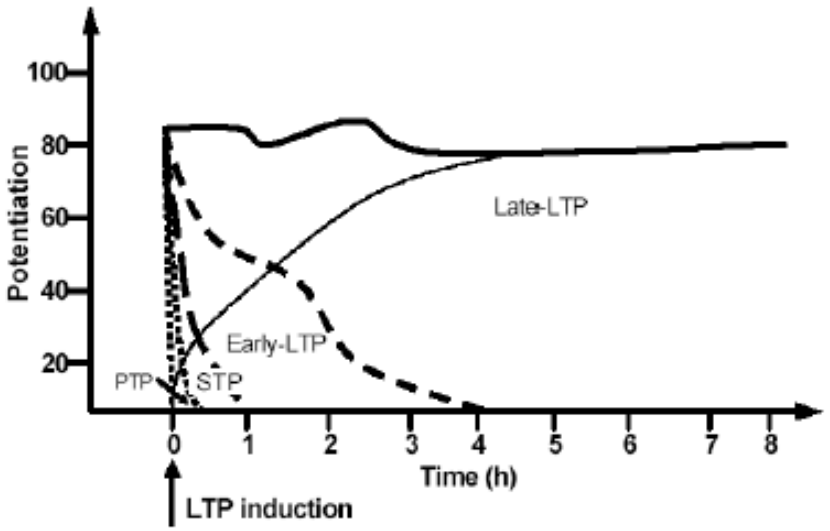



**Fig. 2. The multiple phases of LTP**. See text for a detailed description.

The early-phase of LTP is transient and protein synthesis- independent induced by second messenger cascades, activated by $Ca^{2+}$ influx, and maintained by activated kinases like CaMKII, tyrosine kinase, (Malenka and Nicoll, 1999;Soderling and Derkach, 2000). Late-LTP begins gradually during the first 2-3 h and can last for 6-10 h in hippocampal slices in vitro and for days to months in vivo (Krug et al., 1989;Frey et al., 1995;Otani and Abraham, 1989;Abraham et al., 2002;Kandel, 2001;Reymann et al., 1985). A further major difference between early-LTP and late-LTP is that late-LTP requires protein synthesis (Krug et al., 1984;Frey et al., 1988;Otani et al., 1989). Application of suppressors of RNA-translation during LTP-induction resulted in a

decremental early-LTP while late-LTP was prevented (Krug et al., 1984;Stanton and Sarvey, 1984;Deadwyler et al., 1987;Abraham and Kairiss, 1988;Frey et al., 1988;Frey et al., 1996;Mochida et al., 2001).

The phases and mechanisms of LTD are less extensively studied as compared to LTP. Recently it was reported that LTD within the hippocampal CA1 region in vitro shares similar properties like LTP (Sajikumar and Frey, 2003;Sajikumar and Frey, 2004a). It was shown that different forms of LTD can be induced depending on the induction protocol, in a way as it is the case for the different LTP phases. A reliable long-lasting LTD (late-LTD) up to 8 h can be induced using a strong low-frequency stimuation (SLFS) consisting of of 2700 pulses. Early-LTD, a transient form of LTD lasting less than 2-3 h could be induced using weak low-frequency stimulation (WLFS) consisting of 900 pulses. Like in LTP, early-LTD is protein synthesis-independent while late-LTD is dependent on ongoing protein synthesis (Sajikumar and Frey, 2003). The posttranslational modification of protein-phosphorylation or dephosphorylation of serine and threonine residues is usually considered essential for the initiation and maintenance of long-term potentiation (LTP) and long-term depression (LTD) in neural plasticity.

### 1.6. Cellular mechanisms of LTP and LTD

#### 1.6.1. The involvement of protein kinases and phosphatases

Protein phosphorylation is a key biochemical process involved in synaptic plasticity that operates through a tight balance between the action of protein kinases and protein phosphatases (PPs), which plays an important role in regulating synaptic plasticity in the mammalian hippocampus.

Long-lasting changes in synaptic strength, such as LTP, require intertwining biochemical cascades for their induction and maintenance. Induction of early-phase LTP (early-LTP) in the CA1 region of the hippocampus requires $Ca^{2+}$ influx through the NMDA-type glutamate receptor (N-methyl-D.-aspartate-Receptor) to activate $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) (Lisman and Zhabotinsky, 2001). To convert early-LTP into late-phase LTP (late-LTP), inhibition of protein phosphatase 1 (PP1) is essential to prolong CaMKII activation and phosphorylation of downstream substrates (Blitzer et al., 1995;Colbran and Brown, 2004). PP1 is inhibited during late-LTP by stimulation of $Ca^{2+}$/calmodulin-dependent adenylyl cyclases, raising cAMP levels to activate protein kinase A (PKA) (Wong et al., 1999;Nguyen and Kandel, 1996). Thus, activation of CaMKII combined with inhibition of PP1 "gates" early-LTP into late-LTP at least in juvenile animals (Atkins et al., 2005), but in adult animals this pathway is achieved via a heterosynaptic stimulation of dopaminergic and glutamatergic receptors in CA1.

### 1.6.2. The role of $Ca^{2+}$/calmodulin-dependent protein kinase type II

Calcium/calmodulin-dependent protein kinase II (CaMKII) - a major member of the postsynaptic density - is a $Ca^{2+}$/calmodulin-activated dodecameric enzyme, which is necessary for LTP induction (Malenka et al., 1989;Silva et al., 1992). It is persistently activated by stimuli that elicit LTP, and can, by itself, enhance the efficacy of synaptic transmission. The analysis of CaMKII autophosphorylation and dephosphorylation indicates that this kinase could serve as a molecular switch that is capable of long-term memory storage (Lisman and McIntyre, 2001;Giese et al., 1998). Consistent with such a role, mutations that prevent persistent activation of CaMKII block LTP, experience-

dependent plasticity and behavioral memory (Silva et al., 1992). These results make CaMKII a leading candidate in the search for the cellular and molecular basis of memory.

The Lisman model of LTP (Lisman and McIntyre, 2001) proposes that patterns of synaptic activity that produce low levels of NMDA receptor activation and small increase in intracellular $Ca^{2+}$ depress synaptic strength via a cascade of protein phosphatase activation (Mulkey et al., 1993). This cascade of protein phosphatase activation is thought to entail a $Ca^{2+}$- and calmodulin-dependent activation of calcineurin, that dephosphorylates the PP1 regulatory protein inhibitor-1 (Mulkey et al., 1994). Dephosphorylation of inhibitor-1 activates PP1 that in turn dephosphorylates CaMKII. In contrast, stronger levels of NMDA receptor activation and larger increase in intracellular $Ca^{2+}$ induce LTP by increasing levels of autophosphorylated CaMKII via a simultaneous activation of CaMKII and downregulation of PP1. In the model, a large increase in $Ca^{2+}$ is thought to suppress PP1 activation by stimulating $Ca^{2+}$- and calmodulin-sensitive isoforms of adenylate cyclase (AC) and by activating PKA that suppresses PP1 activation by opposing calcineurin-mediated dephosphorylation of inhibitor-1.

A major target for $Ca^{2+}$ is CaMKII, and among its many actions are the phosphorylation of GluR1 at serine 831 (which increases the channel conductance of the AMPA receptor;(Lee et al., 2000)), and the insertion of the receptor into the postsynaptic membrane through an indirect mechanism; (Hayashi et al., 2000). From a network perspective, the multiple effects of activated CaMKII define it as a ´node´, a point where a signal is split and directed to multiple targets (Schmitt et al., 2005).

### 1.6.3. The interaction of three major postsynaptic signaling pathways in LTP

$Ca^{2+}$/calmodulin protein kinase II (CaMKII), mitogen-activated protein kinase (MAPK), and adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase (PKA) are all required for the induction of LTP (Frey et al., 1993;English and Sweatt, 1997;Bortolotto and Collingridge, 1998). The influx of $Ca^{2+}$ through N-methyl-D-aspartate-type receptors (NMDA-R) or voltage-dependent $Ca^{2+}$ channels (VDCC) can engage signaling cascades that activate these kinases. MAPK and CaMKII can promote the phosphorylation of each other, and MAPK is required for an increase in CaMKII levels produced by LTP-inducing stimulation (Giovannini et al., 2001). PKA activity promotes CaMKII phosphorylation by indirectly inhibiting the protein phosphatase PP1, which would otherwise limit the degree or persistence of CaMKII activation by dephosphorylating the kinase (Atkins et al., 2005). The phosphorylation and inhibition of CaMKII by PKA is likely to be involved in modulating the balance between cAMP- and $Ca^{2+}$-dependent signal transduction pathways (Matsushita et al., 2001). Though PKA was initially identified as having an important signaling role in the protein synthesis-dependent late stages of LTP (Frey et al., 1993), more recent evidence suggests that PKA also provides a mechanism for suppression of protein phosphatase activation in the early stages of LTP induction (Blitzer et al., 1995;Winder et al., 1998;Blitzer et al., 1998). Activation of the cAMP-PKA signaling pathway regulates both activity-dependent changes in synaptic strength and CaMKII phosphorylation in a chemical LTP induction protocol (Yamamori et al., 2004). Adenylyl cyclase (AC), protein phosphatase 2b (PP2b), (also called calcineurin); I-1P, phosphorylated protein phosphatase inhibitor-1, Ras, Rap1, Raf-1, B-Raf, and MAPK/ERK kinase (MEK) are all components of the MAPK cascade.

### 1.6.4. Role of MAPK signalling in synaptic plasticity

In mammalian cells, three major groups of MAPK have been identified: extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPK. It is well documented that ERK is typically stimulated by growth-related signals, whereas the JNK and p38 MAPK cascades are activated by various stress stimuli (Kelly et al., 2003). Studies have indicated that MAPK are expressed abundantly in the central nervous system (CNS) and that ERK is involved in long-lasting neuronal plasticity, including long-term potentiation and memory consolidation (English and Sweatt, 1997).

The ERK cascade, like the other MAPK cascades, is distinguished by a characteristic core cascade of three kinases. The first kinase is a so-called MAPK kinase kinase (MAPKKK, Raf-1 and B-Raf in the erk cascade) which activates the second, a MAP kinase kinase (MAPKK, mitogen-activated protein (MAP) kinase kinase 1 (MKK1 or MEK) in the ERK cascade), by serine/threonine phosphorylation. MAPKKs (MEKs) are dual specificity kinases which in turn activate a MAP kinase (p44 MAPK = ERK1, p42MAPK = ERK2) by phosphorylating both a threonine and a tyrosine residue. The ubiquitous Raf-1 pathway is activated by Ras, which is stimulated by growth factor tyrosine kinase receptors, PKC also activates this pathway by interacting with either Ras or Raf-1. Activation of Raf-1 leads to activation of MEK and consequently the ERKs. The Ras/Raf-1 pathway is inhibited by PKA, which prevents Raf-1 activation and attenuates its activity. In an important breakthrough, Gibson and coworkers discovered that cAMP can be positively coupled to ERK activation in neurons via Rap-1 and B-Raf (Widmann et al., 1999). The B-Raf pathway is stimulated by cyclic AMP-dependent protein kinase (PKA) and signals through the Ras homolog, Rap1.

There is also strong evidence that activation of MAPK is necessary for late-LTP and long-term memory (English and Sweatt, 1997). The first suggestion that ERK participates in LTP was provided by the observation that mRNA levels for ERK2 was elevated in dentate granule cells 24 h after induction of LTP at the perforant path granule cell synapse in vivo (Thomas et al., 1994). A similar profile of mRNA expression after LTP induction was noted for both B-Raf and Raf-1, which is consistent with the activation of either a Ras- or a Rap-dependent pathway upstream of ERK activation during LTP. ERK appears to be critical for expression of both NMDA receptor-dependent and NMDA receptor-independent LTP in area CA1, because delivery of LTP inducing stimulation in presence of MEK inhibitors attenuates LTP. Additionally, MAPK has been shown to be required for long-term memory (Adams and Sweatt, 2002). Inhibition of MAPK impairs long-term (spatial and fear conditioning), but not short-term memory (Blum et al., 1999).

### 1.6.5. Role of Protein kinase C

Protein kinase C (PKC) is a heterogeneous family of ten or more isoforms which plays an important role in neuronal signal transduction. Isoforms from all subclasses are prominently expressed in the rat hippocampus, as demonstrated by immunoblot with isozyme-specific antisera: conventional ($Ca^{2+}$/diacylglycerol (DAG)-dependent), novel ($Ca^{2+}$-independent, DAG-dependent), and atypical ($Ca^{2+}$/DAG-independent). In addition, the zeta isoform is also found as the free, constitutively active catalytic domain, protein kinase Mzeta (PKMζ) (Hernandez et al., 2003;Ling et al., 2002).

Inhibitors of protein kinase C block different phases of hippocampal long-term potentiation (Reymann et al., 1988a;Reymann et al., 1988b). PKC activation is not

essential for the initial phase of LTP, but is a necessary condition for a medium and a late, protein synthesis-dependent phase in this monosynaptic pathway, i.e. for the maintenance of synaptic LTP (Reymann et al., 1988a;Reymann et al., 1988b). But contrary to these views on the subject some results show that postsynaptic PKC is essentially involved in both the initial induction and the subsequent maintenance of LTP, (Wang and Feng, 1992).

PKC isoform consists of an amino-terminal regulatory domain, containing an autoinhibitory pseudosubstrate sequence and second-messenger binding sites, and a carboxy-terminal catalytic domain (Nishizuka, 1995;Ohno and Nishizuka, 2002). PKC is normally held in an inactive basal state by interactions between these two domains. Second messengers activate PKC by binding to the regulatory domain and causing a conformational change that temporarily releases the autoinhibition.

PKM, in contrast, consists of an independent PKC catalytic domain, which, lacking PKC's autoinhibitory regulatory domain, is autonomously active (Schwartz, 1993). In brain, only a single isozyme, the atypical ζ, forms a stable PKM (Sacktor et al., 1993). In LTP, PKMζ increases by new protein synthesis through increased translation from a PKMζ mRNA, producing the independent ζ catalytic domain (Hernandez et al., 2003). The persistent activity of PKMζ is both necessary and sufficient for maintaining LTP (Ling et al., 2002).

### 1.6.6. cAMP-dependent protein kinase (PKA) and synaptic plasticity

Cyclic-AMP dependent protein kinase (PKA) is a serine-threonine kinase that has been strongly implicated in the expression of specific forms of long-term potentiation (LTP), (Frey et al., 1993;Huang and Kandel, 1994) and LTD, (Nguyen and Woo, 2003)

and hippocampal long-term memory. The principal target for cAMP in mammalian cells is cAMP-dependent PKA, which is ubiquitously expressed and mediates intracellular signal transduction and intercellular signal transmission in invertebrates and vertebrates.

The hippocampal cAMP/PKA signalling cascade is principally activated by two mechanisms. The first involves calcium and calmodulin (Ca/CaM). Influx of calcium stimulates Ca/CaM-sensitive adenylyl cyclase, which synthesises cAMP (Eliot et al., 1989). One route of $Ca^{2+}$ influx is through NMDA receptors. Activation of these receptors, can increase cAMP levels in area CA1 of the hippocampus (Chetkovich et al., 1991). The second mechanism for activation of cAMP/PKA signalling involves binding of chemical transmitters and hormones to their receptors, followed by stimulation of adenylyl cyclase by guanine nucleotide-binding regulatory proteins (G-proteins) (Tang and Gilman, 1991). These G-proteins interact with adenylyl cyclase on the inner membrane surface to activate the production of cAMP.

The dependence of late-LTP, in hippocampal slices and behavioral memory, on PKA activity suggests that increasing cAMP signaling might increase behavioral memory by raising the probability that long-lasting synaptic plasticity would occur after synaptic stimulation (Barad et al., 1998). Administration of cAMP analogs such as Sp-cAMPS alone can cause long-lasting potentiation in rats that occludes subsequent electrical induction of late-LTP (Frey et al., 1993), suggesting that simply elevating cAMP throughout the hippocampus or brain might occlude rather than enhance synapse-specific strengthening. Given the significant role of cyclic nucleotides in signal-transduction pathways, it is not surprising that their metabolism and synthesis is highly regulated. Such metabolism is achieved by a large number of enzymes, the

phosphodiesterases (PDE), which catalyze the conversion of cAMP and cGMP into 5′-AMP and 5′-GMP, respectively, via hydrolysis of the 3′-phosphoester bonds.

### 1.7. Phosphodiesterases

Cyclic nucleotide phosphodiesterases (PDE) are a large family of enzymes composed of at least 14 transcription units, many with alternately spliced isoforms. PDEs are comprised of three domains: an N-terminal regulatory domain (residues 1-151 in PDE4B), a catalytic domain (residues 152-489 in PDE4B) and a C-terminal domain (residues 490–568 in PDE4B). N-terminal domains of most PDE families contain unique regulatory domains harboring binding sites for small messenger molecules such as $Ca^{2+}$/calmodulin, cGMP and/or recognition sites for protein kinases including the $Ca^{2+}$/calmodulin-dependent kinase and the protein kinases A. The catalytic domain is the most conserved domain among the PDE families. The PDEs have been grouped into seven families based on their regulation and substrate specificity, two of which, type IV and type VII, have cAMP as their nearly exclusive substrate.

The PDE4 isozymes are cAMP-specific, high-affinity PDEs. Members of this family are found in many tissues in both soluble and membrane-associated forms and are abundant in the central nervous system. PDE4A and PDE4B are expressed at relatively high concentrations in hippocampus, cerebral cortex and striatum and represent the majority of the membrane-bound form of PDE4 in these brain regions. Multiple isozymes have been identified, and at least four separate genes exist, which are highly conserved across several mammalian species. The PDE4 isozymes are regulated by phosphorylation and by binding cAMP. In addition, expression of certain PDE4 genes is regulated significantly by activation of the cAMP intracellular pathway.

Ahmed and Frey identified a specific type IV phosphodiesterase gene, PDE4B3, the first cAMP-specific phosphodiesterase to be associated with LTP in hippocampal CA1 area in vitro (Ahmed and Frey, 2003). They showed that PDE4B3 is modulated during LTP phases. Its activation is NMDA-receptor dependent and its transcription is transiently up-regulated 2 h after tetanization. Protein expression peaks 6 h after LTP induction and is rapidly down-regulated at 8 h, whereas cAMP levels decrease during LTP phases. Immunohistochemical studies identified that the majority of type IV phosphodiesterase protein staining is localized to the cell bodies and dendrites of neurons in hippocampal CA1. But in contrast with area-CA1, PDE4B3-levels in area dentata are characterized by a translational, but not transcriptional regulation within the first 8 h of LTP. Spatial-temporal changes of PDE4B proteins after LTP-induction occurs within the area dentata and was restricted to the soma protein fraction, whereas the substrate, cAMP-levels fluctuate in different compartments, depending upon possibly modulatory inputs. These results may add further support to the hypotheses that different hippocampal structures exhibit different processes in maintaining LTP. Moreover PDE4B3 mRNA is not translocated during LTP out of the soma into dendrites of area CA1, PDE4B proteins and cAMP-levels change in different tissue fractions may have a role in synaptic plasticity and cellular memory formation. Phosphodiesterases inhibitors can increases the cAMP levels by decreasing their breakdown. Rolipram is a well-documented isozyme-selective inhibitor of PDE4.

### 1.7.1. Rolipram

The type 4 cAMP-specific phosphodiesterases (PDE4s) are $Mg^{2+}$-dependent hydrolases that catalyze the hydrolysis of 3', 5'-cAMP to AMP. Since the first finding that

the antidepressive drug 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone (rolipram) specifically inhibits the cAMP-specific PDE4, selective inhibition of PDE types has received particular attention in connection with the development of novel drugs. Rolipram is a PDE inhibitor which is selective for the $Ca^{2+}$/ calmodulin-independent and cAMP-specific isozyme of PDE4 (Beavo, 1988). Rolipram is particularly interesting because many pharmaceutical companies are devising a rolipram-like drug that targets the brain's memory centers, to improve memory and to alleviate diseases like alzheimers. It has been found that rolipram increases cAMP levels in vitro (Donaldson et al., 1988) and in vivo (Schneider, 1984). Administration of cAMP analogs can induce late-LTP (Frey et al., 1993). In contrast to the artificial raise of cAMP by the analogs used in the latter study, PDE-inhibitors maintain the basal cAMP concentration within a physiological range by inhibiting their metabolism (Barad et al., 1998) However rolipram has been associated with side effects, such as emesis and nausea. Although it is not certainly clear whether these side effects are caused by direct interaction between rolipram and the PDE4 or by separate effects on central nervous system, but, recent investigations indicate that the described side effects are caused by interaction of rolipram with high-affinity rolipram binding site (HARBS), one of the two pharmacological distinct conformational states of PDE4 isoenzymes, while several therapeutically relevant effects of PDE4 inhibitors are related to inhibition of PDE4 in its low-affinity rolipram binding conformation. Therefore, extensive studies have been performed to develop rolipram-like drugs, which lack these side effects but retain PDE4-inhibitory properties by increasing the specificity of the inhibitors for the low-affinity rolipram binding site.

### 1.7.2. Rolipram binding site

Rolipram was found to bind in the same site as cAMP. The pyrrolidinone group of rolipram could adopt two different orientations to fit the electron density. The phenyl group is sandwiched between the side-chains of Phe446 and Ile410. Asn395 and Tyr233 formed the bottom of the pocket. The methoxy group was completely buried at the back of the binding site in a small pocket made up of Tyr403, Tyr233, Thr407, Pro396, Gln443, Asn395, Ile410 and Trp406. The cyclopentoxy group was oriented at the front of the pocket, partially solvent-exposed, but in close proximity to Phe414, Phe446, Met411, Met431, Ser442, and Gln443. The methoxy and cyclopentoxy oxygen atoms each made hydrogen bonds with the side-chain NH2 of Gln443. The hydrogen bonds were in the same plane as the phenyl group. The orientation of the Gln443 side-chain was fixed by an additional hydrogen bond between its carboxyl oxygen and the hydroxyl group of Tyr403. Rolipram binds to the high-affinity and low-affinity states of the protein with a $K_i$ of 5-10 nM and 350-400 nM, respectively. The high-affinity binding state requires both the N-terminal domain and the catalytic domain, while the low-affinity binding state requires only the catalytic domain. The truncated protein (152-528) used for structural studies described below contains only the low-affinity binding state ($K_i$=350–400 nM). Well characterised mutations that affect rolipram binding are primarily located in the binding pocket and are predicted to be in contact with cAMP substrate (Atienza et al., 1999).

### 1.7.3. Rolipram and memory

Rolipram, a selective inhibitor of type 4 cAMP-specific phosphodiesterase (PDE4), produces an increase in brain cAMP levels via the inhibition of its degradation

(Schneider, 1984). Behavioral studies show that rolipram inhibits locomotor activity and rearing induced by methamphetamine and produces biphasic effects on schedule-controlled behavior, increasing response rate at lower doses and decreasing response rate at higher doses (O'Donnell and Frith, 1999;Iyo et al., 1995). Rolipram also exhibits antidepressant-like effects in animal models and in patients with depressive disorders (O'Donnell and Frith, 1999;O'Donnell, 1993;Hebenstreit et al., 1989).

Imanishi and colleagues (Imanishi et al., 1997) found an ameliorating effect of rolipram on learning and memory impairment in rodents. Rolipram also has been shown to reverse the impairment of either working memory or reference memory induced by the muscarinic receptor antagonist scopolamine (Egawa et al., 1997;Imanishi et al., 1997;Zhang and O'Donnell, 2000).

Inhibition of the phosphodiesterase 4 (PDE4) enzyme reverses memory deficits produced by infusion of the MEK inhibitor U0126 into the CA1 subregion of the rat hippocampus thus PDE4 is likely to be an important link between the cAMP/PKA and MEK/ERK signalling pathways in the mediation of memory (Zhang et al., 2004). In mouse hippocampal CA1, rolipram reinforced a transient into a lasting potentiation (Barad et al., 1998). The dependence of late-LTP, in hippocampal slices and behavioral memory, on PKA activity suggests that increasing cAMP signaling might increase behavioral memory by raising the probability that long-lasting synaptic plasticity would occur after synaptic stimulation. Moreover it was speculated that rolipram, by generally raising cAMP concentration throughout the brain, may enhance memory by a different mechanism perhaps by consolidating changes at recently stimulated synapses "tagged" by endogenous signalling mechanisms (Barad et al., 1998).Thus PDE mediates the synthesis of PRPs in an early-LTP input which otherwise is incapable of synthesising

proteins. A fundamental question in the synaptic plasticity field is how these newly synthesized proteins are specifically targeted to the tetanized synapses without affecting nearby un-tetanized synapses. Are the gene products produced in the cell body and transported specifically to, or are they produced locally at the tetanized synapses? Synaptic tagging hypothesis provides a clear answer to these questions (Frey and Morris, 1998a;Frey and Morris, 1997).

### 1.8. Synaptic tagging

Gene expression and protein synthesis that mediate the long-term changes of LTP generally take place in the cell body or, for protein synthesis, in dendritic compartments, i.e. far away from the stimulated synapse. However also late-LTP is synapse-specific, i.e. LTP induced at one synapse does not propagate to adjacent inactive synapses. Therefore, the cell is posed with the difficult problem of synthesizing plasticity-related products in the nucleus or cell body, but ensuring they only reach synapses that have received LTP-inducing stimuli. As a possible solution to this targeting problem, the 'synaptic tag hypothesis' (Frey and Morris, 1997;Frey and Morris, 1998a) proposed that the persistence of LTP is mediated by the intersection of two dissociable events. The first event involves the generation of a local ‘synaptic tag’ at specific synapses in association with and perhaps causally related to the induction of LTP. The second involves the production and diffuse distribution of ‘plasticity-related proteins’ (PRPs) that are captured and utilised only at those synapses possessing a tag.

The ´synaptic tagging´ hypothesis describes a mechanism, how input specificity is achieved during a protein synthesis-dependent stage (Frey and Morris, 1997;Frey and Morris, 1998a;Frey and Morris, 1998b;Martin and Kosik, 2002). Late-LTP was induced

on one pathway (S1), and the protein synthesis inhibitor anisomycin was then bath applied just before the second pathway (S2) was tetanised. Normally, only early-LTP would be induced and late-LTP inhibited in the presence of anisomycin. However, the LTP induced on S2 remained potentiated for up to 8 h post-tetanus. Similarly, “weak” tetanic stimulation that normally induces only early-LTP could be ‘transformed’ into late-LTP heterosynaptically if a “strong” tetanus was delivered to an independent input to the same population of CA1 pyramidal cells shortly before or shortly after the weak tetanus (Frey and Morris, 1998b). Similar findings reflecting synaptic capture, but at the single-cell level, have been observed in Aplysia neurons in culture (Martin et al., 1997).

The synaptic-tag hypothesis also makes a number of other predictions. One is that the successful induction of late-LTP at a synapse will depend on the intersection of two parameters: the decay time course of the tag and the intracellular kinetics of relevant protein synthesis and distribution; however, which of these is initiated first is unimportant. The synaptic-tag hypothesis can also help explain the observation that the induction of early-LTP on one pathway precludes the induction of further early-LTP on that same pathway for a period thereafter, but that early-LTP can be induced on a pathway displaying late-LTP. Young et al showed that late-LTP-associated transcription and the expression of prolonged potentiation can be differentially regulated by previous synaptic activity (Young and Nguyen, 2005).

The synaptic tag hypothesis allows us to think about the properties of LTP in a new way. The usual way of thinking about associativity is in terms of the heterosynaptic interaction of two or more inputs, over a short time scale (less than one second), mediated via the voltage dependence of the NMDA receptor. The synaptic tag idea points to a secondary form of associativity in which one input can influence another over

a much longer time scale (about 90 min). Moreover depotentiation after 5 min can effectively reset the tag but 10 or 15 min is unable to reset the tag complex (Sajikumar and Frey, 2004b). Input specificity is usually considered in relation to the compartmentalization of $Ca^{2+}$ transients within dendritic spines and thus local $Ca^{2+}$-dependent phosphorylation. However, the input specificity of late-LTP is determined by local tags that sequester proteins manufactured a relatively long way away. Finally, persistence can be variable; whether or not early- LTP is transformed into late-LTP will depend on the history of activation of the neuron, during both the immediate past and the time that follows shortly after. This history includes heterosynaptic activation of aminergic as well as glutamatergic input pathways, the former being particularly important in freely moving animals. Recently Sajikumar and Frey (Sajikumar and Frey, 2004a) have reported that synaptic tagging occurs during LTD with a similar time course as in LTP. Synaptic tagging has been described also by other laboratories and is now a widely studied model for processes involved in the associative interaction of neurons in neuronal nets during memory formation (Frey and Morris, 1997;Frey and Morris, 1998a;Martin, 2002;Martin and Kosik, 2002;Sajikumar and Frey, 2004a;Navakkode et al., 2004;Sajikumar et al., 2005b;Fonseca et al., 2004;O'Carroll and Morris, 2004;Young and Nguyen, 2005). Synaptic tagging encourages us to think of LTP and LTD in the context of the entire neuron; it is a step towards a better understanding of the cellular and molecular basis of memory.

### 1.8.1. The identity of the 'synaptic tag´

What is the molecular identity of the putative synaptic tag? The tag does not necessarily has to be a single molecule, however, experimental data indicate that the

tag must satisfy a number of criteria: (1) the tag is induced in a protein synthesis-independent manner, (2) the tag possesses a lifetime of 1-2 hr, (3) the tag is induced both by early-LTP/ -LTD and by late-LTP/ -LTD, (4) the tag is induced in an input-specific and physically immobile manner, (5) the tag interacts with the proteins required for late-LTP/-LTD to facilitate capture/tagging, and (6) distinct tags are created as a consequence of LTP/LTD induction. A number of possible postsynaptic modifications have been enumerated as candidates for the synaptic tag (Frey and Morris, 1998a;Martin and Kosik, 2002). One possible candidate is the phosphorylated state of an early-LTP-associated kinase with duration of about four to six hours. For example, it is known that LTP requires the activation of various kinases, such as CaMKII, while LTD requires the activation of various phosphatases, such as calcineurin (CaN). Another possibility could be a change in cytoskeletal dynamics. There is evidence that cytoskeletal changes occur during LTP and LTD, these changes, along with changes in the molecular motors that interact with the cytoskeleton, could form the mechanism behind the tagging process. Alterations in membrane receptor number, molecular architecture of the synapse, localized protein degradation, or conformational changes in particular molecules, among other possibilities, could also form the basis for the tag. BDNF, Sp-cAMPS, and DHPG which are sufficient to induce late-LTP may also provide some clues to the nature of the tag (Hegde, 2004). The atypical protein kinase C known as protein kinase Mζ (PKMζ), the persistent activity of which requires protein synthesis, has been shown to be another possible candidate. But recently we could identify PKMζ as the first LTP specific plasticity related protein, not a tag molecule (Sajikumar et al., 2005b). Inhibition of PKA before and during the tetanic stimulation blocked the late-LTP in the CREB transgenic mice, suggesting that PKA is required for tagging the synapse.

Another potential candidate for a synaptic tag that has recently received significant attention is the actin microfilament network at the synapse. The actin network in neurons is extremely dynamic, and these dynamics have been shown to change with activity. Studies have shown that ubiquitin-mediated proteolysis of the regulatory subunit of PKA results in a persistently active kinase, and that this degradation involves the transcriptional induction of an ubiquitin carboxy-terminal. Together, these findings raise the possibility that local activation of PKA and local regulation of the ubiquitin-proteasome pathway can serve as synaptic tags that combine with transcriptional events (for example, the induction of the ubiquitin carboxy-terminal hydrolase) to produce persistent increase in synaptic strength due to local protein synthesis (Hegde, 2004).

### 1.8.2. Cross-tagging

Recently, the 'synaptic tagging' model has been expanded to include interactions between LTP and LTD, referred to as synaptic ´cross-tagging´. In cross-tagging, a late LTP/-LTD in one synaptic input S1 transforms the opposite, protein synthesis-independent early LTP/-LTD in an independent input S2 into its long-lasting form. Thus heterosynaptic induction of either LTD/LTP on two sets of independent synaptic inputs S1 and S2 can lead to late-associative interactions: early-LTD in S2 was transformed into a late-LTD, if late-LTP was induced in S1 (Fig. 3). The synthesis of process-independent PRPs by late-LTP in S1 was sufficient to transform early- into late-LTD in S2 when process-specific synaptic tags were set. Cross-tagging not only expands the repertoire of interactions between pathways but raises the fundamental question of whether the function of a newly synthesized PRP is to generally prolong the action of a

weak stimulus, or whether there are separate PRPs to specifically prolong potentiation or depression.

Based on synaptic tagging- and cross-tagging-hypotheses, Susumu Tonegawa proposed a “clustered plasticity model” for long term memory engrams in which he proposed, for long-term memory engrams, the memory representation at the single-cell level is composed of a pattern of synaptic potentiation and depression clustered in one or a few dendritic branches, and not dispersed to all the dendritic compartments. In a nut shell, the inevitable diffusion of newly made proteins through dendrites from the site of synthesis and the similarity of the protein repertoire accompanying late-LTP and late-LTD provide a molecular basis for the tagging and cross-capture hypothesis for late-LTP and late-LTD.

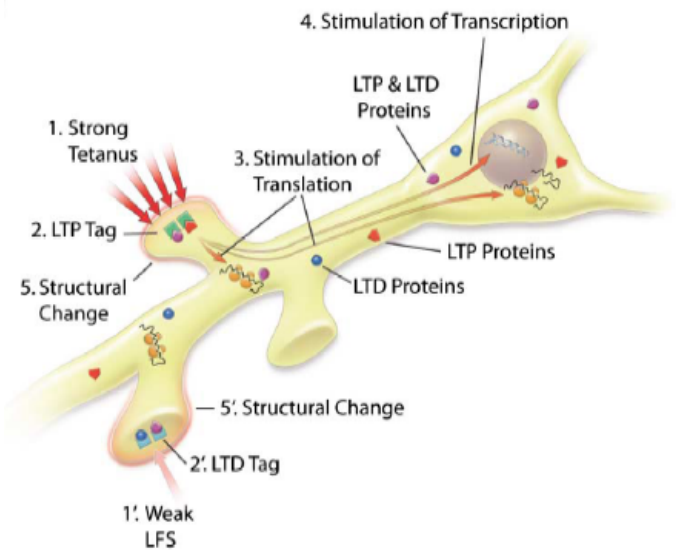



**Fig. 3. Model for conversion of early-LTD to late-LTD via synaptic capture (from Kelleher et al, 2004b).**

When four tetanic trains are delivered to a synapse (1), an LTP tag (2) is formed at the synapse (this tag may also be induced by a single tetanic train), and translation and transcription (3 and 4) are induced. The

newly synthesized proteins can support expression of both late-LTP and late-LTD and are thus presumed to include products necessary for late-LTP, late-LTD, or both. These newly synthesized proteins are also available to other synapses, but only the synapse bearing the LTP tag captures the proteins necessary for late-LTP, causing that synapse to express late -LTP and the accompanying structural changes (5). If a second synapse then receives weak-LFS (1′) in close temporal proximity to stimulation of the first synapse, transcription and translation are not induced, but an LTD tag is created (2′). This synapse then captures the proteins necessary for late-LTD (which were synthesized in response to late-LTP induction at the first synapse), resulting in structural changes (5′) and expression of late-LTD at the second synapse. A similar process also occurs when early-LTP is converted to late-LTP. Since the tag has a half-life of approximately 1-2 h, the weak stimulation can occur either before or after the strong stimulation (from Kelleher et al, 2004).

### 1.9. Aims of this dissertation

In the first part of my dissertation the main aim was to study the role of rolipram, a selective cAMP phosphodiesterase inhibitor, on late plastic events during functional CA1 plasticity in vitro in hippocampal slices. First, I studied the role of PDE inhibition on LTP, and could show that rolipram reinforces an early form of LTP into a late form of LTP (RLTP). In a further series of experiments, I studied the properties of RLTP like protein-synthesis and NMDA-receptor dependence and whether RLTP can interact with processes of synaptic tagging. Further, I studied whether RLTP was influenced by inhibitors of the D1/D5 receptor.

We have earlier shown that PKMζ represents a plasticity-specific-protein (PRP) which is both necessary and sufficient for LTP, but not for LTD. So my next main aim was to study whether the action of PDE is specific like PKMζ or it can act as a process-unspecific PRP necessary for mediating processes of both plasticity forms. So I studied the role of PDE inhibition on LTD and its properties. Further I could also show that RLTD

can interact with processes of synaptic tagging which is dependent on D1/D5-receptors unlike in case of RLTP. In addition I was interested to investigate the mechanism by which rolipram reinforces early-LTD into late-LTD. For that, I studied whether RLTD requires the activation of ERK1/ERK2. Then I further delineated the specific pathways by which ERK1/ERK2 is activated during RLTD i.e. whether the activation of ERK1/ERK2 occurs by a Rap/PKA or Ras/Raf-1 pathway.

The second part of my dissertation deals with the question of what exactly is the putative nature of the synaptic tag. I studied the role of two promising candidates CaMKII and MAPK during the processes of synaptic tagging in LTP and LTD. I could show that CaMKII plays an important role in mediating the setting of tags in LTP, but not in LTD. MAPK was shown to mediate setting of tags in LTD, but not for LTP. Further I confirmed these data with cross-tagging experiments using CaMKII and MAPK inhibitors.

# 2. Materials and methods

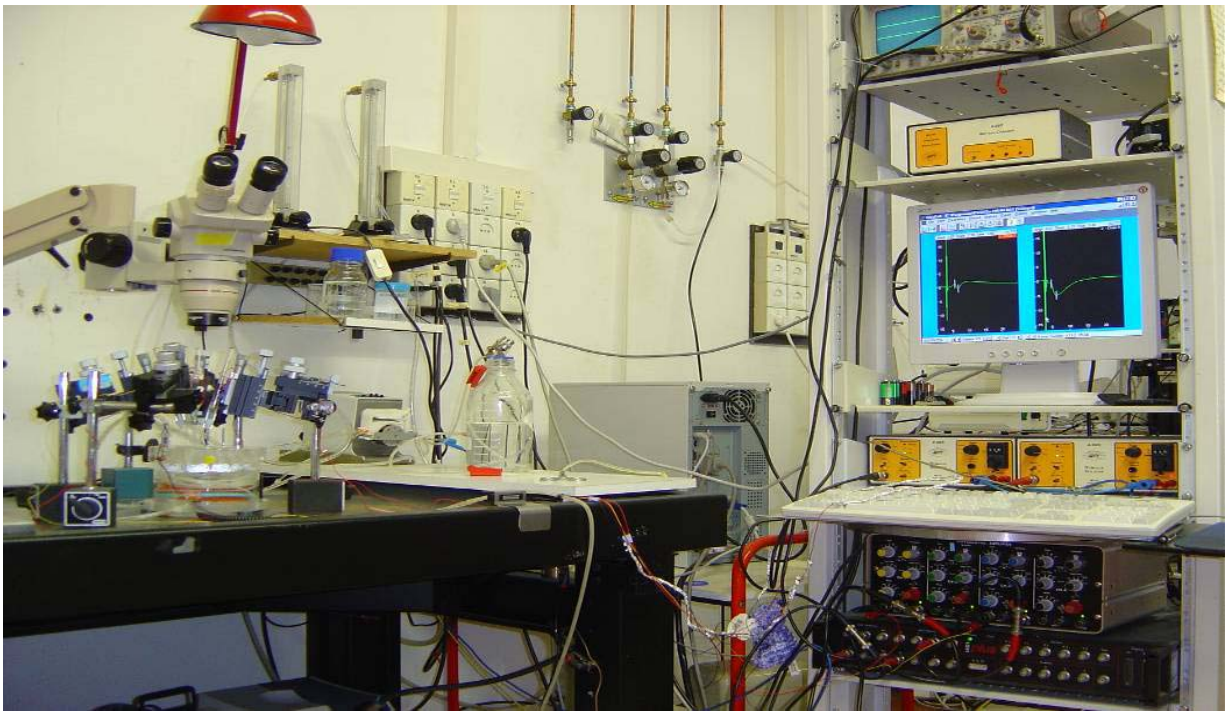
## 2.1. Hippocampal slice preparation

All experiments were performed in right hippocampal slices (400 μm thick) prepared from 7 weeks old male Wistar rats (total number of animals: 310). The animal was stunned by a blow behind the foramen magnum and decapitated (cervical dislocation). Following decapitation, the skin and fur covering the skull were cut away and an incision was made on both sides. The bone covering the brain was prised away and dura removed before transferring the brain into chilled and carbogenated (carbogen: gas consisting of 95% $O_2$ and 5% $CO_2$) artificial cerebrospinal fluid (ACSF) (about 4°C) (Reymann et al., 1985). Cold solution was used to slow down the metabolism of the tissue, to limit the extent of excitotoxic and other kinds of damage occurring during the preparation of slices (Reymann et al., 1985). Chilling the petridish and tissue slicer support on ice may help reduce tissue deterioration. Brain is placed in a petriplate on filter paper and the cerebellum and frontal cortex is dissected away. Divide the remaining part of the brain in the central sulcus by a deep cut using a scalpel and the hippocampal commissure was cut and the right hippocampus was taken out on to the stage of manuel tissue chopper (Cambden, UK), and 400 μm thick slices were cut at 70° transverse to the long axis from the middle third of the right hippocampus. After sectioning, the slices were picked up by a wet artist's brush, floated in a petri dish containing the cooled and carbogenated ACSF, and immediately transferred to the nylon net in the experimental chamber maintained at 32$^{o}$C by a wide bored pipette. One of the critical points which elapse between the removal of the brain and the placing of the slices in the chamber is that slice preparation should be performed in less than 3 min

and favourably at a temperature of 4$^{o}$C to minimize cellular metabolism and to avoid irreversible intracellular phase changes. It is well known from studies investigating ischemic and/or hypoxic influences on brain function that ischemic episodes with a duration of longer than 3 min as well as glutamate receptor-dependent calcium release during preparation can result in an irreversible prevention of protein synthesis in nervous tissue (Erdogdu et al., 1993;Djuricic et al., 1994;Djuricic et al., 1995). Furthermore, to obtain these morphological and functional characteristics we use always a cleaned new razor blade for each preparation of not more than 3-4 slices from a single hippocampus from one animal to obtain hippocampal CA1-slices (dorsal part of the right hippocampus of males). When slices are taken out with proper care the responses, observed on stimulation are similar to those seen in intact animals. Slices were incubated within an interface chamber (Fig. 4) at 32°C (carbogenated incubation medium contained 124 mM NaCl, 4.9 mM KCl, 1.2 mM $KH_2PO_4$, 2.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 24.6mM $NaHCO_3$, 10 mM D-glucose). Supply of oxygen was achieved by controlling the gas flow over the surface of the slice (carbogen flow rate: 32 l/h) thus preventing the drying out of the slices (Sajikumar et al., 2005a).

Slices were preincubated for at least 4 h, a quite unusual long period, but it has been shown by the following reasons to be critical for a stable long-term recording as well as the study of late plasticity for up to 16 h, under conditions which resemble the functionality of studies in vivo.Temperature conditions are also crucial for mammalian slice preparation in vitro. Studies from Micheva and Smith revealed that subphysiological temperatures might dramatically affect functional plasticity in mammalian presynaptic terminals (Micheva and Smith, 2005).

**A**



**B**

**C**

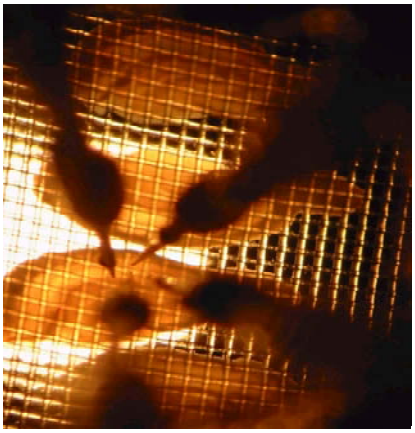


**Fig. 4. Interface chamber and electrical set-up for long term extra cellular recording.**
**(A)** An overview of recording chamber and its electrical set-up. **(B)** Interface chamber with manipulators. **(C)** Microscopic view of a hippocampal slice located with electrodes.

Hippocampal slices in vitro are characterized by a very low spontaneous activity which may result from an almost 'absolute rest' during preincubation. Biochemical studies have shown that metabolic stability is reached in slices after 2-4 h, i.e., metabolite levels require 2-4 h to stabilize, and these levels are then maintained for at

least 8 h of incubation (Whittingham et al., 1984). This includes parameters for the activity of enzymes, second messengers, pH, and others. Interestingly, the value for bio-active molecules which stabilizes at a very low level, if strong electrical stimulation was not delivered to the tissue. We suppose that in addition to processes of the acute slice preparation, low electrical activity may result in the delayed but prolonged metabolic stability at a low level after about 4 h if no stimulation is applied to the tissue. This may lead to a reduction of PRPs to an amount near zero if the half life of the proteins is considered with about 2 h. Thus, starting with functional experiments after a preincubation time of 4-5 h may rectify all slices and neurons to a low but very comparable basal metabolic and plasticity level. Tetanization for instance, would then activate a machinery of processes ‘from zero’ (a situation never occurring in behaving animals) which is mechanistically more useful to determine time constants during plastic events, than it would be the case by using freely behaving untreated rats. If in intact rats protein synthesis is blocked by a pharmacological reversible inhibitor a similar situation as in slices can be created revealing similar time constants for early-LTP in vitro. Unfortunately, currently available reversible protein synthesis inhibitors reduce the synthesis of macromolecules in the intact animal for several hours, making this preparation probably unusable to directly study processes of synaptic tagging with the methods used so far. Thus, slice preparations represent an ideal, however also partially artificial model to determine properties of tagging and late-associativity. Although, most of the problems concerning brain slice incubation are known for a long time, most laboratories start their ‘physiological’ slice experiments after a very short preincubation period of even less than 1 h. Knowing the metabolic instability during that period we prolonged the preincubation of hippocampal slices to at least 4 h to obtain comparable

and more physiological results in describing functional processes in slice preparations. This requirement is supported by additional data such as measuring basal endogenous protein phosphorylation patterns and the translocation of different protein kinase C isoenzymes (α, β and γ) to the membrane as markers of their activation in tissue obtained from hippocampal slices in vitro or from intact, untreated rats. Studies revealed that only slices incubated in the same way as described here showed comparable patterns of phosphorylation and enzyme translocation as detected in the intact animal (Angenstein and Staak, 1997). Although one could argue that specific modifications of slice preparation may circumvent distinct problems raised above, to maintain the complex slice physiology at a level which allows reliable studies of functional plasticity favours a more simple method: to wait (Sajikumar and Frey, 2004a).

Following the preincubation period, the test stimulation strength was determined for each input to elicit a population spike of about 40 % (for LTD studies) or 25 % (for studies conducted to investigate LTP) of its maximal amplitude determined by slice specific input-output relationship. For stimulation, biphasic constant current pulses were used. The baseline was recorded for at least 60 min before LTP/LTD induction. Four 0.2 Hz biphasic, constant-current pulses (0.1 ms per polarity) were used for testing 1, 3, 5, 11, 15, 21, 25, 30 min post-tetanus or 21, 25, 30 min post-LFS and thereafter once every 15 min up to 8 h. Since the two recorded parameters showed either similar time course in the experiments (if the population spike was not abolished after induction of LTD at all), for clarity only the fEPSP data are shown. A detailed description of the experimental protocol for the preparation, incubation and investigation of reliable rat hippocampal CA1 late-LTP/LTD is shown in Fig. 5 (Sajikumar et al., 2005a).

## 2.2. Experimental protocol

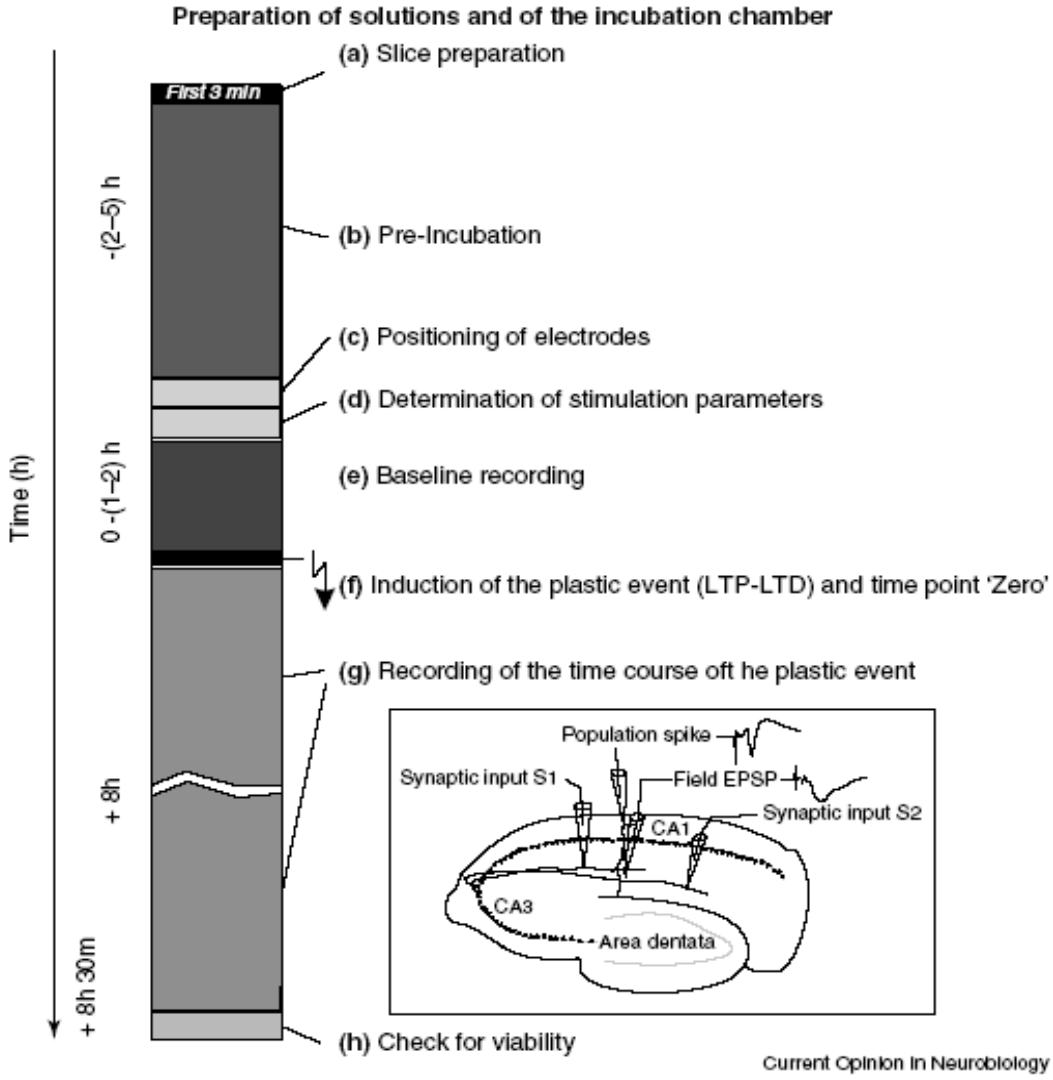



**Fig. 5. Experimental protocol for the preparation, incubation and investigation of reliable rat hippocampal CA1 late-LTP/LTD.**

The scheme represents a general protocol for how to perform experiments investigating long-lasting functional plasticity in hippocampal slice preparations in vitro. **(a)** Slice preparation takes three minutes. In the first minute of the experiment the rat is killed by a blow to the back of the neck and the right hippocampus is removed. In the second and third minutes the hippocampus is cooled in carbogenated artificial cerebro-spinal fluid (ACSF) at 4°C, and three to four slices are cut and gently positioned in the

incubation chamber at 32°C. **(b)** In the next three to four hours the slices are pre-incubated at 32°C. **(c)** At this stage, electrodes are gently positioned in the slices and there is a rest period of 30 min. We use electrolytically, sharpened stainless steel electrodes(input resistance: 5 MΩ; AM-Systems, USA) that are cleaned with alcohol and ACSF shortly before they are gently and slowly positioned in the middle of the hippocampal slice (about 200 µm from the surface) to reach the neuronal area that has been best preserved (Pohle et al., 1986). We stimulate with biphasic constant current pulses to guarentee stable stimulation for the duration of the experiment. **(d)** Approximately four and half hours after slice preparation the parameters of stimulation are determined according to an input-output relatioship. At this time point the baseline recording begins. Recorded potentials were amplified by a custom made amplifier (INH, Magdeburg, Germany) **(e)** The baseline recording continues for one or two hours and can vary, for example, if the stability of baseline recordings has not yet been achieved or if pharmacological substances have been administered. During the baseline recording input-output stability should be should be checked at a few stimulus intensities. **(f)** Time point zero. At this point the LTP or LTD is induced. **(g)** For the next 6 or 8 hours the recording takes place. Recording takes place at two points in the same neuronal population. **Inset:** positioning of electrodes in a transverse hippocampal slice to stimulate two separate synaptic inputs, S1 andd S2, to the same neuronal population. The population spike and the field EPSP are recorded. Anlog traces represent typical control potentials. The analog signals were then digitised using a CED 1401 A/D converter and analysed with custom-made software (PWIN, Magdeburg, Germany). (h) After 8 hours there is another 30 minutes of recording during which the input-output relationship is monitored and LTP or LTD is induced again to check the viability of the slice. The Time course shown here only represents the case for LTP or LTD in a synaptic input S1 and a stability control recorded in a second, independent input S2.

All experiments were carried out in accordance with the European Communities Council Directive of 24th November 1986 (86/609/EEC). It is also certified that formal approval to conduct the experiments described has been obtained from the animal subjects review board of the institution/local government which can be provided upon

request. All efforts were made to minimize the number of animals used and their suffering.

### 2.3. Stimulation Protocols: Induction of late-LTD, early-LTD, late-LTP and early-LTP

For inducing late-LTD, a strong low-frequency stimulation protocol (SLFS) which consisted of 900 bursts (one burst consisted of 3 biphasic, constant current stimuli at a frequency of 20 Hz, interburst interval: 1 s, i.e. f=1 Hz, stimulus duration 0.2 ms per half-wave; a total number of stimuli of 2700) was found to be the most effective protocol (Sajikumar and Frey, 2003;Sajikumar and Frey, 2004a). This stimulation pattern produced a stable LTD in vitro for at least 8 h. For inducing a transient early-LTD a weak low-frequency stimulation protocol (WLFS) consisting of 900 pulses (f=1 Hz, impulse duration 0.2 ms per half-wave, a total number of stimuli of 900, as ever: biphasic, constant current stimuli) was determined to be the most efficient in inducing early-LTD (Sajikumar and Frey, 2003;Sajikumar and Frey, 2004a). Late-LTP was induced using three stimulus trains of 100 pulses (‘strong’ tetanus: f=100 Hz, stimulus duration 0.2 ms per polarity with 10 min intertrain-intervals) (Frey and Morris, 1997;Frey and Morris, 1998b). In experiments with induction of early-LTP, a single tetanus with 21 pulses was used (`weak’ tetanus: f=100 Hz, stimulus duration 0.2 ms per polarity, population spike threshold stimulus intensity for tetanization (Frey and Morris, 1997;Frey and Morris, 1998b).

## 2.4. The nature of field potentials

In extracellular recording the potential difference is measured between two electrodes, one of which is placed within the tissue of interest and the other which is outside the tissue and acts as a ´reference electrode´. The voltage at the reference electrode is by convention zero. During activity of the nervous tissue the current ´*I*´ that flows between parts of a cell (due to movement of ions), through the external resistance ´R´ produces a potential difference ´V´, therefore the change of potential against time can be recorded. The potential difference that can be recorded extracellularly due to the activity of one cell is very small. However, the laminated structure of the hippocampal formation, where many neurons are tightly packed together in the same orientation allows the recording of quite large responses. When many neurons are simultaneously activated, the change in the potential in each of them is in the same ´direction´ and thus, they summate. The absolute amplitude of the potentials is dependent on the value of the external resistance so that in an interface chamber, where the slices are partly surrounded by air (high resistivity) the potentials are much larger than in submerged chambers. Since field potentials are recorded from a population of neurons, changes can reflect not only changes in the amplitude of the responses in the individual cells but also the number of neurons involved and the synchrony of their activity.

In the hippocampus two main types of field potentials are recorded: the field excitatory post synaptic potential (fEPSP) and the population spike (PS). At low stimulation intensities (below threshold), the fEPSP is a reflection of the individual EPSPs of the neurons as well as IPSPs due to feed forward inhibition. Above threshold, IPSPs due to recurrent inhibition and a component due to neuronal firing may also be present. Since inhibition usually occurs with a delay with respect to the onset of the

EPSPs, the slope of the fEPSP is considered to be a good measure of activity at the excitatory synapses, although the amplitude of the fEPSP shows similar changes. When measured in the dendritic region, the fEPSP is by convention negative (sink), whilst in the cell body layer the potential recorded is positive (source) (Bliss and Gardner-Medwin, 1971;Bliss and Richards, 1971). The PS which is usually recorded in the cell body layer is a component potential, reflecting the changes in the potential due to the firing of the action potential by the neurons, superimposed upon the reversed fEPSP originating in the dendrites. The amplitude of the PS is a measure between the negative peak of the potential and the positive peak preceding it. Since action potentials are ´all-or-none´ the PS reflects the number of neurons involved and the synchrony of their firing (Andersen et al., 1969).

**2.5. Pharmacology**

Rolipram (Tocris), a type IV-PDE inhibitor, was used at a concentration of 0.1 µM dissolved in ACSF and 0.1% dimethyl sulphoxide (DMSO). D-2-amino-5-phosphonopentanoic acid (AP-5; Sigma) was used at a concentration of 50 µM (dissolved in ACSF) to block the NMDA-receptor. Anisomycin (Sigma), a reversible protein synthesis-inhibitor, was used at a concentration of 25 µM (a concentration that blocked at least 85% of $^{3}$H-leucine incorporation into hippocampal slices; (Frey et al., 1991a)). The selective dopaminergic D1/D5-receptor SCH23390 was used at a concentration of 0.1 µM (Tocris; dissolved in ACSF). Emetine (Tocris Cookson) was used at a concentration of 20 µM (dissolved in ACSF and 0.1% DMSO). MEK inhibitor U0126 (Promega) was used at a concentration of 1µM (dissolved in DMSO to a stock concentration of 10 µM). PD 98059 (Calbiochem) was used at 10 µM concentration.

Manumycin A (Streptomyces parvulus (Calbiochem), a selective inhibitor for farnesyltransferase, was dissolved in 0.02% DMSO in ACSF. Lethal Toxin-82 (LT-82) (Clostridium Sordelli), donated by Prof. Michel Popoff, France was dissolved ACSF.

For tag candidate search studies the CaMKII inhibitors KN-62 ((1-[NO-bis-1, 5-isoquinolinesulfonyl]-N-methyl-L-tyrosyl- 4-phenylpiperazine)) and AIP (autocamtide-2-related inhibitory peptide) (Calbiochem) was used at a concentration of 5 μM. It was prepared by dissolving in DMSO (the final concentration of DMSO was 0.1 %), as a stock solution (10 mM) and was stored at -20°C. The required volume containing the final concentration of 5 μM was dissolved in ACSF immediately before bath application. Stock solutions of MAPK antagonist, U0126, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) were prepared in DMSO at a 10 mM stock solution and the required volume containing the final concentration of 20 μM was dissolved in ACSF immediately before bath application.

**2.5. Statistics**

The average values of the population spike (mV) and slope function of the field EPSP (mV/ms) per time point were subjected to statistical analysis (Wilcoxon signed rank test, when compared within one group or the Mann-Whitney-U-test when data were compared between groups ($p<0.05$ considered as being statistically significant different)).

# 3. Results

## 3.1. Rolipram-induced reinforcement of early-LTP (RLTP) and its properties

Induction of late-LTP in S1 (STET; Fig. 6A, filled circles) using high-frequency stimulation (HFS) resulted in late-LTP that was significantly different for 6 h, compared with the control input S2 (open circles). Control stimulation of S2 showed relatively stable potentials for the time course investigated. Induction of early-LTP in synaptic input S1 (Fig. 6B, filled circles) resulted in a transient statistically significant form of LTP with a duration of 2-3 h before returning to baseline values where the potentials remained stable for the time of recording. A control input S2 without application of high-frequency stimulation remained stable at baseline level for the whole experimental session (open circles in Fig. 6B). Application of rolipram, a phosphodiesterase (PDE) inhibitor 30 min before until 30 min after the induction of early-LTP in input S1 (Fig. 6C, filled circles) converted the transient early form of LTP into a statistically significant late form with a duration of at least 6 h (rolipram reinforced LTP or RLTP), the latest time point we have recorded (Fig. 6C, filled circles). Control responses obtained from S2 (open circles in Fig. 6C) remained stable at baseline levels. Analyses of the input-output relationship before the application of rolipram and tetanization and 6 h after LTP induction revealed no differences when compared with rolipram-untreated slices. A comparable EPSP-spike-shift was seen in the tetanized input. The control input remained stable over the investigated 7 h. We also did not observed any effects of rolipram on LTP-induction by analysing the tetanization trains under the influence of the drug at the used concentration. Next, we investigated as to whether the PDE-inhibitor had to be present during the induction of early-LTP to be effective in generating RLTP or whether it would

have been sufficient to apply rolipram after the induction of early-LTP. In the latter case it could act via late-associative properties on the earlier tetanization of input S1 in a non-specific way. Fig. 6D represents the time course of the Field EPSP when rolipram was applied shortly after the induction of early-LTP (15 min after tetanization in S1). In this case, rolipram was ineffective in affecting early-LTP (filled circles in Fig. 6D). Next, we examined whether NMDA-receptor activation was required for RLTP. As shown in Fig. 6E, co-application of 50 μM AP-5 prevented the induction of early-LTP and RLTP. In this series, after recording a baseline for 50 min, AP-5 was applied for 10 min before co-applied with rolipram for another 1h. The following series of experiments studied the possible requirement of macromolecular synthesis for RLTP. The reversible protein synthesis inhibitor anisomycin was applied 10 min before co-application with rolipram for a further 6h. Fig. 6F (filled circles) shows that anisomycin did not prevent early-LTP but RLTP, thus the reinforcement of early-LTP by rolipram seems to be protein synthesis-dependent. In all series of experiments the control input S2 (open circles) was not influenced by the drugs or tetanization.

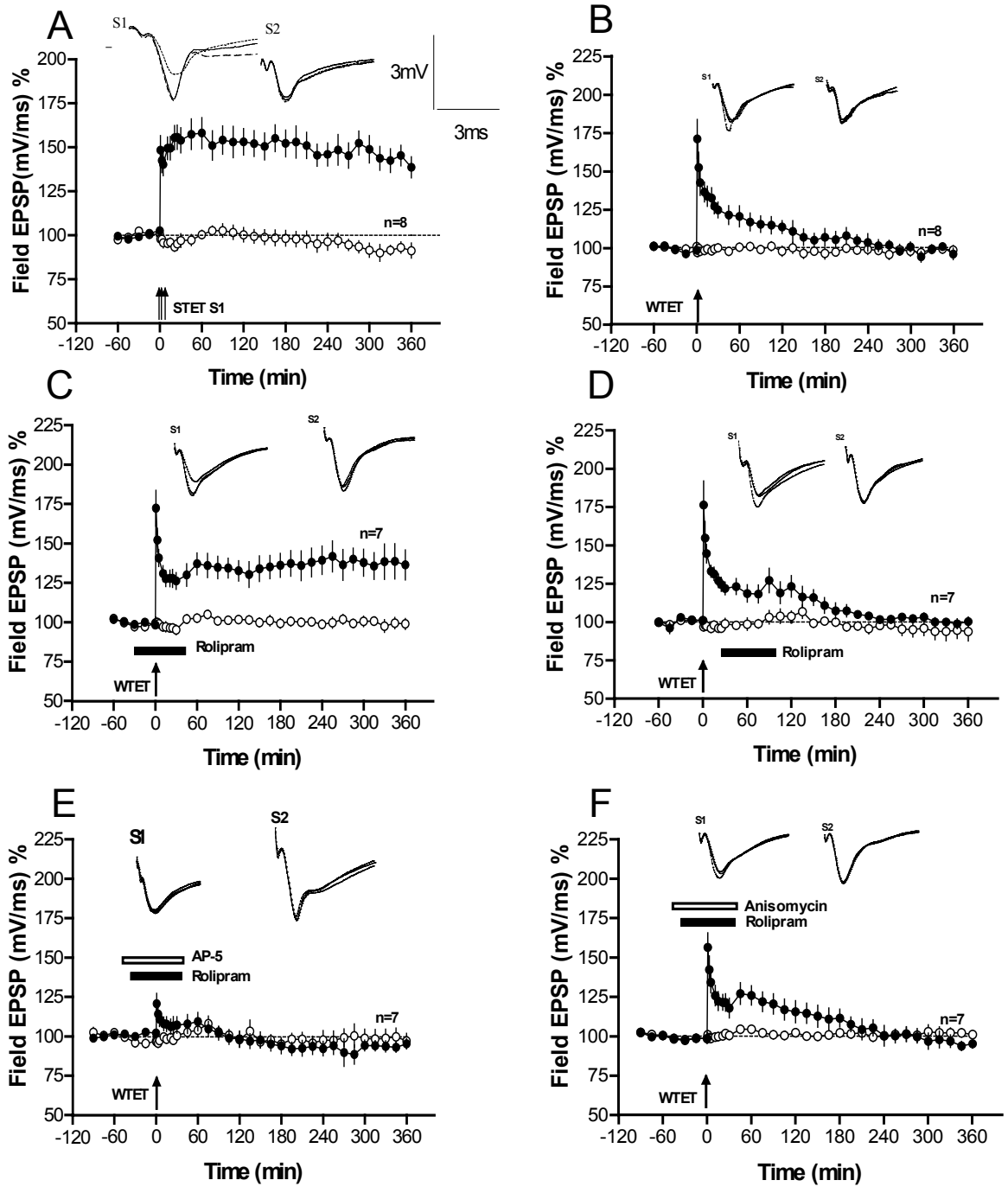



**Fig. 6. Rolipram-induced reinforcement of early-LTP (RLTP) and its properties.**

**(A)** Time course of the slope of the Field EPSP after induction of late-LTP in S1 (filled circles, n=8). **(B)** Time course of the slope of the Field EPSP after induction of early-LTP in S1 (filled circles). Open circles represent a control stimulated synaptic input S2; n=8. **(C)** Transformation of early- into late-LTP by

rolipram. Application of rolipram, 30 min before stimulation of S1 (filled circles) with a tetanization protocol which normally would induce early-LTP (washout of the drug: 30 min after tetanization; open circles: control input); n= 7. **(D)** The same experiment as in (C) with the exception that rolipram was applied 15 min after LTP-induction for 1 h; n=7. **(E**) The influence of the NMDA-receptor blocker AP-5 on RLTP: after recording a baseline for 50 min, AP-5 was applied for 10 min before co-applied with rolipram for another 1h. Tetanization with a protocol which normally would induce early-LTP in S1 (filled circles) and control recording of S2 (open circles); n=7. **(F**) The same as in (E) but instead of AP-5, the reversible protein synthesis inhibitor anisomycin was applied 10 min before co-application with rolipram for a further 1 h; n=7.

Analog traces always represent typical Field EPSPs 30 min before (dotted line), 30 min (hatched line) and 6 h (closed line) after tetanization of input S1 and corresponding control S2. Arrows indicate the time point of tetanization of the corresponding synaptic input. Triplet arrows indicates strong tetanisation (STET) while single arrows represent weak tetanisation (WTET) to induce early-LTP at the corresponding input. Bars represent the period of drug application. Scale bar: 3mV/3ms (valid for all single analog examples represented).

### 3.2. PDE inhibition by rolipram and processes of synaptic tagging during LTP

RLTP seems to mimic protein synthesis-dependent late-LTP. Thus the question arose as to whether processes of 'synaptic tagging' can be detected, since only those inputs showed RLTP in which early-LTP was induced together with the application of rolipram (see Fig. 6C). Thus, a first set of controls studied the possible interference of the subsequent induction of early-LTP in two separate synaptic inputs S1 and S2. As shown in Fig. 7A early-LTP of the two inputs was not influenced if the time interval between subsequent tetanization was 40 min. However, when early-LTP was induced in S1 followed by the application of rolipram 15 min after tetanization of S1, i.e. at a time point where rolipram was ineffective in affecting the potentiation in that input (see Fig. 6D), and a subsequent induction of early-LTP in S2 was induced 40 min after

tetanization of S1 - but now under the influence of rolipram - both inputs S1 and S2 paradoxically expressed late-LTP (Fig. 7B). We then investigated whether these rolipram-induced, associative prolongations of early-LTP require the synthesis of macromolecules. In the series shown in Fig. 7C we have repeated the experiment represented in Fig. 7B with one exception: here, emetine (5 min after tetanization of S1) was applied shortly before rolipram (15 min after tetanization). We have also tested a second, structurally different inhibitor of protein synthesis; anisomycin to confirm our results (Fig. 7D). Interestingly in these cases, under protein synthesis inhibition the associative reinforcement of early-LTP in S1 and S2 was prevented. These data support the involvement of phosphodiesterases in processes of 'synaptic tagging'.

It has been shown recently, that protein synthesis-dependent processes of 'synaptic tagging' in area CA1 require specifically the heterosynaptic activation of D1/D5-receptors during induction (Sajikumar and Frey, 2004a). Thus, we were interested as to whether the action of rolipram still requires D1/D5-receptor-activation or as to whether PDE-processes act downstream of D1/D5-receptors. Therefore, we have now applied the selective D1/D5-receptor antagonist SCH23390 shortly before the application of rolipram (Fig. 7E) - in the same manner as in the set with anisomycin (Fig. 7D). As shown in Fig. 7E, 'synaptic tagging' and the expression of RLTP in the two inputs S1 and S2 was not negatively influenced by the blockers of the dopaminergic receptors supporting an action of the PDE downstream of the D1/D5-receptors. Interestingly, a tendency of enhanced potentiation was detected under these circumstances.

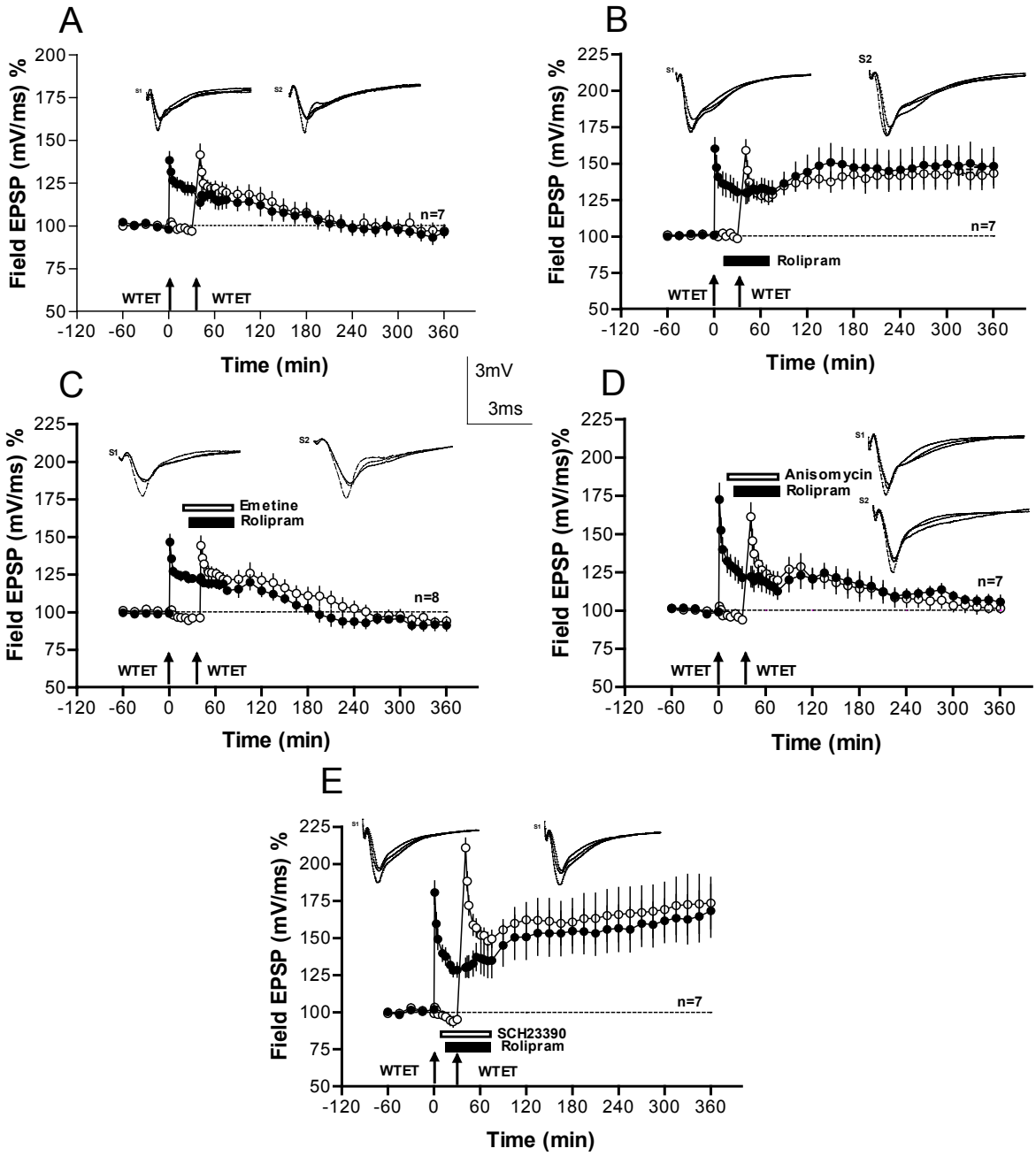



**Fig. 7. PDE inhibition by rolipram and processes of synaptic tagging during LTP**

**(A)** Time course of the slope of Field EPSPs after subsequent induction of early-LTP in S1 (filled circles) and S2 (open circles) with a time interval between tetanization of S1 and S2 of 40 min; n=7. **(B)** Paradoxical transformation of early- into late-LTP in S1 (filled circles) by subsequent induction of RLTP in S2 40 min later (open circles). Same stimulation protocol as in (A) with the exception that 15 min after

tetanization of S1 rolipram was added to the bath medium for 1h before washout; n=7. **(C)** and **(D)** RLTP and synaptic tagging depends on protein synthesis. The same procedure as in (B), however, after the additional application of emetine 5 min after tetanization of S1; n=8. (D) represents the effect of a structurally different protein synthesis inhibitor, anisomycin on RLTP, suggesting a specific action on protein synthesis by the drugs used; n=7. **(E)** The action of rolipram is downstream of the D1/D5-receptors. When SCH23390 was applied instead of anisomycin (as in (D)) neither the paradoxical transformation of early- into-LTP in S1 (filled circles) by subsequent RLTP-induction in S2 (filled circles) nor RLTP in S2 were blocked; n=7.

Analog traces always represent typical Field EPSPs 30 min before (dotted line), 30 min (hatched line) and 6 h (closed line) after tetanization of input S1 or, in cases in which S2 was also tetanized, before or after the tetanization of that input. Single arrows represent weak tetanisation (WTET) to induce early-LTP at the corresponding input. Bars represent the period of drug application. Scale bar: 3mV/3ms (valid for all single analog examples represented).

### 3.3. Rolipram-induced reinforcement of early-LTD (RLTD) and its properties

In a first control set of experiments we have induced late-LTD in a synaptic input S1 by the application of a strong low-frequency stimulation (SLFS) which resulted in robust late-LTD of the Field-EPSP in S1 with a duration of at least 6 h (Fig. 8A, filled circles). A separate, synaptic control pathway S2 (Fig. 8A, open circles) remained stable at baseline values. A statistically significant difference between S1 and S2 was observed for the measured 6 h after SLFS in S1. This type of LTD was protein synthesis-dependent (Sajikumar and Frey, 2003;Sajikumar and Frey, 2004a). Induction of early-LTD in synaptic input S1 (Fig. 8B, filled circles) resulted in a transient early form of LTD with a duration of 2-3 h before returning to baseline values at which the potentials remained stable for the time of recording. A control input S2 (Fig. 8B; open circles) remained stable at baseline levels for the entire experimental session (statistically

significant different between S1 and S2 for 2 h after WLFS to S1). Application of rolipram (0.1 μM) 30 min before until 30 min after the induction of early-LTD in input S1 converted the transient form of early-LTD in to a statistically significant late form with a duration of up to 6 h (Fig. 8C, filled circles). Control responses from S2 remained stable at baseline levels (Fig. 8C, open circles). Analysis of the input-output relationship before the application of rolipram and WLFS and 6 h after LTD induction revealed no differences when compared with rolipram-untreated slices. A comparable and similar EPSP/spike shift was observed in the LTD-induced input. We then investigated whether the PDE inhibitor has to be present during the induction of early-LTD to be effective in generating RLTD or whether it is sufficient to apply rolipram after WLFS. As shown in Fig. 8D (filled circles) rolipram application 25 min after the induction of early-LTD in S1 was unable to induce RLTD in that input. The control input S2 again remained stable throughout the recorded time period of 6 h (Fig. 8D, open circles).

### 3.4. Properties of rolipram-reinforced early-LTD

We investigated whether NMDA-receptor activation is necessary for RLTD. After recording the baseline for 50 min, AP-5 (50 μM) was bath-applied for 10 min before being co-applied with rolipram for another 1 h (Fig. 8E). When WLFS was applied to S1 (filled circles) in presence of AP-5 together with rolipram the induction of early-LTD as well as RLTD was prevented. In the next series of experiments we studied whether protein synthesis is required for the reinforcement of early- into late-LTD. Application of 20 μM emetine (Fig. 8F) or 25 μM anisomycin (Fig. 8G) in a similar manner as in Fig. 8E prevented any lasting form of LTD in S1 (filled circles). Potentials in the control input

remained stable (open circles in Figs. 8F and G). Thus, reinforcement of early-LTD by rolipram was protein synthesis-dependent.

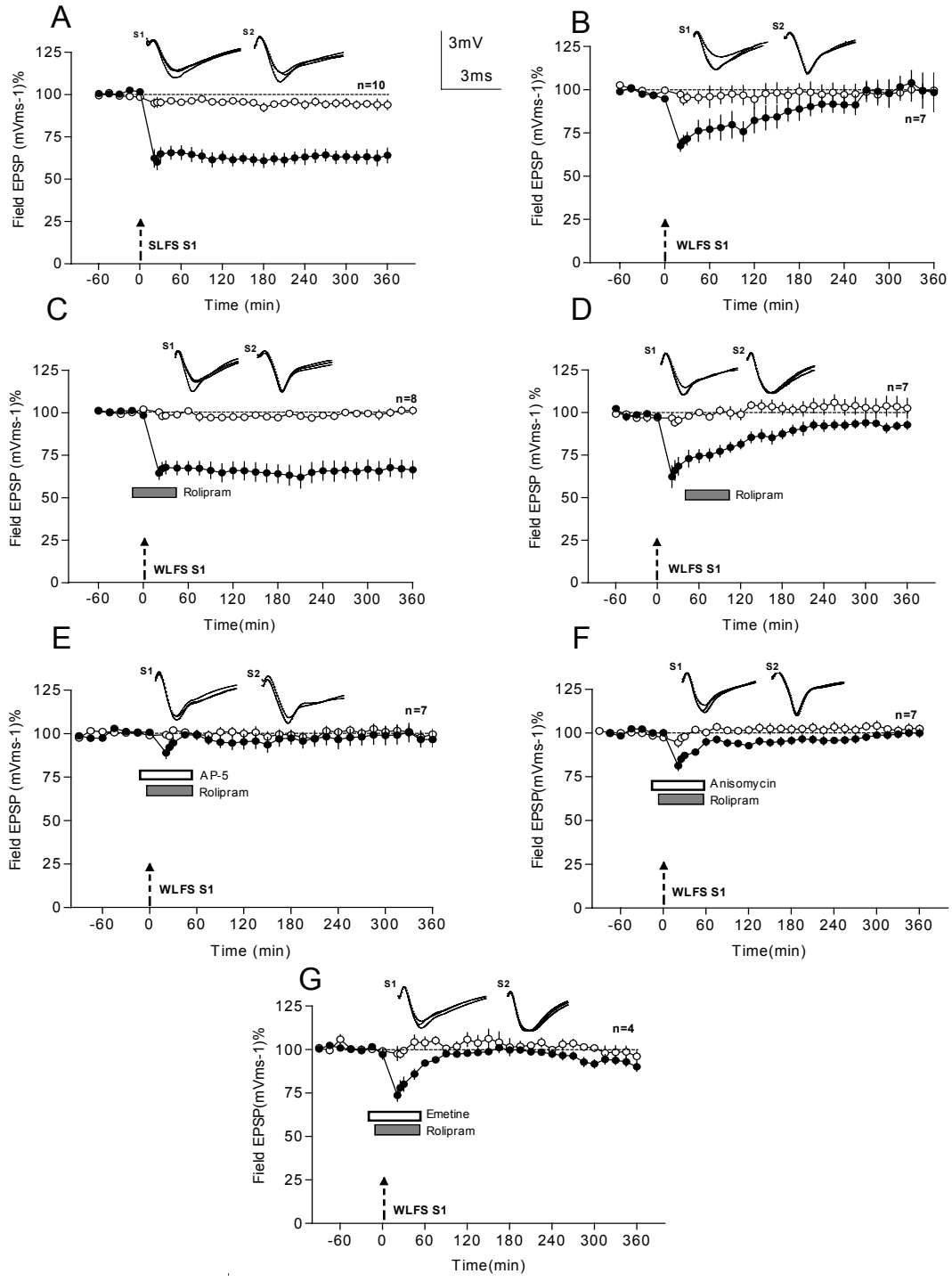



**Fig. 8. Rolipram-induced reinforcement of early-LTD and its properties. (A)** Induction of late-LTD in S1 (filled circles) using a repeated LFS protocol (i.e., strong low-frequency stimulation (SLFS); broken

arrow) resulted in late-LTD which is significantly different for the 6 h investigated when compared with a control input S2. Control stimulation of S2 revealed relatively stable potentials for the time course investigated (open circles); n=10. **(B)** A WLFS protocol (broken arrow) was used which elicited transient early-LTD of the Field EPSP with a duration of 185-210 min; n=7. **(C)** Transformation of early- into late-LTD by rolipram. Application of rolipram (0.1 μM) 30 min before WLFS of S1 (filled circles) that normally would induce early-LTD (washout of the drug, 30 min after WLFS; open circles, control input) ; n=8. **(D)** The same experiment as in (c) with the exception that rolipram was applied 25 min after LTD and elapsed for 1 h; n=7. **(E)** The influence of the NMDA receptor blocker AP-5 on RLTD. After recording a baseline for 50 min, AP-5 (50 μM) was applied for 10 min before being co-applied with rolipram for another 1hour. WLFS in presence of AP-5 prevented the early-LTD (filled circles), the control recording of S2 remained stable during the recorded time period (open circles); n=7. **(F)** Same as in (E) but instead of AP-5, the reversible protein synthesis inhibitor anisomycin (25 μM) was applied 10 min before co-application with rolipram for an additional hour; n=7. **(G)** Represents the effect of a structurally different protein synthesis inhibitor, emetine (20 μM) on RLTD, suggesting a specific action on protein synthesis by the drugs used; n=4.

The analog examples given in the figures represent adequate potentials 30 min before (dotted line), 30 min after (broken line) as well as 6 h after the induction of the event (here after induction of SLFS in S1; closed line) in input S1 and S2, respectively. Scale bar: 3 mV/3 ms (valid for all single analog examples presented). Broken arrows indicate the time point of low frequency stimulation. Bars represent the period of drug application.

### 3.5. PDE inhibition by rolipram and processes of synaptic tagging during LTD

Next, we studied whether the protein synthesis-dependent RLTD was characterized by processes of synaptic tagging, because only those inputs expressed RLTD which received WLFS, i.e. in which early-LTD was induced. RLTD was therefore input-specific and is dependent on protein synthesis. We have reported earlier that the induction of conventional early-LTD in two inputs S1 and S2 do not interfere with each other, (Sajikumar and Frey, 2004). For investigating the processes of synaptic tagging

during RLTD, early-LTD was induced in synaptic input S1, after 25 min rolipram (0.1 μM) was applied (a time point where it was ineffective in affecting the first input; see Fig. 8D) for 1 h. Fifty five min after induction of early-LTD in S1 (filled circles), early-LTD was induced in input S2 (open circles), but now it was under the influence of rolipram. Paradoxically, in both inputs a late form of LTD was observed (Fig. 9A) which supports our hypothesis that synaptic tagging takes place during RLTD. In the next series of experiments we investigated whether synaptic tagging in LTD in area CA1 required the heterosynaptic activation of glutamatergic and dopaminergic D1/D5-receptors. To investigate that, the experiment described in Fig. 9A was repeated with the exception that shortly before application of rolipram the D1/D5-receptor-specific antagonist SCH23390 (0.1 μM) was applied 10 min before being co-applied with rolipram (Fig. 9B). SCH23390 prevented synaptic tagging and the rolipram-induced transformtion of early- into late-LTD in both inputs S1 and S2. Thus, the action of rolipram in the hippocampal CA1 of adult rats was dependent on D1/D5-receptor activation. In a similar manner, co-application of the protein synthesis inhibitors anisomycin or emetine with rolipram also prevented processes of synaptic tagging induced by rolipram and the transformation from early-into late-LTD (Fig. 9C and D).

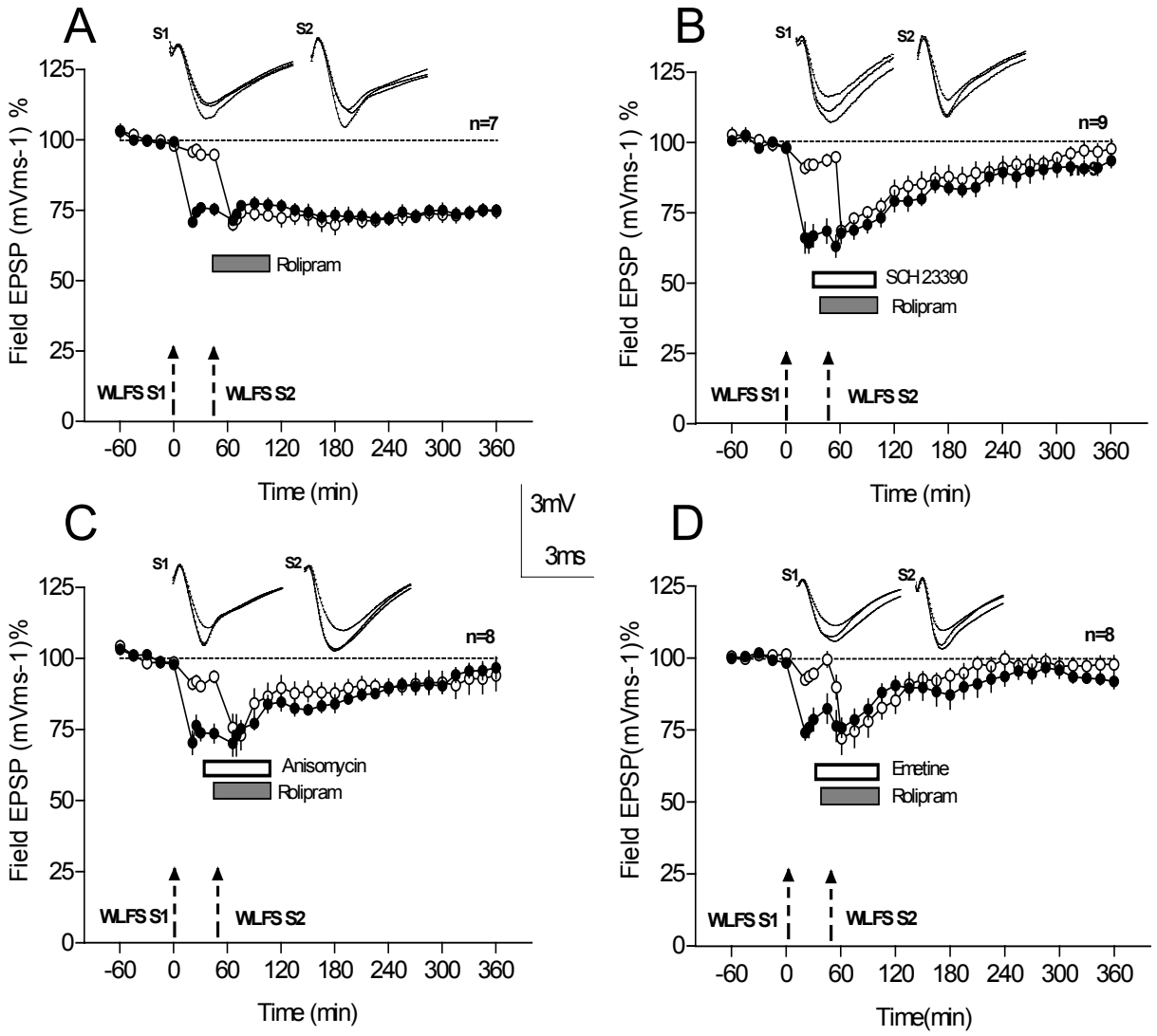



**Fig. 9. PDE inhibition by rolipram and processes of synaptic tagging during LTD**

**(A)** Transformation of early- into late-LTD in S1 (filled circles) by subsequent induction of RLTD in S2 55 min later (open circles; n=7). WLFS was induced in S1 and 25 min after WLFS of S1, rolipram was added to the bath medium for 1 h. **(B)** The same experiment as in (A) with the exception of the additional application of D1/D5 receptor antagonist SCH23390 (0.1 μM) 15 min after WLFS of S1 (n=9). Here synaptic tagging by RLTD is prevented. **(C)** and **(D)**, experiment presented in (B) was repeated using anisomycin (25 μM; n=8) and emetine (20 μM; n=8), protein synthesis inhibition prevents synaptic tagging in RLTD.

Analog traces always represent typical Field-EPSPs 30 min before (dotted line), 30 min (hatched line) and 6 hr (closed line) after WLFS of input S1, or in cases in which WLFS was delivered to S2. Broken

arrows indicate the time point of the WLFS of the corresponding synaptic input. Scale bar: 3 mV/3 ms (valid for all single analog examples presented). Bars represent the period of drug application.

### 3.6. Reinforcement of early-LTD by rolipram through MAPK activation.

Zhang et al. (2004) found in behavioral experiments a functional link between MAPK/ ERK and cAMP signalling pathways in the mediation of long-term memory, in which PDE4 is likely to be involved. Moreover, ERK activation is necessary for the persistent maintenance and expression of NMDA receptor-dependent LTD in area CA1 of the adult hippocampus in vivo (Thiels et al., 1996). The main question arose if MAPK activation is also essential for RLTD? As shown in Figs. 10E and F, the MEK inhibitors U0126 (1 μM) or PD098059 (1 μM) effectively blocked RLTD, which suggests that MAPK mediate RLTD-processes. In each set of experiments a baseline was recorded for 50 min and then one of the MEK inhibitors, either U0126 (1 μM; Fig. 10E) or PD098059 (1 μM; Fig. 10F) was applied for 10 min before co-applied with rolipram for another 60 min. MEK inhibitors prevented RLTD without affecting early-LTD in S1 when WLFS was applied 30 min after application of rolipram to S1 (filled circles in 10E and F). The control inputs S2 in the two sets of experiments remained stable throughout the recorded time period (Fig. 10E and F, open circles).

The previous sets of experiments revealed that MEK activation is essential for RLTD. Now the question arose which signaling cascade is involved in the activation of MEK? First, we have investigated the Ras-mediated cascade. Application of manumycin-A, a Ras inhibitor (2 μM), 10 min before co-application with rolipram for 30 min prior to early-LTD did not prevent the expression of RLTD. i.e. the transformation of early- into late-LTD in input S1 (Fig. 10G, filled circles) excluding the Ras signaling cascade as a candidate for the activation of MEK during RLTD. The next series of

experiments were conducted to examine if the Rap-mediated pathway of activating MEK may take place in RLTD. Lethal Toxin-82 (LT-82; 200 ng/ml), an inhibitor of the Rap1-B-Raf pathway, was applied in a similar manner as described in Fig. 10H. In contrast to manumycin-A, LT-82 blocked RLTD (Fig. 10G and H, filled circles). We can conclude that the prevention of RLTD was due to Rap1-B-Raf-inhibition by LT-82 because Ras inhibition by manumycin did not prevent RLTD. In both cases the control input S2 remained stable at baseline levels throughout the recorded time period of 6 hr (Fig. 10G and H, open circles).

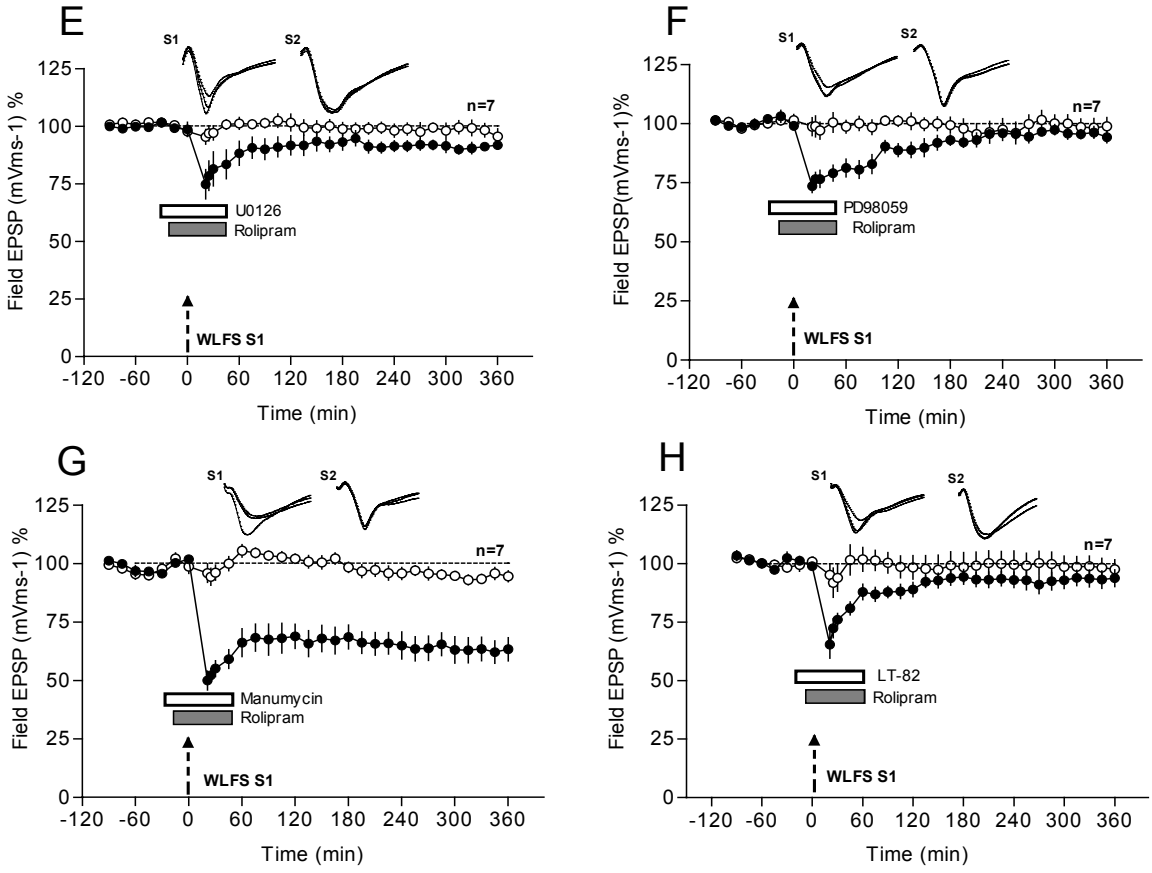



**Fig. 10. The reinforcement of early-LTD by rolipram through MAPK activation**

**(E)** The influence of the MEK inhibitor, U0126 on RLTD (n=7). After recording a baseline for 50 min, U0126 (1 μM) was applied for 10 min before being co-applied with rolipram for another hour. WLFS in S1 in presence of MEK inhibitor U0126 prevents RLTD (filled circles), control recording of S2 (open circles)

remained stable during the recorded period. **(F)** The same experiment as in (E) however instead of U0126 a different MEK-inhibitor, PD98059 (1 μM; n=7) was applied, confirming the results obtained in (E). **(G)** Effect of Ras inhibition by mamumycin-A on RLTD (n=7). After recording a baseline for 50 min, mamumycin A (2 μM) was applied for 10 min before being co-applied with rolipram for another hour. WLFS in S1 in presence of manumycin had no effect on RLTD (filled circles), control potentials from S2 remained stable (open circles). **(H)** Effect of Lethal Toxin-82 (LT-82; n=7) on RLTD. The same experiment as in (G) but with the presence of a Rap inhibitor LT-82 (200 ng/ml), which prevents the RLTD (filled circles).

The analog examples given in the figures represent adequate potentials 30 min before (dotted line), 30 min after (broken line) as well as 6 h after the induction of the event (here after induction of SLFS in S1; closed line) in input S1 and S2, respectively. Scale bar: 3 mV/3 ms (valid for all single analog examples presented). Broken arrows indicate the time point of low frequency stimulation. Bars represent the period of drug application.

### 3.7. Role of CaMKII and MAPK on LTP and LTD

The role of CaMKII and MAPK on LTP was previously reported (Yang et al., 2004;Bortolotto and Collingridge, 1998). We reproduced these experiments in our experimental conditions before investigating its role during the processes of synaptic tagging in LTP/LTD or in synaptic cross-tagging between LTP and LTD.

Induction of late-LTP in S1 in presence of CaMKII inhibitor, KN-62 (open rectangle, 5 μM, Fig. 11A, filled circles) by using a STET resulted in early-LTP lasting 2-3 h. We have used a very low concentration of 5 μM KN-62 since we were interested to investigate a concentration which resets the synaptic tags. The control inputs showed normal response over the investigated time period of 8h (Fig. 11A, open circles). We next investigated whether 5 μM KN-62 has any effect on LTP maintenance. As it is

shown in the Fig. 11B, application of KN-62 fifteen minutes after (for the next 1 h) the induction of LTP did not affect its maintenance (Fig. 11B, filled circles).

We next investigated whether CaMKII inhibition by KN-62 has any role in LTD induction and its maintenance. Fig. 11C shows that late-LTD is dependent on CaMKII activation. As it is shown, application of 5 μM KN-62 before and after the induction of late-LTD in S1 prevents the induction of LTD (Fig. 11C, filled circles). Interestingly the early form of LTD was also prevented leaving a depression lasting 45 min. In Fig 11D, similar to Fig. 11B we applied KN-62 fifteen minutes after (elapsed for the next 1 h) the induction of late- LTD. As it is shown in Fig. 11D neither the depressed synaptic input S1 or the control input S2 did not showed any effect (Fig. 11D, filled and open circles).

Next, we were interested to know the role of MAPK on LTP and LTD. We used a specific inhibitor of MAPK, U0126 at a concentration of 20 μM. In Fig. 11E, application of 20 μM U0126, 30 min before and 30 min after the induction LTP in S1 prevents its late maintenance with out affecting the early phase of LTP lasting 2-3 h. Responses obtained from S2 (open circles) remained relatively stable potentials at control levels. Application of U0126 fifteen minutes after the induction of LTP for the next 1 h did not affect the maintenance of potentiated synaptic input S1 or the control synaptic input S2 (Fig. 11F, filled and open circles).

The last set of experiments in this series investigated the role of MAPK on LTD. As it is shown in Fig. 11G application of 20 μM U0126 before and after the induction of late-LTD in S1 prevents the induction of LTD (filled circles). Interestingly here the early form of LTD was also prevented like in case of late-LTD in presence of KN-62 shown in Fig. 11C. Application of U0126 fifteen minutes after (for the next 1 h) the induction of late- LTD did not affected either the depressed synaptic input S1 or the control input S2

(Fig. 11H, filled and open circles) maintaining a stastically significant depression in S1 up to 8 h.

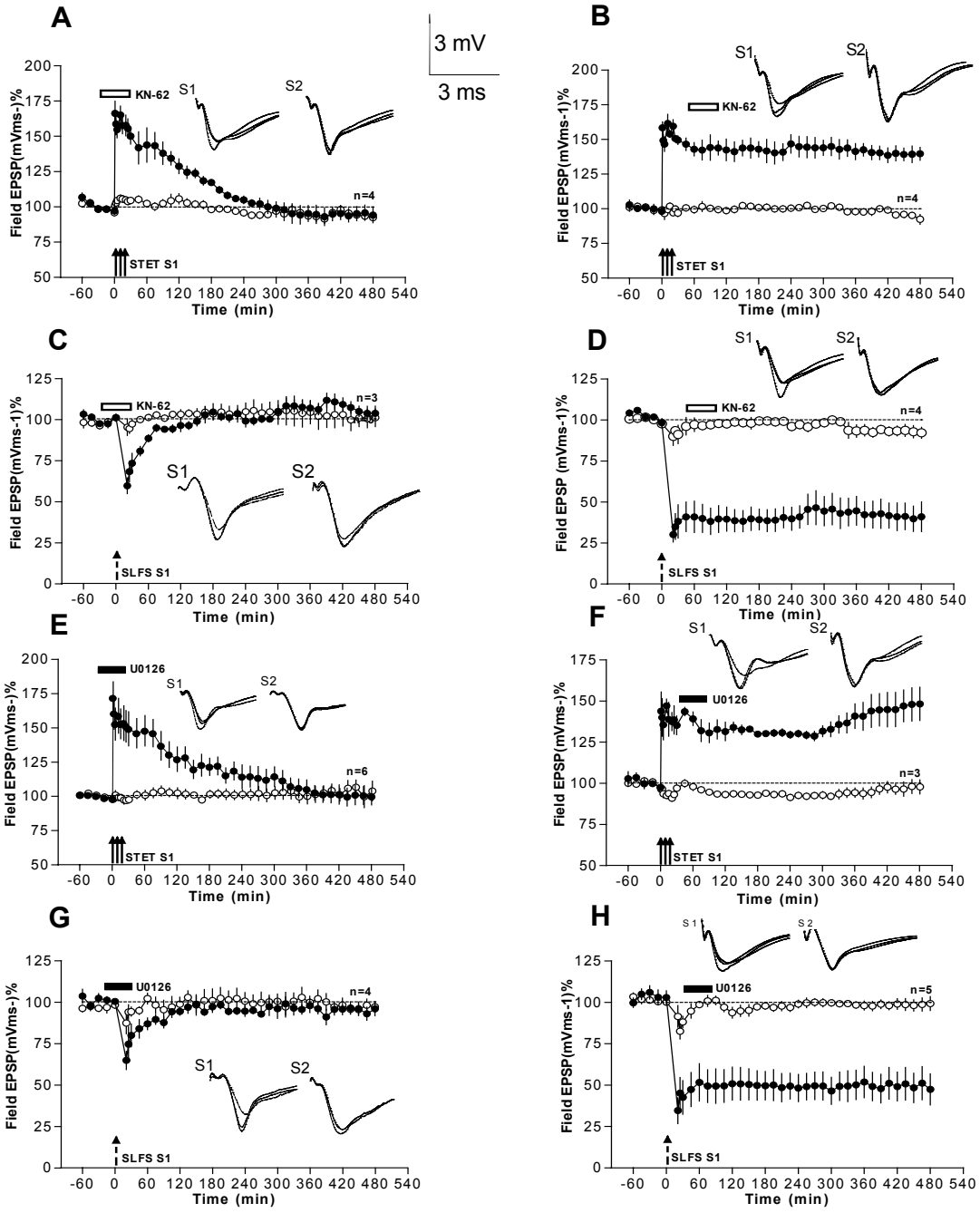



**Fig. 11. Role of CaMKII and MAPK on LTP and LTD.**

**(A)** Time course of the slope of the field EPSP after induction of late-LTP in S1 (filled circles). Open circles represent a control stimulated synaptic input, S2 (n = 4). Application of a CaMKII inhibitor KN-62 (open rectangle, 5 μM) 30 min before stimulation of S1 with a strong tetanization protocol that normally would

induce late-LTP (washout of the drug, 30 min after tetanization) is prevented resulting in an early-LTP. **(B)** The same experiment as in A with the exception that KN-62 was applied 15 min after LTP induction for 1 h (n = 4). **(C)** Application of the CaMKII inhibitor KN-62, 30 min before and after the induction of late-LTD in S1 prevented the induction of LTD (filled circles). Responses obtained from S2 (open circles) remained relatively stable potentials at control levels (n = 3). **(D)** The same experiment as in D with the exception that KN-62 was applied 15 min after LTD induction for 1 h (n = 4). **(E)** Time course of the slope of the field EPSP after the induction of late-LTP in S1 (filled circles) in presence of a MAPK inhibitor U0126 (filled rectangle, 20 μM, drug applied 30 min before and elapsed 30 min after the induction LTD in S1). Responses obtained from S2 (open circles) remained relatively stable potentials at control levels (n = 6). **(F)** The same experiment as in E with the exception that U0126 was applied 15 min after LTP induction for 1 h (n = 3). **(G)** Application of the MAPK inhibitor U0126, 30 min before and after the induction of late-LTD in S1 prevented the induction of LTD (filled circles). Responses obtained from S2 (open circles) remained relatively stable potentials at control levels (n = 4). **(H)** The same experiment as in F with the exception that U0126 was applied 15 min after LTD induction for 1 h (n = 4).

Triplets of filled arrows indicate the time point of strong tetanization (STET) for inducing late-LTP and broken arrows represents strong low frequency stimulation (SLFS) of the corresponding synaptic input for inducing late-LTD. Analog traces represent typical Field EPSPs 30 min before (dotted line), 3 min (broken line) and 8 h (closed line) after STET or SLFS.

### 3.8. Role of CaMKII and MAPK on synaptic tagging in LTP and LTD.

An earlier set of experiments showed that 5 μM KN-62 or 20 μM U0126 prevented the late phase of LTP and LTD, if applied during their induction, whereas application of either CaMKII or a MAPK inhibitor after the induction of either LTP or LTD does not have any role in its maintenance, therefore it is possible to induce early-LTP/-LTD in the second synaptic input in presence of CaMKII or MAPK inhibitor without affecting the PRPs which is triggered by the induction of late-LTP/-LTD in the synaptic input S1 thus,

enable us to investigate whether CAMKII or MAPK mediate setting of the synaptic tags in LTP/-LTD respectively.

Fig. 12A shows synaptic tagging in LTP in presence of the CaMKII inhibitor, KN-62. Induction of late-LTP in synaptic input S1, fifteen minutes after induction of late-LTP in S1, 5 μM KN-62 was applied and 45 min later ( i.e., 30 min after drug application) an early-LTP was induced in the second synaptic input S2. KN-62 was washed out 1 h after its application. As it is seen in the Fig. 12A, the maintenance of late-LTP was unaffected (filled circles) in S1 while the conversion of early into late-LTP in S2 (Fig. 12A; open circles) through processes of synaptic tagging was prevented by the CaMKII inhibitor revealing that setting of the ‘tag’ or ‘tag complex' in LTP tagging is a CaMKII-dependent process. The potentials in S2 reached near baseline levels immediately resembling a short-term potentiation (STP) and then slowly resume or resemble an early-LTP. Unlike early-LTP, STP does not set a tag (Frey and Morris, 1997), therefore CaMKII inhibitor resets the tag, but not the plasticity-related proteins.

The next main questions were: Are LTP-tags and LTD-tags similar or not? Does CaMKII also mediate the setting of synaptic ‘tags’ in LTD? Keeping these questions in mind we investigated the role of CaMKII during LTD tagging. As it is shown in Fig. 12B late-LTD was induced in S1 (filled circles), 45 min thereafter, early-LTD in S2 was induced but now under the influence of KN-62 (drug applied 30 min before and 30 min after the induction of early-LTD in S2). Here the tagging interactions were not influenced by the CaMKII inhibitor, i.e., the conversion of early- to late-LTD was intact. These results revealed that the setting of the LTD-tags is a CaMKII- independent process and thus, the tags for LTP and LTD seem to be different.

If CaMKII mediates the setting of synaptic tags in LTP, but not for LTD, then which molecule can mediate the specificity of the tag for LTD? We next investigated the role of MAPK. In a first series of experiments we investigated its role for LTP tagging. Fig. 12C represents a similar experiment as Fig. 12A, but with the exception that instead of the CaMKII-inhibitor KN-62, 20 μM of the MAPK inhibitor U0126 was used. Here the early -LTP in S2 (Fig. 12C open circles) was transformed into late-LTP showing the tagging interactions between late-LTP in S1 (filled circles) and early-LTP in S2. This set of experiments revealed that the setting of the LTP-tag is independent of MAPK-activity. Fig. 12D shows the role of U0126 on LTD-tagging. The same experiment as in Fig. 12B, but instead of KN-62 MAPK inhibitor, U0126 was applied. Here the transformation of early- into late-LTD is prevented, showing that the setting of the LTD-tag is a MAPK-dependent process.

The persistence of LTP or LTD is dependent on the setting of the tag during early-LTP/-LTD and the availability of PRPs. Earlier experiments from our laboratory have shown that late-LTP can be induced also during protein synthesis inhibition if a synaptic tag is set and PRPs are available, e.g. provided the induction of a late plasticity form in another synaptic input within a distinct time window (Frey and Morris, 1997). We have shown that CaMKII and MAPK inhibitors prevent late-LTP and LTD (see Fig. 11A, D, E, and G) and that CaMKII and MAPK mediates the setting of tags in LTP and LTD respectively. Therefore, we further studied their role during the subsequent induction of late-LTP in both S1 and S2, whereas the CaMKII inhibitor was applied during the induction of late-LTP in S2. Fig.12E shows that induction of late-LTP in S1 (filled circles) and S2 (open circles), but S2 in the presence of KN-62. Here induction of late-LTP in S2 in presence of KN-62 prevents the setting of the tag and thus prevent the expression of

late-LTP in S2. This experiment again confirms the results obtained in Fig. 12A, that setting of LTP tag is mediated by the CaMKII. Fig. 12B showed that the setting of the LTD-tag is a CaMKII-independent process. We verified the validity of this experiment by inducing late-LTD in S1 (Fig. 12F, filled circles) in the absence of KN-62, fifteen minutes after the induction of late-LTD in S1 the KN-62 was applied and 45 minutes after late-LTD in S1, late-LTD in S2 was induced (open circles). Here the persistence of late-LTD in S2 was intact, supporting the fact that CaMKII inhibition did not affect the setting of the LTD-tags in S2.

Fig. 12C represents that MAPK do not mediate the setting of tags in LTP. Thus, we next investigated the role of MAPK during LTP-tagging similar to the experiments described above for CaMKII (Fig 12E and F). Thus, instead of KN-62 the MAPK inhibitor U0126 was used. Interestingly, the persistence of late-LTP in S2 (Fig. 12G open circles) was not prevented indicating that MAPK are not involved in LTP-tagging, whereas they mediate setting of tags in LTD (Fig. 12H).

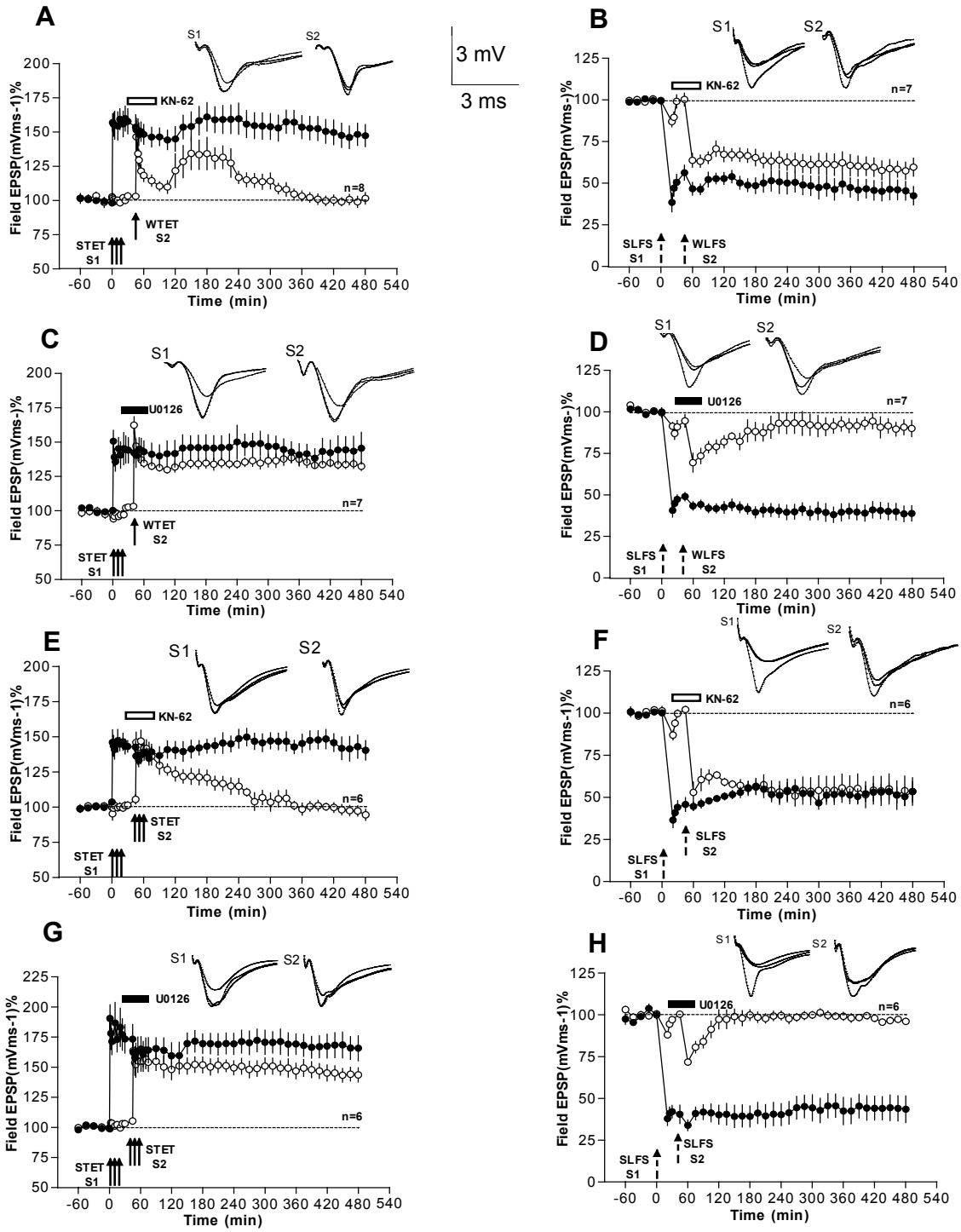



**Fig. 12. Role of CaMKII and MAPK on synaptic tagging in LTP and LTD.**

**(A)** Induction of late-LTP in S1 (filled circles) followed by early-LTP in S2 (open circles) 45 min after tetanization of S1. Early-LTP in S2 was induced in the presence of the CaMKII inhibitor KN-62 (open rectangle, 5 μM, drug applied 30 min before and 30 min after the induction early-LTP in S2, n=8). **(B)**

Late-LTD was induced in S1 without drug application (filled circles). Fifteen minutes after late-LTD induction in S1, KN-62 (5 μM) (open rectangle) was added and elapsed for the next 1 h and 45 min after late- LTD of S1 early-LTD was induced in input S2, but now under the presence of KN-62 (open circles) (n=7). **(C)** The same experiment as in (A) with the exception that the induction of early-LTP in S2 (open circles) was in the presence of the MAPK inhibitor U0126 (n=7). **(D)** The same experiment as in B with the exception that the induction of early-LTD in S2 (open circles) was in the presence of the MAPK inhibitor U0126 (n=7). (**E)** Late-LTP was induced in S1 without drug application (filled circles). Fifteen minutes after SLFS in S1, KN-62 was added and 45 min after LTP of S1, late-LTP was induced in S2 but now under the influence of KN-62 (open circles). Under these conditions late-LTP in S2 was prevented (n=6). **(F)** Late-LTD was induced in S1 without drug application (filled circles). Fifteen minutes after SLFS in S1, KN-62 was added and 45 min after LTD of S1, late-LTD in S2 was induced but now under the influence of KN-62 (open circles). Under these conditions late-LTD on S2 was still observed (n=6). **(G)** The same experiment as in E with the exception that the induction of late-LTP in S2 (open circles) was in presence of the MAPK inhibitor U0126. Under these conditions late-LTP in S2 was still observed (n=6). **(H)** The same experiment as in F with the exception that the induction of late-LTD in S2 (open circles) was in the presence of the MAPK inhibitor U0126. Under these conditions late-LTD in S2 was prevented (n=6).

Triplets of filled arrows indicate the time point of STET for inducing late-LTP and hatched arrow represents SLFS of the corresponding synaptic input for inducing late-LTD. Analog traces represent typical Field EPSPs 30 min before (dotted line), 30 min (dotted line) and 8 h (closed line) after STET or SLFS.

### 3.9. Role of CaMKII and MAPK during synaptic cross tagging.

We investigated the role of CaMKII or MAPK inhibition on processes of cross-tagging. Fig. 13A represents data where late-LTD was induced in S1 (filled circles). Fifteen minutes later, KN-62 was applied for the next 1h. Then, 45 minutes after the induction of late-LTD in S1, early-LTP was induced in S2 (open circles) in the presence of KN-62. In this case, early-LTP was not transformed into late-LTP by processes of cross-tagging, confirming the findings described Fig. 12A and E. CaMKII mediates the

setting of LTP-specific tags and if blocked, cross-tagging can not occur. Fig. 13B represents a similar experiment as Fig. 13A but by using a different CaMKII inhibitor, AIP at a concentration of 5 μM, which revealed the same results.

Next, we changed the order of the induction of LTP and LTD. Fig. 13C and D allowed us to confirm that CaMKII does not mediate the setting of tag in LTD. Induction of late-LTP in S1 (filled circles) followed by early-LTD in S2 (open circles) but the latter in the presence of KN-62 did not prevent cross-tagging. Early-LTD was transformed into late-LTD. Results shown in Fig. 13D confirm these experiments using a second structurally different inhibitor, AIP.

Fig. 13E-H represents the role of MAPK on cross-tagging by using the same experimental designs. In Fig. 13E and F are similar to Fig. 13C and D but now with the use of the MAPK inhibitors U0126 or PD98059. Here the conversion of early-LTD into a late-LTD in S2 is prevented by both MAPK inhibitors (open circles). In an opposite series of experiments, i.e, an experimental design similar to that in Fig. 13A and B the role of MAPK for cross-tagging was investigated. As it is shown in Fig. 13G and H, U0126 or PD98059 did not prevent the setting of the tag in S2 , thus it could utilize the proteins synthesized from the late-LTD synaptic input S1 (filled circles) resulting in the transformation of a transient early-LTP into a late-LTP lasting 8 h (open circles).

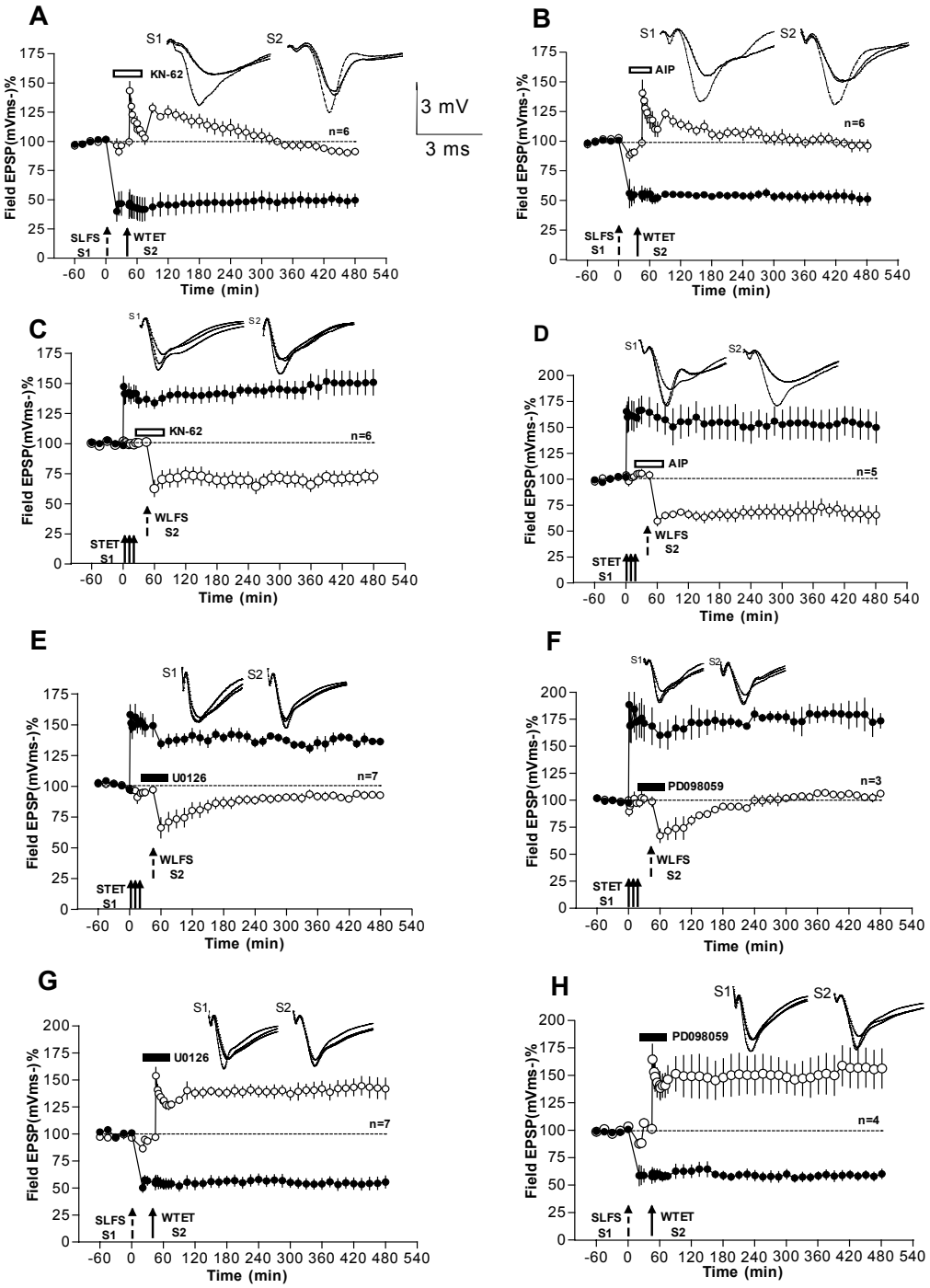



**Fig. 13. Role of CaMKII and MAPK on 'cross-tagging'**

**(A)** Late-LTD was induced in S1 without drug application (filled circles). Fifteen minutes after SLFS in S1, KN-62 was added and 45 min after late-LTD of S1, early-LTP in S2 was induced but now under the influence of KN-62 (open circles; (n=6)). **(B)** The same experiment as in (A) with the exception that the

induction of early-LTP in S2 (open circles) was in presence of second structurally different CaMKII inhibitor AIP (5 μM; n=6). **(C)** Induction of late-LTP in S1 (filled circles) was followed by early-LTD in S2. Here, early-LTD was transformed into late-LTD indicating cross-tagging between late-LTP and early-LTD irrespective of the inhibition of CaMKII by KN-62 (open circles) (n=6). **(D)** The same experiment as in (C) with the exception that the induction of early-LTD in S2 (open circles) was in the presence of another CaMKII inhibitor AIP (n=5). **(E)** Induction of late-LTP in S1 (filled circles) was followed by early-LTD in S2, but the latter in the presence of the MAPK inhibitor U0126. Here, early-LTD was not transformed into late-LTD (open circles; n=7). **(F)** The same experiment as in E with the exception that the induction of early-LTD in S2 (open circles) was in presence of another MAPK inhibitor PD98059 (n=3). **(G)** Late-LTD was induced in S1 without drug application (filled circles). Fifteen minutes after SLFS in S1, U0126 was added and 45 min after late-LTD of S1, early-LTP in S2 was induced but now under the influence of U0126 (open circles). Under these conditions early-LTP on S2 was transformed into late-LTP (n=7). **(H)** The same experiment as in (G) with the exception that the induction of early-LTP in S2 (open circles) was in presence of the MAPK inhibitor PD098059 (n=4).

Triplets of filled arrows indicate the time point of STET for inducing late-LTP and hatched arrow represents SLFS of the corresponding synaptic input for inducing late-LTD. Analog traces represent typical Field EPSPs 30 min before (dotted line), 30 min (dotted line) and 8 h (closed line) after STET or SLFS.

# 4. Discussion

## 4.1. RLTP and synaptic tagging

My initial studies were concentrated on the effects of rolipram, a phosphodiesterase (PDE) inhibitor, on long-term potentiation (LTP) and synaptic tagging. These studies were based on the findings that rolipram ameliorates impairments of learning and memory in rats and mice, and suggest that rolipram might act by elevating cAMP levels (Imanishi et al., 1997). Ahmed and Frey (Ahmed et al., 2004;Ahmed and Frey, 2003;Ahmed and Frey, 2005b;Ahmed and Frey, 2005a) identified a type IV-specific phosphodiesterase gene, PDE4B3, the first cAMP-specific phosphodiesterase to be associated with LTP and is specifically regulated during hippocampal CA1 LTP. Barad et al. have demonstrated that in mice low doses of rolipram can act to potentiate and extend LTP at the CA3-CA1 synapse in response to a stimulus that normally induces LTP lasting less than 1.5 h and improves memory (Barad et al., 1998). Targeting the degradative enzyme, PDE, may be particularly useful because partial inhibition of degradation may be undetectable at basal levels of substrate, when the degradative enzyme is likely to be present in great excess, and some mechanisms may compensate for low levels of inhibition (Dessauer et al., 1996). Because rolipram is a competitive inhibitor with cAMP for PDE, our results are most consistent with a model in which basal adenylyl cyclase activity is reduced to match that of the inhibited PDE through phosphorylation by PKA.

The notion that PDE4B3 is specifically regulated during different phase of LTP let us speculate that PDEs could act as PRPs or can regulate the synthesis of PRPs (see (Frey and Morris, 1998a;Sajikumar and Frey, 2004a)) whose synthesis is induced by the

induction of long-lasting plastic events. A PDE would represent an ideal general PRP since its direct effect on specifically regulating PKA-activity could drive a synapse either to increase or decrease its efficacy, thus expressing LTP or LTD, respectively (Sajikumar and Frey, 2004a).

We found that the type IV-specific PDE inhibitor rolipram can prolong an early form of LTP- which normally decays within 3-4 h - to a long-lasting form of LTP with duration of at least 6 h, the latest time point we have investigated (Fig. 6B and C). These results obtained in hippocampal slices from rats confirm the findings in mice published by Barad et al., 1998. We could also find that application of rolipram after the induction of LTP had no effect (Fig. 6D). Therefore the action of rolipram is synapse- and activity-specific. We used a concentration of rolipram which will not effect the basal cAMP concentration because toxicity appeared at a dose that raised basal cAMP levels as reported by Barad et al (Barad et al., 1998).

In addition to these data, we now describe that this reinforcement of early-LTP by rolipram is dependent on the activation of the NMDA-receptor and on protein synthesis (Fig. 6E and F). Stimulation of N-methyl-D-aspartate (NMDA) receptors on neurons activates both cAMP and cGMP signaling pathways. These results are strengthened by the fact that the PDE4 inhibitor, rolipram enhanced the ability of NMDA to increase cAMP in the neurons and PDE inhibitor-augmented effects of NMDA on cAMP and cGMP formation were antagonized by 5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate (MK-801), verifying NMDA receptor mediation (Suvarna and O'Donnell, 2002).

Furthermore, the induction of late-LTP requires the activation of protein kinase A (PKA) through cAMP -dependent processes (Frey et al., 1993). The substrate of the

type IV-PDE is cAMP. Thus, rolipram, as a PDE inhibitor, may enhance the translational machinery by increasing cAMP-levels and PKA-activity. These findings suggested that amplification of signals through the cAMP pathway might lower the threshold for generating long-lasting long-term potentiation. Monti et. al showed recently that rolipram delivery increased the basal rat hippocampal expression and phosphorylation of CREB, as well as the expression of the cAMP-dependent, memory-related protein, Arc (Monti et al., 2005). Furthermore, the increase of CREB phosphorylation and Arc expression consequent to the learning experience was enhanced in rolipram-treated rats, compared to controls. In addition, PDE-related processes which could counteract with potentiation-related intracellular processes would be depressed. Thus, early-LTP- a normally transient form of LTP, which is characterised by the setting of protein-synthesis-independent tags (Frey and Morris, 1997;Frey and Morris, 1998a) can be transformed into a protein synthesis-dependent late-LTP.

It was speculated that rolipram, by generally raising cAMP concentration throughout the brain, may enhance memory by a different mechanism perhaps by consolidating changes at recently stimulated synapses "tagged" by endogenous signalling mechanisms. Frey et al have demonstrated that blockade of either D2 or D1 receptor decreases the magnitude of late phases of LTP, 2 h or more after the induction (Frey et al., 1991b;Frey et al., 1990). This late phase seems to involve the effects of cAMP on protein synthesis (Frey et al., 1993). D1/D5-receptor agonists that are positively coupled to adenylyl cyclase specifically induce a slowly developing long-lasting potentiation of the field excitatory postsynaptic potential in the CA1 region of the hippocampus that lasts for > 6 h, an effect that is blocked by inhibitors of protein synthesis and NMDA-receptor (Huang and Kandel, 1995). This potentiation is also

blocked by the specific D1/D5-receptor antagonist SCH23390 and is occluded by the potentiation induced by cAMP agonists. Earlier works by Otmakhova and Lisman indicates that dopamine receptor agonists (such as Bromo-APB) or antagonists (such as SCH23390) may enhance or limit, respectively, the extent of early-LTP after a tetanus of intermediate intensity (40 pulses at 100 Hz) (Otmakhova and Lisman, 1996).

D1/D5-receptors seem to be implicated more strongly in the mechanisms of reinforcement (Otmakhova and Lisman, 1996) and they increase cAMP (Kebabian and Calne, 1979) which is important for early-LTP (Blitzer et al., 1995). Immunohistochemical localisation of dopamine D1/D5-receptors shows that there is heavy staining along pyramidal cells of the CA1 (O'Carroll and Morris, 2004). It was therefore of interest to study the effect of dopamine on RLTP, a rapidly developing, activity-dependent, synapse-specific modification.

The normal role of this dopaminergic activation might be to activate second-messenger systems, such as the PKA/cAMP cascade or the ERK/MAPK pathway, and thereby regulate downstream gene transcription or translation (Rosenblum et al., 2002;Frey et al., 1993). The multiple tetanic trains produce a transient rise in cAMP (Chetkovich et al., 1991) and a consequent activation of PKA (Blitzer et al., 1995) are consistent with such a possibility.

Our studies on RLTP show that the action of rolipram was downstream of the D1/D5-receptor. Our earlier hypothesis suggested that a synergistic action of glutamatergic and dopaminergic processes specifically regulate the synthesis of candidate PRPs, such as of the PDE4B3 (Sajikumar and Frey, 2004a). This hypothesis is now strongly supported by our here presented experiments: Weak tetanization of a synaptic input S1 which would normally result only in early-LTP was transformed into

late-LTP if a subsequent RLTP was induced in input S2 (Fig. 7B). The explanation of that transformation could be as follows: early-LTP was induced in synaptic input S1, sufficient to activate a 'synaptic tag' in this input. However, the tag as such is unable to convert early-LTP into late-LTP (Frey and Morris, 1997). But when a subsequent weak tetanization was delivered to a synaptic input S2 in the presence of rolipram both inputs now expressed late-LTP. Each of the tetanization of S1 and S2 activated their synapse-specific tags, but only the weak tetanization of S2 under the influence of rolipram (i.e. PDE inhibition), resulted in the activation of synapse-non-specific synthesis of PRPs which now could be captured by the 'synaptic tags' in S1 and S2, thus being able to express late-LTP in the two inputs. Application of rolipram after the induction of early-LTP and without subsequent tetanization of a second synaptic input was unable to cause the same effect (Fig. 6D). Early-LTP induction must be co-applied with PDE-repression to result in RLTP. Thus, induction of early-LTP does, not only lead to the setting of a 'tag' but is also involved in initiating synergistic events - together with PDE-inhibition - which finally results in the synthesis of PRPs required for the expression of late-LTP. However, we cannot exclude that enzymes other than PDEs are also involved in regulating the synthesis of PRPs. Tagging of proteins in S1 whose synthesis was initiated by the RLTP-dependent processes in input S2; 40 min after weak tetanization of S1 supports our assumption that PDE may represent one PRP or may regulate the synthesis of PRPs. Fig. 7C and D shows that synaptic tagging by rolipram is prevented by protein synthesis inhibitors.

Our results that rolipram reinforces early-LTP by increasing the cAMP concentration is further strengthened by the findings that rolipram, is able to restore the cAMP/PKA/ pathway activity and LTP (Vitolo et al., 2002;Gong et al., 2004). Rolipram

has been shown to reverse memory deficits produced by pharmacological blockade, such as *N*-methyl-D-aspartate (NMDA) (Zhang et al., 2000) or muscarinic blockade (Imanishi et al., 1997;Zhang and O'Donnell, 2000), and those induced by MAPK inhibitors (Zhang et al., 2004). Agents such as caffeine that have nonspecific phosphodiesterase inhibitory activity as well as other activities can also improve cognition in specific conditions (Nicholson, 1990). There is evidence that PKA activation in the hippocampus improves memory,(Ramos et al., 2003). Inhibition of cyclic AMP (cAMP)-specific PDE4 enhances memory in rodents further adds to the pharmacological value of the study (Zhang et al., 2005). MEM1018 and MEM1091 are newly developed PDE4 inhibitors that had not been evaluated as yet for their effects on working and reference memory but Zhang et al showed that MEM1018 and MEM1091 enhance memory in a manner generally similar to rolipram (Zhang et al., 2005).

**4.2. RLTD and Synaptic tagging**

We investigated the role of rolipram during LTD and synaptic tagging. The type IV-specific PDE inhibitor rolipram can reinforce a normally transient early-LTD into a long-lasting form of LTD with duration of at least 6 h which we named RLTD (Fig. 8C). Thus, rolipram - in addition to the reinforcment of a transient early-LTP into late-LTP in mice or in rat hippocampal slices in vitro (Barad et al., 1998;Navakkode et al., 2004) - also revealed similar effects for LTD (Fig. 8C). The synapse specificity of rolipram on activated synapses was confirmed by the findings that application of rolipram after the induction of LTD had no effect on its maintenance (Fig. 8D). We also showed that this reinforcement of early-LTD by rolipram was dependent on the activation of NMDARs and protein synthesis (Fig. 8E-G).

Hippocampal LTD, similar to persistent hippocampal LTP, appears to require protein synthesis from local mRNA and for its prolonged maintenance probably also *de novo* mRNA-synthesis (Frey and Morris, 1998a;Kauderer and Kandel, 2000;Sajikumar and Frey, 2003;Sajikumar and Frey, 2004a;Kelleher, III et al., 2004b;Kelleher, III et al., 2004a). Therefore, a mechanism must exist which transduces the local translational process to a transcriptional one. The MAPK/ERK-cascade is thought to participate in such a signal transduction process during LTP (Giovannini et al., 2001;English and Sweatt, 1997). It remained to be investigated till then, if the MAPK/ERK-cascade may fulfil similar function during LTD.

### 4.3. RLTD and the role of MAPK/ERK cascade

We could show that RLTD was prevented by inhibitors of MAPK, U0126 and PD98059 (Fig.10E and F). Therefore for the first time we reported that the MAPK/ERK cascade is involved in maintaining LTD in rat hippocampal slices in vitro. The cAMP/PKA-mediated activation of the ERK cascade may be an important pathway in regulating local protein synthesis, as well as gene expression during functional plasticity and memory formation. However, the specific pathways by which cAMP/PKA are coupled to extracellular signal regulated kinases are not yet fully understood. In a recent report, Morozov et al discussed distinct pools of p42/44MAPKs during different forms of LTP with their specific signalling pathways (Morozov et al., 2003). One pathway for LTP-maintenance involves the binding of the Raf family proteins (Raf-1, A-Raf and B-Raf) by activated Ras (Marshall, 1995). The major function of Ras in activating Raf is to relocate Raf to the cell membrane, where it is activated by a yet unknown mechanism and then in turn activates MEKs. Using mice genetically engineered to express the

neurofibromatosis mental retardation syndrome, Costa and co-workers provided evidence that a Ras/ERK hyperactivation contributes to hippocampus-dependent learning and related memory deficits as well as to a disruption of CA1-LTP (Costa et al., 2002). There is also growing evidence that the NMDAR/PKC-dependent Ras/Raf-1 pathway of MAPK/ERK-activation is one of the most important pathways for maintaining hippocampal CA1-LTP (English and Sweatt, 1997). However, it remains unclear which pathway of MAPK/ERK-activation is used for the prolonged, protein synthesis-dependent late-LTD in CA1. In the dentate gyrus, the Ras-pathway seems also to be important for LTD (Murray and O'Connor, 2004).

Here, we could show that RLTD, i.e. a prolonged LTD which seems to resemble the late phase of LTD, can be prevented by LT-82, an inhibitor of Rap1/B-Raf-pathway of MAPK activation triggered by dopaminergic D1/D5-receptor activation (Fig. 10H, and Fig. 14). Interestingly, manumycin-A, an inhibitor of the Ras/Raf-1-mediated MAKP pathway did not prevent RLTD (Fig. 10G and Fig. 14) indicating that MAPK activation is not mediated via Ras but Rap1 signalling. These results suggest that MAPK/ERK-regulated processes during LTD in adult animals are mediated by D1/D5-receptor-mediated cAMP/PKA-dependent processes.

Rap1 exerts a dual regulation of p42/p44 MAPK by either inhibiting Raf1 or activating B-Raf. In addition, Rap1 can also antagonise Ras (Altschuler and Ribeiro-Neto, 1998). Because Ras is anchored to the plasma membrane, whereas Rap is anchored to the membrane of endosomal compartments (Kim et al., 1990;Pizon et al., 1994;Resh, 1996), Rap1 and Ras may regulate distinct and different pools of MAPK (see above) resulting in different functions.

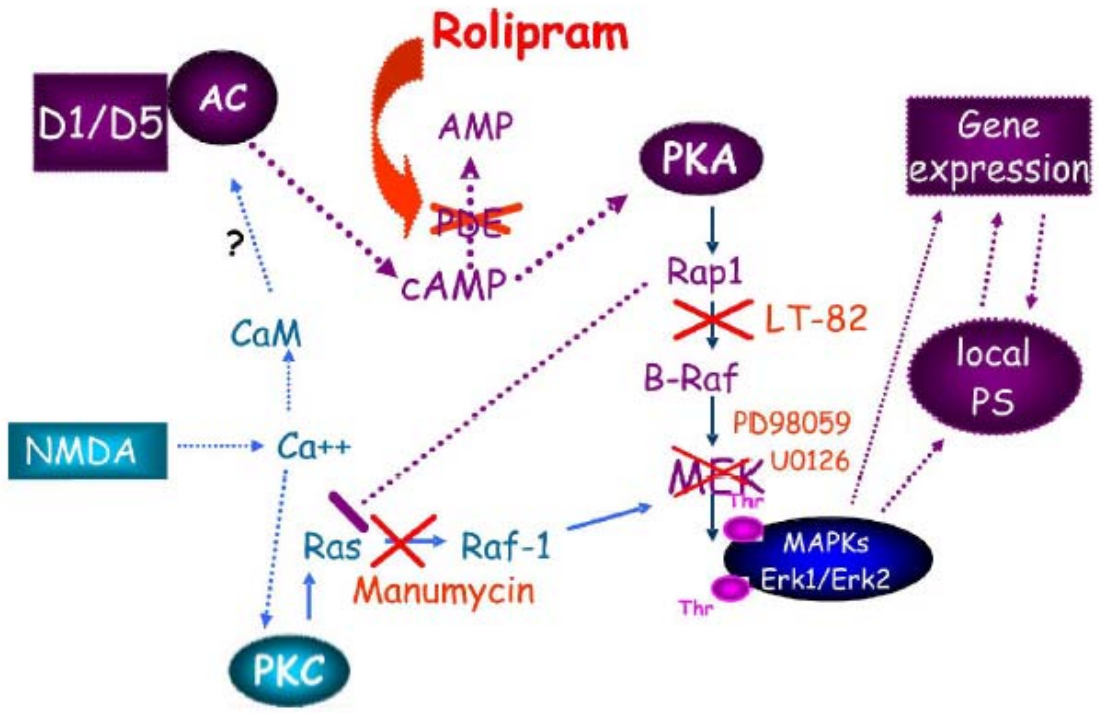



**Fig. 14. Schematic illustration of the possible action of rolipram during LTD, i.e. during the transformation of early-LTD into late-LTD in hippocampal CA1 from adult rats**(adapted from Sweatt, 2001)**.**

Inducion of electrically induced early-LTD by field electrodes in hippocampal CA1 neurons from adult rats activates glutamatergic NMDAR as well as dopaminergic D1/D5-receptors. For RLTD we could exclude the NMDAR-PKC/Ras mediated activation of MAKP/ERK. The interaction of NMDAR-mediated Ca2+/calmodulin-initiated processes with dopaminergically induced processes remain speculative but favour a role in marking/tagging the synapse for LTD. D1/D5-receptor-dependent processes mediate a cAMP/PKA-dependent, Rap1/B-Raf-mediated activation of MAPK/ERK resulting in protein synthesis and the transformation of early-LTD into late-LTD.

If RLTD represents conventional late-LTD, it should also be characterized by processes of synaptic tagging. If so, then for a protein synthesis-dependent stage,

synapse-specific tags must be set in the activated synapse population that then can capture PRPs whose synthesis took place non-specifically in response to the activated synapses. We have shown that the induction of early-LTD in one synaptic input S1 can be input-specifically reinforced by rolipram to a late-LTD, which is dependent on D1/D5-receptor activation as well as protein synthesis (Figs. 9 A-D), supporting synaptic tagging during RLTD.

Although, we provide evidence that the D1/D5-cAMP/PKA-Rap1/B-Raf mediated pathway of MAPK/ERK-activation seems to be one of the effective pathways during RLTD, there remain still many open questions. For instance, although rolipram seems specifically to induce late-LTD we cannot fully exclude that other, additional mechanisms may take place during conventional, electrically-induced LTD, other than that described here for RLTD. The requirement of a coactivation of NMDAR and D1/D5-receptors for RLTD also remains unclear. We suggest that through NMDAR-interactions the stimulated input was tagged to interact in an input-specific manner with the PRPs, allowing the synapse population to express synapse-specific late-LTD.

In addition, our results contribute to how the synthesis of PRPs, i.e., the pre-requisite for a cellular long-lasting memory trace, might be regulated via the activation of modulatory, non-glutamatergic inputs, if this activation occurs within a distinct time window around the induction of normally transient plastic phenomena such as early-LTP or early-LTD at a glutamatergic input (Frey and Morris, 1998a;Frey et al., 2001;Sajikumar and Frey, 2004a;Korz and Frey, 2003;Navakkode et al., 2004).

We had suggested that the synthesis of both process-specific and process-non-specific PRPs occurs during the induction of either late-LTP or late-LTD (Sajikumar and Frey, 2004a;Kelleher, III et al., 2004b;Sajikumar et al., 2005b). Recently, we identified

PKMζ as the first LTP-specific PRP (Sajikumar et al., 2005b). Furthermore, we have suggested that PDE4B3 could represent a process-non-specific PRP which regulates the synthesis of secondary PRPs maintaining either LTP or LTD (Ahmed and Frey, 2005b;Ahmed and Frey, 2005a;Navakkode et al., 2004;Sajikumar and Frey, 2004a;Sajikumar et al., 2005b). In addition, such regulatory PRPs might be involved in gene expression resulting in a local pool of mRNA whose products are specific for either LTP or LTD and which guarantee a prolonged maintenance of LTP or LTD beyond 8 h (Kelleher, III et al., 2004a;Kelleher, III et al., 2004b). Our data here provide more evidence for a specific role of PDEs in the regulation of processes required for the prolonged maintenance of hippocampal CA1-LTD.

Our results support further the requirement of heterosynaptic activation of processes which are critical for the expression of a long-lasting memory trace. These results were confirmed by a number of other laboratories (Chen et al., 1995;Huang and Kandel, 1995;O'Carroll and Morris, 2004), which let us hope that the focus of research in synaptic plasticity will in addition to glutamatergic mechanisms also include the description of modulatory processes, required for the consolidation of a memory trace at the cellular and networks level in the adult brain.

### 4.4. Synaptic tagging and cross-tagging: Revealing the nature of synaptic tags specific for LTP or LTD

One of the major question in studying processes of synaptic tagging is: What is the nature of the synaptic tag? Two protein kinase families highly expressed in brain that have been implicated in molecular mechanisms regulating LTP are the $Ca^{2+}$/calmodulin-

dependent kinase II (CaMKII) and ERK1/ERK2 (extracellular signal-regulated kinases) (Soderling et al., 2001;Sweatt, 2004;Schmitt et al., 2005).

CaMKII is required for LTP induction and can, by itself, induce the downstream processes that potentiate transmission which led us to select CaMKII as a potential candidate for tag. More recently Hudmon et al showed that CaMKII holoenzymes were shown to be capable of associating with one another (or self-associate) in response to $Ca^{2+}$ stimulation. Therefore CaMKII may form a scaffold that, in combination with other synaptic proteins, recruits and localizes additional proteins to the postsynaptic density. They also discussed the potential function of CaMKII self-association as a ´tag´ of synaptic activity (Hudmon et al., 2005). According to them the unique multimeric structure of the enzyme would thus provide a geometrically constrained scaffold onto which other synaptic proteins could be assembled, making CaMKII self-association an ideal synaptic tagging mechanism.

The ERK-MAPK signaling pathway is a highly conserved kinase cascade linking transmembrane receptors to downstream effector mechanisms (Chang and Karin, 2001). Previous studies have demonstrated the general involvement of the ERK signaling pathway in synaptic plasticity, learning and memory (English and Sweatt, 1997;Impey et al., 1998). The impact of MAPK cascade in synaptic plasticity led us to choose it as another possible tag candidate.

Our initial studies at least partially confirmed the results obtained from others that CaMKII or MAPK inhibition interfere with the induction of LTP and LTD (Mayford et al., 1995;Stevens et al., 1994;Schnabel et al., 1999;English and Sweatt, 1997;Kelleher, III et al., 2004a) (Fig. 11 A,C,E and G ). Extracellular bath application of the selective CaMKII inhibitor KN-62 to hippocampal slices in vitro blocked the induction of LTD and

of induction of LTP (Stanton and Gage, 1996;Bortolotto and Collingridge, 1998). Moreover, the concentration of MAPK inhibitor, U0126 which we used (Fig. 11 E), blocked the late phase of LTP leaving the early phase intact (Sweatt, 2004;Thomas and Huganir, 2004), which also supports the findings in Aplysia that ERK activation is necessary for long-term facilitation (LTF) but not for the short term facilitation (STF) (Martin et al., 1997). CaMKII or MAPK inhibition after the induction of LTP or LTD had no effect on its maintenance (Fig. 11B,D,F and H) which supports the findings reported earlier that inhibition of ERK activation after the induction of LTP does not reverse LTP in either area CA1 or the dentate gyrus (Coogan et al., 1999;English and Sweatt, 1997;Kelleher, III et al., 2004a;Impey et al., 1998;Kanterewicz et al., 2000).

Here we report for the first time that p42/44 MAPK activation is essential for LTD in CA1 region of the hippocampus in vitro (Fig. 11G). Different stimulation protocols may activate different ERK signal cascade (Thiels and Klann, 2001), as in strong low frequency stimulation which we used in our studies seemed to activate p42/44MAPK. One hypothesis to consider is that ERK is a general regulator of neuronal protein synthesis in response to different extracellular stimuli, and the specificity of the response (LTP or LTD) may be determined by the pattern of synaptic activity. Alternatively, the activation of ERK may be differentially regulated by NMDARs and mGluRs via different small GTPases. Recent work suggests that Rap1 regulates a specific subcellular fraction of ERK activity in hippocampal neurons (Morozov et al., 2003).

It was proposed earlier that spine morphological changes may contribute to the setting of tags in LTP (Frey and Morris, 1998a). We extend our hypothesis that the enhanced spine density in the activated synapses can hold the CaMKII in the post synaptic density thus enhancing the AMPARs conductance, because CaMKII drives the

insertion of new AMPA receptors into synapses (Wu et al., 1996;Lisman and Zhabotinsky, 2001). We have recently reported that an atypical PKC isotype, PKMζ acts as an LTP-specific PRP (Sajikumar et al., 2005b). There are considerable evidence that PKMζ increases the AMPA receptor responses (Ling et al., 2002;Osten et al., 1996), thus we propose that the activated synapses holding the CaMKII enhances the AMPAR responses to a further level so that the tagging of PKMzeta from a strongly tetanized input to weekly tetanized inputs occurs in LTP tagging.

Depotentiation can reset the tag if it is applied within 5 min after the induction of early-LTP (Sajikumar and Frey, 2004b). Our present results are consistent with these findings that CaMKII inhibition results a depotentiation like effects in early LTP thus may contribute the resetting of synaptic tags in LTP (Fif. 12A). Induction of late-LTP in S1 followed by weak tetanus to induce early-LTP in presence of CaMKII inhibitor KN-62 prevents LTP tagging. Therefore tagging of proteins from a strongly tetanised input to weakly tetanised input requires the mediation of CaMKII. We have shown earlier that short term potentiation or STP is incapable of generating a synaptic tag (Frey and Morris, 1997), therefore the depotentiating effect shows that CaMKII inhibition completely degrades the tag. To further confirm our results we induced late-LTP in both synaptic inputs S1 and S2, but S2 in presence of CaMKII inhibition. In this set of experiments also we could not observe a tagging interaction between the inputs S1 and S2. We further tested the role of CaMKII inhibition in LTD. Induction of late-LTD followed by early-LTD in the presence of KN-62 had no effect on LTD tagging (Fig.12 B) which was further confirmed by inducing late-LTD in both synaptic inputs but S2 under the influence of KN62 (Fig.12 F). Thus, our results provide strong evidence for a specific role of CaMKII in the setting of LTP-specific tags (Fig. 12 A and E).

In a recent review Thomas et al. speculated that, whether translocation of ERK can 'tag' the activated synapses by phosphorylation of specific substrates (Thomas and Huganir, 2004). Our results allow us to point out a critical role for ERK1/ERK2 for mediating the setting of LTD tags (Fig. 12D and H) but not for LTP tags (Fig. 12 C and G). Gallagher et al. demonstrated a role for ERK in mGluR-LTD in area CA1 of the rat hippocampus (Gallagher et al., 2004). ERK is specifically important in mGluR- and protein synthesis-dependent LTD. We confirmed these findings by inducing late-LTD in both inputs, however having the second input under the influnece of U0126. Late-LTD in S2 was prevented (Fig. 12H). The setting of LTP-tags was independent of MAPK (Fig. 12C and G). Thus, these results support our assumtion that the setting of LTD-tags is mediated by MAPK (Fig. 12D and H)

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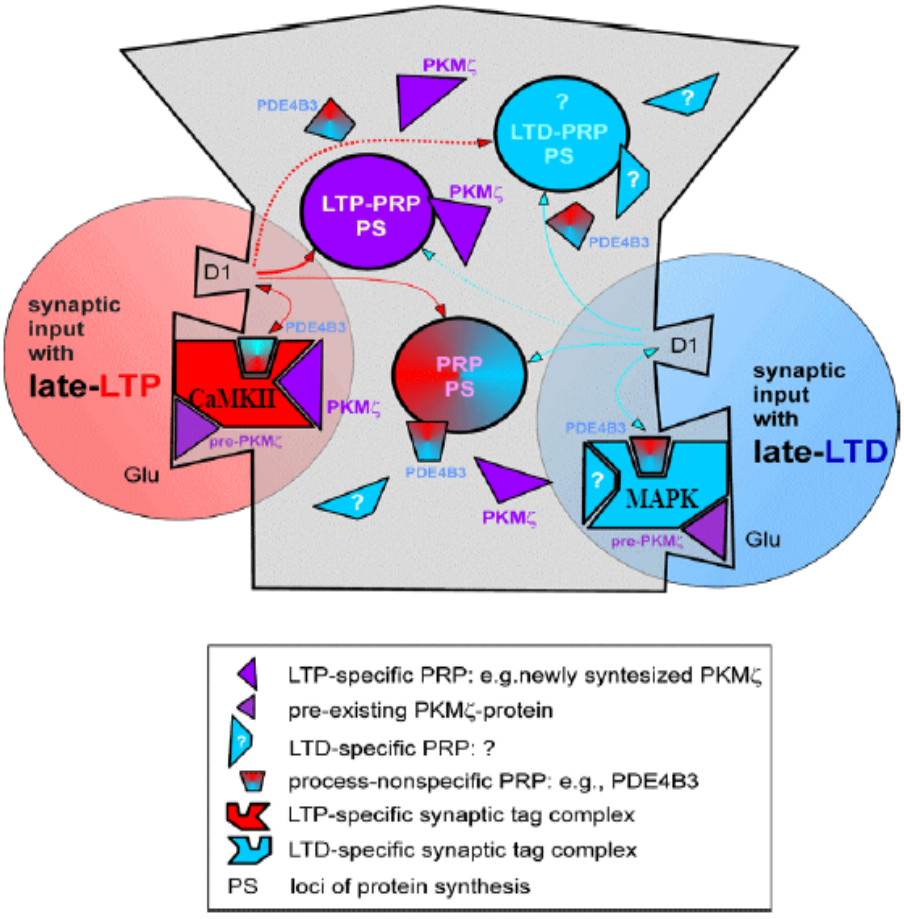
**4.5. Role of PKMζ, PDE4B3, CaMKII and MAPK in synaptic tagging/cross tagging in a CA1 pyramidal neuron**

Rolipram can prolong an early-LTP into late-LTP (RLTP) and it reinforces early-LTD into late-LTD (RLTD) at the same concentration. Therefore, unlike PKMζ, which is both necessary and sufficient for LTP but not for LTD, PDE4B3 is the first known process-non-specific regulatory protein which regulates the synthesis of process-non-specific PRP or is involved in the regulation of the synthesis of process-specific PRPs, (Navakkode et al., 2004;Ahmed et al., 2004;Ahmed and Frey, 2003). An LTD-specific PRP has yet to be identified.

Moreover, we were able to show that CaMKII acts as an LTP-specific tag molecule, although CaMKII is also necessary for the maintenance of both LTP and LTD.

MAPK, on the other hand mediate the setting of LTD-specific tags, although MAPK are also essential for the maintenance of both LTP and LTD (Fig. 16).




**(**Adapted from Sajikumar. S, Navakkode.S, Frey JU. J Neurosci. 2005 Jun 15;25(24):5750-6.)

**Fig. 16. Role of PKMζ, PDE4B3, CaMKII and MAPK in synaptic tagging/cross tagging in a CA1 pyramidal neuron.** Hypothesis of the activity-dependent synthesis of a pool of PRPs that are either specific for LTP (red triangles), specific for LTD (blue trapezoids), or non-specific (mixed red and blue trapezoids). PDE4B3 is the first known non-specific PRP, which could be also important for process-specific aspects such as the regulation of the synthesis of process-specific PRPs (Ahmed and Frey, 2003; Navakkode et al. 2004; Ahmed at al., 2004). PKMζ is the first identified LTP-specific PRP. An LTD-specific PRP has yet to be identified. CaMKII acts as the process-specific tag molecule in LTP. MAPK represents a process-specific molecule that mediates the setting of LTD tag. We suggest that not only

does the process-specific tag consists of a complex machinery of molecules specific for LTP (red symbol at the synapse) or LTD (blue symbol at the synapse in Fig. 16; Frey and Morris, 1998; Sajikumar and Frey, 2004), but also PRPs represent a pool of proteins expressing their effector roles by selective interactions with these process-specific tag complexes, in addition to basic short-term plasticity functions. D1-dopaminergic D1/D5-receptor; Glu- glutamatergic synapse.

## 5. Conclusions

The principal findings of this dissertation are

1) Rolipram, a selective cAMP phosphodiesterase inhibitor, reinforces an early form of LTP, which lasts less than 2-3 h into a long-lasting LTP (RLTP) which is stable up to a recorded time period of 6 h in hippocampal CA1 region in vitro.
2) RLTP is protein synthesis- and NMDA-receptor- dependent, similar to conventional late-LTP.
3) RLTP can interact with processes of synaptic tagging.
4) The action of rolipram is downstream of D1/D5-receptor activation during LTP.
5) cAMP-dependent processes are directly involved in the synthesis of plasticity-related proteins and PDE represents one PRP mediating LTP.
6) Rolipram reinforces an early form of LTD which lasts for less then 2-3 h into a long-lasting form (RLTD) which is stable for 6 h recorded time period. Thus for the first time we report a reinforcement-effect in LTD.
7) RLTD is protein synthesis- and NMDA-receptor-dependent similar to late-LTD.
8) RLTD, similar to RLTP, can interact with processes of synaptic tagging.
9) Synaptic tagging by RLTD is dependent on the activation of D1/D5-receptors.
10) Mitogen-activated protein kinases (MAPK; ERK1/ERK2) mediate the reinforcement-effect of rolipram in LTD.
11) Activation of MAPK by rolipram is mediated through the rap-signalling pathway rather than ras signalling pathway during LTD.
12) The cAMP-dependent processes are directly involved in the synthesis of plasticity-related proteins (PRPs) for both LTP and LTD.

13) Thus for the first time we report a process-unspecific PRP, i.e. PDE that regulates opposing forms of plastic events like LTP and LTD.
14) CaMKII or MAPK (ERK1/ERK2) inhibition during the induction of LTP or LTD prevents their maintenance.
15) CaMKII or MAPK (ERK1/ERK2) inhibition after the induction of LTP or LTD has no effect on their maintenance.
16) CaMKII mediates the setting of tags specific for LTP, but not for LTD.
17) MAPK (ERK1/ERK2) mediates the setting of tags specific for LTD, but not for LTP.

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# Appendices

## I. Zusammenfassung

Gedächtnisformierung und -konsolidierung unterliegen sehr wahrscheinlich langfristigen plastischen Prozessen, welche die synaptische Übertragungsrate zwischen Neuronen modulieren. Die bisher am besten untersuchten Modelle synaptischer Plastizität sind die Langzeitpotenzierung (LTP) und die Langzeitdepression (LTD). Die bisherige Kenntnis der zellulären und molekularen Mechanismen die der LTP und LTD unterliegen, wurden überwiegend an hippokampalen Schnittpräparaten in vitro gewonnen.

Meine ersten Studien konzentrierten sich auf den Effekt von Rolipram, einem Hemmer der Typ IV-spezifischen cAMP-Phospodiesterase (PDE), auf späte Phasen funktioneller plastischer Prozesse in der CA1 Region hippokampaler Schnittpräparate der Ratte. Ich konnte zeigen, das die frühe Phase der LTP (early-LTP) die normalerweise innerhalb von 2 h - 3 h auf baseline-Niveau abklingt in eine späte Form der LTP (late-LTP) transformiert wird (> 6 h), wenn während der Tetanisierung Rolipram appliziert wurde. Diese Rolipram-verstärkte LTP (RLTP) ist NMDA-Rezeptor- und proteinsynthese-abhängig. Da die Aktivierung der cAMP- Signalkaskade während der Induktion eine late-LTP Dopaminrezeptoraktivität (D1/D5) in der CA1-Region erfordert (Frey et al., 1989; Frey et al., 1990), überprüften wir, ob die RLTP von dopaminerger Aktivität abhängt. Da die RLTP ebenso wie die konventionelle späte LTP von Proteinsynthese abhängt, untersuchte ich, ob Rolipram Prozesse des synaptic tagging beeinflußt. Hemmung der PDE und nachfolgende Induktion einer RLTP in einer Population von Synapsen (S1) erlaubte die Transformation einer early-LTP in eine late-LTP in einer zweiten, von der ersten unabhängigen Synapsenpopulation (S2) derselben

Neuronenpopulation. Dieses Ergebnis unterstützt unsere Hypothese, daß cAMP-abhängige Prozesse unmittelbar in die Synthese plastizitätsrelevanter Proteine (PRPs) involviert sind.

In neueren Studien wurden Interaktionen zwischen LTP und LTD über synaptic tags nachgewiesen (Sajikumar & Frey, 2004a) und als „cross-tagging“ in die Literatur eingeführt. Da der tag der durch die Induktion der frühen Phase einer der beiden Formen der synaptischen Plastizität von PRPs profitieren kann, deren Synthese durch die Induktion der späten Phase von LTP oder LTD angestossen wurde, stellte sich die Frage nach der Rolle prozeß-unspezifischer und –spezifischer Proteine. Wir konnten PKMζ als erstes LTP-spezifisches Protein nachweisen, das sowohl nötig als auch hinreichend für die Aufrechterhaltung der late-LTP aber nicht der late-LTD ist (Sajikumar et al., 2005). Da wir zeigen konnten, daß eine PDE-Inhibierung zur Verstärkung der early-LTP führt, stellte sich nun die interessante Frage, ob es auch zu einer LTD-Verstärkung führen kann (RLTD.

In der vorliegenden Arbeit zeige ich, daß in der CA1-Region hippokampaler Schnittpräparate adulter Ratten die Gabe von Rolipram während des Tetanus ebenso zu einer Transformation einer early-LTD in eine late-LTD führt. Zudem konnte ich zeigen, daß diese Transformation NMDAR- und proteinsyntheseabhängig ist, sowie eine dopaminerge Aktivierung erfordert und somit ähnlichen Mechanismen zu unterliegen scheint, wie eine elektrisch induzierte late-LTD. Daher stellte sich die Frage, ob synaptic tagging während einer RLTD nachzuweisen ist. Ich konnte zeigen, das in S1 eine LTD-Verstärkung zu beobachten ist, wenn in S2 eine early-LTD in Anwesenheit von Rolipram induziert wird. Somit wären die Kriterien von synaptic tagging für die RLTD erfüllt.

Während die molekularen Mechanismen verschiedener Formen der LTP relativ gut untersucht sind, ist dies für die LTD nicht der Fall. Wir begannen daher, die Mechanismen des Rolipram-Effektes auf die LTD genauer zu untersuchen. Wir konnten durch Einsatz von Inhibitoren (U0126 und PD98059) der mitogen-aktivierten Proteinkinase (MAPK) zeigen, daß die durch extrazelluläre Signale regulierte Kinase (ERK1/ERK2) induzierte Signalkaskade in RLTD involviert ist. Außerdem studierten wir die Rolle spezifischer Kaskaden durch die ERK1/ERK2 während RLTD aktiviert wird und fanden eine spezifische Rolle der Rap/PKA abhängigen Kaskade. Wir konnten unter Einsatz von spezifischen Inhibitoren des Ras/Raf-1 Signalweges (Manumycin) und des Rap/B-Raf Pfades (LT-82) zeigen, das die MAPK-Aktivierung während RLTD ausgelöst wird durch die synergistische Interaktion der der NMDAR und D1/D5-Rezeptor abhängigen Rap/B-Raf Signalwege, jedoch nicht des Ras/Raf1 Pfades. Zusammenfassend zeigen diese Ergebnisse, daß PDE4B3 ein prozeß-unspezifisches Protein ist, welches die Ausbildung einer LTP und/oder LTD reguliert.

In einem nächsten Schritt interessierte uns die grundlegende Frage: was ist der synaptic tag? Gibt es spezifische tags für LTP und LTD? Viele Forschergruppen haben spekuliert, das der tag die aktivierte, phosphorylierte Form eines Proteins sein könnte oder auf Änderungen des Zytoskeletts wie etwa spine-Durchmesser oder Aktin-Filamente beruhen könnte. Wir studierten die Rolle zweier vielversprechender Kandidaten CaMKII und ERK1/ERK2 (MAPK) als LTP- oder LTD-spezifische tags. Zunächst bestätigten wir die Ergebnisse anderer Labors, daß die Inhibierung der CaMKII oder MAPK nach Induktion von LTP oder LTD keine Rolle auf deren Aufrechterhaltung hat. Wir konnten erstmals zeigen, das p42/44 MAPK auch für die Aufrechterhaltung der LTD benötigt wird.

Ebenso konnten wir durch Einsatz eines spezifischen Hemmers (KN-62) zeigen, das das Setzen eines tags oder tag-Komplexes während LTP jedoch nicht LTD ein CaMKII-vermittelter Prozeß ist. Zusätzlich zu dem klassischen tagging-Experiment, sicherten wir dieses Ergebnis ab, indem in beiden Inputs (S1 und S2) eine late-LTP induzierten, in S2 allerdings in Gegenwart von KN-62. Während in S2 late-LTP geblockt wird, bleibt late-LTP in S1 erhalten. Induktion von late-LTD in beiden Inputs in Gegenwart von KN-62 in S2 hatte jedoch keinen Einfluß auf deren Aufrechterhaltung.

Nach Klärung der Rolle von CaMKII im Setzen des tags während LTP stellte sich die Frage nach einer spezifischen Kinase die das Setzen eines LTD-tags vermittelt. Wir wiederholten die gleichen Experimente wie für CaMKII oben beschrieben mit zwei spezifischen MEK-Hemmern (U0126 und PD98059). Wir konnten zeigen, daß im Gegensatz zu CaMKII, MAPK keinen Einfluß auf das Setzen LTP-spezifischer tags hat, jedoch die Aktivierung LTD-spezifischer tags vermittelt.

Als nächstes untersuchten wir die Frage, ob mit cross-tagging die weitere Prozeß-Spezifität der tags charakterisiert werden kann. Hierzu induzierten wir late-LTD in S1 und nach 45 min early-LTP in S2 in der Gegenwart eines CaMKII-Hemmers (KN-62 und Autocatamide 2-inhibitory peptide, AIP). Es zeigte sich keine Transformation in eine late-LTP in S2, es fand also kein cross-tagging statt. Die Durchführung des gleichen Experimentes in Gegenwart von MAPK-Hemmern (U0126 und PD98059) führte zudem zu keiner Transformation der early-LTD in eine late-LTD, was die spezifische Rolle für MAPK während LTD-taggings bestätigt. Zusammenfassend konnten wir zeigen, das LTP-tagging durch CaMKII vermittelt wird, während MAPK in LTD-tagging involviert ist.

## II. Selbständigkeitserklärung

### Erklärung

Hiermit erkläre ich, daß ich die von mir eingereichte Dissertation mit demThema

**´ Investigation of cellular mechanisms of hippocampal LTP and LTD´**

selbständig verfaßt, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr.rer.nat.) an anderen Einrichtungen eingereicht habe.

Förderstedter Str. 90, Magdeburg, 26. 09. 2005

Master of Science – Sheeja Navakkode Gangadharan

## III. Publications

**Navakkode S**, Sajikumar S and Frey JU (2005). The effect of rolipram, a type IV-specific phosphodiesterase inhibitor, on distinct forms of long-term depression and ´synaptic tagging´. (Re-revision in **Journal of Neurosci.**).

Sajikumar S**, Navakkode S**, and Frey JU (2005).Protein synthesis dependent long-term functional plasticity: methods and techniques. **Curr. Opin. Neurobiology.** October issue, In Press (on-line: 7th Sept. 2005).

Sajikumar S**, Navakkode S**, Sacktor TC and Frey JU (2005). Synaptic tagging and cross-tagging: the role of protein kinase M zeta in maintaining long-term potentiation, but not long-term depression. **Journal of Neurosci. 2005 Jun 15; 25(24):5750-6.**

**Navakkkode S**, Sajikumar S and Frey JU (2004). The type IV-specific phosphodiesterase inhibitor, rolipram, and its effect on hippocampal long-term potentiation and synaptic tagging. **Journal of Neurosci 24 (35): 7740-7744.**

**Navakkkode S**, Sajikumar S and Frey JU (2005). D1/D5 receptor agonist induces activity independent long-term potentiation in hippocampal slices in vitro (submitted).

Sajikumar S**, Navakkkode S,** Frey JU (2005). In search for the specificity of 'synaptic tags' during LTP and LTD. (submitted).

## Abstracts presented

**Navakkode S,** Sajikumar S and Frey JU, (2005) The effect of rolipram, a type IV-specific phosphodiesterase inhibitor, on distinct forms of long-term depression and ‘’synaptic tagging’’. Synaptic Function and Plasticity Conference. The University of British Columbia (UBC) Vancouver, Canada**.** July 21-23, 2005.

Sajikumar S**, Navakkode S,** and Frey JU, (2005).Synaptic Function and Plasticity Conference. The University of British Columbia (UBC) Vancouver, Canada**.** July 21-23, 2005 **(Selected for oral presentation).**

**Navakkode S,** Sajikumar S**,** and Frey JU, (2005). XI th Magdeburg International Neurobiological symposium on ´Learning and Memory´: Cellular and Systemic views. The effect of rolipram, a type IV-specific phosphodiesterase inhibitor, on distinct forms of long-term depression and ‘’synaptic tagging’’. May 28-June 1 (2005).

Sajikumar S**, Navakkode S,** Sacktor TC and Frey JU, (2005). XI th Magdeburg International Neurobiological symposium on ´Learning and Memory´: Cellular and Systemic views. In search for the process specific molecules in LTP or LTD. May 28-June 1 (2005).

**Navakkode S,** Sajikumar S and Frey JU (2005) The effect of rolipram, a type IV-specific phosphodiesterase inhibitor, on distinct forms of long-term depression and ‘’synaptic tagging’’. Proceedings of the 30$^{th}$ Göttingen Neurobiology Conference and the 6$^{th}$ Meeting of the German Neuroscience Society 2005.

Sajikumar S, **Navakkkode S**, Frey JU (2005) In search for the specificity of 'synaptic tags' during LTP and LTD. Proceedings of the 30$^{th}$ Göttingen Neurobiology Conference and the 6$^{th}$ Meeting of the German Neuroscience Society 2005.

**Navakkode S** & Frey JU (2004) Role of rolipram, a type-IV-specific phosphodiesterase inhibitor, during “synaptic tagging” in rat hippocampal slices in vitro. 4$^{th}$ Forum of European Neuroscience Society Meeting, Lisbon, Portugal. Abstr. 214.

## IV. Curriculum Vitae


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| Name: | **Sheeja Navakkode Gangadharan** |
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| **Present Position:** | PhD-student |


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| | |
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| 2003 (Jan) - Present<br>Doctoral student | Leibniz Institute for Neurobiology,<br>Magdeburg, Germany.<br>Title of Dissertation:<br>Investigation of Cellular mechanisms of hippocampal LTP and LTD.<br>Supervisor: Prof. Dr. Julietta Uta Frey |
| 2001-2002<br>Junior Research Fellow | Dept. of Human Genetics,<br>National Institute of Mental Health and Neurosciences,<br>P.B. No 2900,<br>Bangalore-560 029, India.<br>Project title: Cytogenetic investigations of neuropsychiatric disorders. |
| 2000-2001<br>Junior Research Fellow | Dept of Molecular Medicine,<br>Sree Chitra Tirunal Institute for Medical Sciences and Technology,<br>Bio Medical Technology Wing,<br>Poojappura, Thiruvananthapuram-695 012, Kerala.<br>Project Title: Transcriptional down regulation of alpha1-4-glucan-6-glycosyl transferase gene in epileptic hippocampus. |

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| MSc in Biotechnology | **Passed in First class with Distinction** from Cochin University of Science and Technology |
| | BSc in Botany with Zoology and Chemistry as Subsidiaries Passed in **First class with distinction and 2nd rank** from Calicut University, Kerala. |
| MSc Project: Dept. of Molecular Medicine | |
| | Sree Chitra Tirunal Institute for Medical Sciences and Technology, Bio Medical Technology Wing, Poojappura, Thiruvananthapuram-695 012, Kerala Project Title: Analysis of Glycogen branching enzyme gene expression in brain: an approach to understand the pathology associated with mesial temporal lobe epilepsy. |
| Pre-Degree in Biological Sciences Calicut | Passed in **First class with Distinction**, Mercy College, University, Kerala. |
| School | Secondary School Leaving Certificate |
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**Awards and Achievements**

- Awarded Joint **CSIR-Junior Research Fellowship (JRF)** and Eligibility for Lectureship-National Eligibility Test (NET) held on 2000 by Council of Scientific & Industrial Research, Human Resource Development Group Examination Unit, PUSA, New Delhi, India. No.2-44/2000 (i)- EU. 11.
- Awarded **Second Rank in B Sc**. Degree Examination, Calicut University.
- Awarded Dr. Mrs Chandrika Sankar Prize for the **Best student** I st year B Sc.
- Awarded Reghunath Vasudevan Memorial Prize instituted by Rekha Vasudevan to the **Best student** in II nd year B Sc.
- Awarded Mr E.J. Antony Memorial Prize to the **Best Student** in II nd year B SC.
- Awarded Mrs P. Sunitha Devi and Mrs. Viji Krishnan memorial prize to the Best **Stuent in B Sc**.
- Awarded the **Gold medal for II nd Rank** in Calicut University.
- Scholarship for Pre-Degree and Bachelors Degree.

## References

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