Late-LTP in Apical CA1 Dendrites of Hippocampal Slices In Vitro

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Binu Ramachandran

Abstract

The major forms of hippocampal synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) are regarded as cellular correlates of learning and memory formation. In recent years, an impressive research effort has been devoted to understanding the cellular and molecular mechanisms of hippocampal synaptic plasticity, particularly LTP.

During my initial studies I could reproduce electrically induced LTP in apical dendrites of pyramidal neurons within CA1 region of hippocampal slices in vitro. Depending on different induction protocols distinct forms of LTP such as a transient, protein synthesis-independent early-LTP (with duration of 3-4 h) or a de novo protein synthesis-dependent late-LTP (lasting for at least 6 h) could be induced. Both forms of LTP required NMDA-receptor activation and especially the late-LTP required synergistic activation of glutamatergic and dopaminergic inputs during its induction.

It has been reported that the LTP in CA1 region is characterized by processes of synaptic tagging. During LTP induction the activated synapses are marked by a “synaptic tag/ tag complex” which can capture plasticity-related proteins (PRPs). During synaptic tagging, early-LTP induced in one synaptic input can be transformed into a late-LTP, if late-LTP was induced in an independent synaptic input of the same neuronal population within a distinct time window. The synthesis of process unspecific plasticity-related proteins (PRPs) by late-LTP induction in the second synaptic input is sufficient to transform/reinforce the early-LTP into a late-LTP, which is marked by a synaptic tag/ tag complex.

Next, I was interested to investigate whether actin network function is essential for the maintenance of LTP in hippocampal CA1 region. It has been reported that the dynamics of actin cytoskeleton is essential for the maintenance of LTP. Here we found that the inhibition of actin polymerization affects the protein synthesis-independent early-LTP and protein synthesis-dependent late-LTP. But interestingly, the application of actin inhibitors after the induction of late-LTP was unable to block LTP at all, suggesting an early mechanism that is required for the induction and maintenance of LTP.

In the last series of experiments I have investigated, whether inhibition of actin network interferes with processes of synaptic tagging. The transformation of early-LTP into late-LTP was blocked by the application of structurally different actin polymerization inhibitors, latrunculin A and cytochalasin D. We suggest that the actin network is required for early “house keeping” processes for inducing and maintaining early-LTP. Furthermore, inhibition of actin dynamics negatively interacts with the setting of synaptic tag complex. We propose actin as a tag-specific molecule in apical CA1 dendrites, where it is directly involved in the tagging/capturing machinery and inhibition of actin network thus prevents the interaction with plasticity-related proteins. This results in the prevention of late-LTP by inhibition of the actin network during LTP induction.

ABBREVIATIONS

ABPs: actin binding proteins

ACSF: artificial cerebrospinal fluid

AMPAR: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor

Ani: anisomycin

AP-5: D-2-amino-5-phosphonopentanoic acid

CA: cornu ammonis

CamKII: calcium/calmodulin-dependent protein kinase II

Cyt D: cytochalasin D

DMSO: dimethyl sulfoxide

E-LTP: early long-term potentiation

EPSP: excitatory post synaptic potential

F-actin: filamentar actin

G-actin: globular actin

HFS: high-frequency stimulation

Lat A: latrunculin A

LTD: long-term depression

LTP: long-term potentiation

L-LTP: late long-term potentiation

mRNA: messenger ribonucleic acid

NMDA: N-methyl D-aspartate

PKC: protein kinase C

PKMzeta: protein kinase M zeta

PRPs: plasticity-related proteins

PS: population spike

PTP: post tetanic potentiation

STET: strong tetanization

STP: short-term potentiation

WTET: weak tetanization

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1. Introduction

1.1. Learning and Memory

The most distinctive feature of the mammalian central nervous system is its ability to adapt to the environment and to improve its performance over time and experience. An important basis for this peculiar property is the plastic nature of the synapses, i.e. the capacity to change their signaling strength, both in short and long term, in response to specific patterns of synaptic activity. The neural changes evoked by the stimuli can persist even for very long times, virtually for the whole life of the individual. This neural plasticity represents the basis of higher brain functions such as learning and memory. Learning is the process by which the brain acquires new information and memory stands for the ability to store or retain the acquired information (Squire, 2004).

Memories are generally classified into two major forms, one for skills and other form for knowledge called non-declarative memory or implicit memory and declarative memory or explicit memory, respectively. Implicit memory refers to information storage to perform various reflexive or perceptual tasks and is recalled unconsciously. The implicit memory is more robust and may last for all our life even in the absence of further practice (Squire, 2004). Implicit memory involves a heterogeneous collection of memory functions and types of learned behaviors such as reflexive conditioning, fear conditioning and priming. The explicit memory is concerned with the factual knowledge of persons, things, notions and is recalled by a deliberate and conscious effort.

Explicit memory can be further classified as episodic and semantic memory. Episodic memory allows us to remember personal events and experience, on the other hand semantic memory is a sort of public memory for facts and notions. The

explicit memory fades relatively rapidly in the absence of recall and refreshing and prone to distortion (Cohen and Squire, 1980; Squire., et al., 1993; Squire, 2004).

Neuropsychological studies on patients, mainly pioneered by Brenda Millner with the famous H.M. case, have shown that the multiple memory systems involve distinct brain areas, especially the medial temporal lobe, and exhibit distinctive features (Scoville and Milner, 1957). Later studies on H.M have confirmed that the hippocampus is essential for the formation of new episodic memories and might also have a role in their long-term storage.

In 1949 Donald Hebb in his book “The Organization of Behavior” proposed that memories are stored in the mammalian brain as stronger synaptic connections between neurons active during learning. The specific mechanism he suggested to bring about these changes in synaptic transmission is relatively simple. He formalized this ideas 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 efficacy, as one of the cells firing B, is increased”
>
> (Hebb, 1949).

In other words, correlation/or association of pre- and post-synaptic activity in two neurons elicits some change in one or both of the neurons such that the synaptic connection between them is strengthened (Hebb, 1949). These kind of modified synapses are referred as “Hebbian synapses” or “Hebb synapses”. Later this became a theoretical foundation for many neurobiological and computational models of “synaptic plasticity” and has revolutionized thinking about the nature of the neural

mechanisms of learning and memory formation. The term “synaptic plasticity” was first introduced by the Polish psychologist Jerzy Konorski to describe, an activity driven persistent changes in synaptic efficacy that assumed to be the basis of information storage in the brain (Konorski, 1948). From later studies evidence has emerged, supporting the view that memories are represented as enduring changes in the functional circuitry of the brain and that synaptic contacts between neurons serve as the pliable substrate for “memory traces”.

In 1973 Bliss and his coworkers discovered that brief high-frequency electrical stimulation of perforent pathway can enhance synaptic transmission for days even weeks in the rabbit hippocampus in vivo (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973). This long lasting form of synaptic plasticity is known as long-term potentiation or LTP. LTP is defined as”persistent increase in synaptic efficacy after a brief tetanic stimulation in the afferent pathway “(Malenka, 1994; Malenka and Nicoll, 1999; Malenka and Bear, 2004). LTP is induced by correlated pre- and postsynaptic activity i.e. it is Hebbian and exhibits several basic properties of learning and memory (Bliss and collingridge, 1993; Matthies et al., 1989, 1990; Teyler et al., 1984).

In recent years, an impressive research effort has been devoted to understand the cellular and molecular mechanisms of various forms of synaptic plasticity, particularly LTP in the hippocampus.

1.2. Hippocampus

The hippocampus plays a critical role in tasks which require the flexible representation of information, i.e. tasks of declarative memory. Damage of the hippocampus can lead to severe memory impairment, e.g. patients with hippocampal lesions show deficits in declarative functions. Two contending theories consider the hippocampus to be either the locus for temporary storage of information to be

consolidated (Squire, 1992) or the locus of permanent information storage through multiple memory traces (Nadel and Moscovitch, 1997; Riedel and Micheau, 2001).

The hippocampus is a specialized region of the limbic cortex, located in the medial temporal lobe. Hippocampus derives its name from the sea horse (hippo= horse, kampos= sea monster; Greek). The hippocampal formation is divided into the hippocampus proper, dentate gyrus and the subiculum. The hippocampus proper is composed of regions with tightly packed pyramidal neurons, mainly as CA1, CA2 and CA3. The CA1-CA3 subfields are called the Cornu Ammonis or Ammon's horn for its resemblance to a ram's horn of the Egyptian God Ammon. The CA1 region is also called the superior region, which composed of tightly packed pyramidal cells. These cells become loosely packed in CA2 and CA3 region (also called the inferior region) and this thinning denotes the boundary between the two areas. The CA3 region marks the transition from the hippocampus proper to the dentate gyrus (Isaacson, 1982). The dentate gyrus is part of the large hippocampal formation (which is often referred to simply as the hippocampus) that includes the dentate gyrus and the subiculum (Giap et al., 2000).

1.2.1. Trisynaptic pathway in Hippocampus

The hippocampus receives highly processed multi-modal information from the association cortices (Amaral and Witter, 1995), that is, inputs from all the sensory modalities, vision, hearing, touch, etc, have already converged and been preliminarily associated with one another by the time they reach the hippocampus. The hippocampus has direct connections to the entorhinal cortex via the subiculum. Outputs from these structures can affect many other areas of the brain. For example the entorhinal cortex projects to the cingulated cortex, which has connections to the temporal lobe cortex, orbital cortex, and olfactory bulb. Thus, all of these areas can be influenced by hippocampal output, primarily from CA1. The entorhinal cortex is a

major source of inputs to the hippocampus collecting information from the cingulated cortex, amygdale, orbital cortex and olfactory bulb (Johnson and Amaral, 1998). The hippocampus receives inputs via the precommissural branch of the fornix from the septal nuclei.

Information flow within the hippocampus formation is classically described as a trisynaptic circuit, signifying a cascade of processing (Amaral, 1993; Amaral and Witter, 1995), although there is also evidence of some feedback processing within the hippocampus (Penttonen et al., 1997). The first synaptic connections to enter the hippocampus arise from layer II of the entorhinal cortex, which sends highly processed sensory information through the perforant path to dentate gyrus. These axons also branch off collaterals to the CA3 region. The second synaptic connections come from the dentate gyrus via the mossy fibres to the CA3. Thus the information from the entorhinal cortex arrives to CA3 both monosynaptically and disynaptically. This information is further processed within CA3 through auto-association fibres, which connect the CA3 pyramidal cells with one another. The third connection in the trisynaptic circuit brings the information from the CA3 cells via the Schaffer collaterals to the CA1 cells. Interestingly, CA1 also receives the information from the entorhinal cortex twice, trisynaptically from CA3 and monosynaptically through a direct connection from layer III of the entorhinal cortex (Amaral, 1993; Amaral and Witter, 1995).CA1 projects its processed information to subiculum, where once again the entorhinal cortex has also sent its information. Finally, the information is returned from CA1 to the entorhinal cortex both monosynaptically through direct projections from CA1 and disynaptically through the subiculum. Fig.1 represents the major intrinsic connections of the hippocampal formation. These simultaneous projections appear to be a guiding principle of the hippocampal circuit, allowing the processed information to be compared with a form of the original information at every step

through parallel processing in addition to the classical serial cascade of processing (Amaral, 1993).

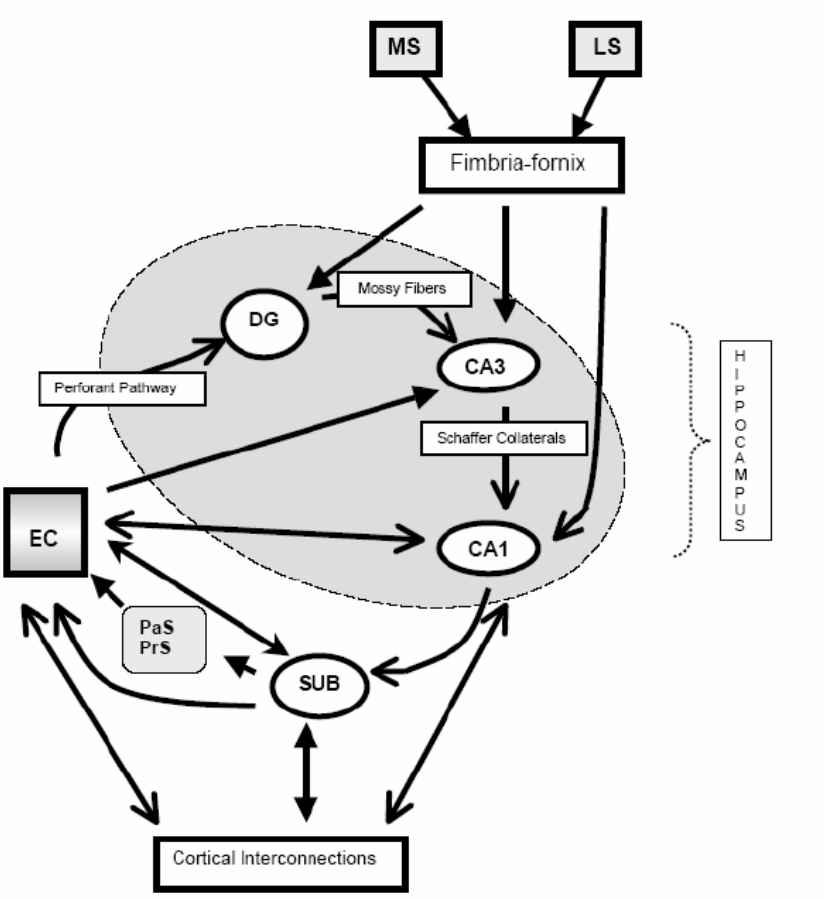


Fig.1.Schematic representation of major intrinsic connections of the mammalian hippocampal formation (adapted from Amaral and Witter, 1995). EC. Entorhinal cortex; DG, dentate gyrus; MS, medial septum; LS, lateral septum; CA1 and CA3, fields of Ammon`s horn; SUB; subiculum; PaS; parasubiculum; PrS: presubiculum.

1.2.2. Pyramidal CA1 neurons

Pyramidal neurons within the CA1 region of the hippocampus are large multipolar neurons with a triangular shaped cell body. The long axon of each pyramidal neuron typically emanates from the base of the soma and branches profusely, making excitatory glutamatergic synaptic contacts along its length. The dendritic tree of a pyramidal neuron has two distinct domains: the basal and apical dendrites, which descend from the base and the apex of the soma, respectively. All pyramidal neurons have several, relatively short basal dendrites. Usually one large apical dendrite connects the soma to a tuft of dendrites. This main apical dendrite bifurcates before giving rise to the tuft at a variable distance from the soma. In some cases the resulting dendrites each bifurcate again. Oblique apical dendrites emanate from the main apical dendrite at various angles.

The distinct morphologies of basal and apical dendrites suggest that inputs to these domains might be integrated differently. Furthermore, different dendritic domain receive distinct synaptic inputs, for instance, CA1 neurons receive input to the distal tuft from the entorhinal cortex through the perforant path and from the thalamus, whereas the remainder of the dendrites receive input from CA3 through the Schaffer collaterals. Furthermore, CA3 neurons that are distant from CA1 project primarily to apical dendrites, whereas CA3 neurons that are closer to CA1 project more heavily to basal dendrites. The functional significance of this arrangement remains mysterious. The CA1 pyramidal neuron receives cortical information by two distinct pathways. The direct input reaches CA1 directly through the perforant path and it conserves the specificity of the informational context. The other pathway (indirect input) connects pyramidal neurons via the dentate gyrus (DG) with the CA3 field and reaches the CA1 region via Schaffer collaterals (SC). The perforant path terminates on distal apical dendrites of CA1 cells, in the stratum lacunosum-moleculare, while schaffer

collateral input terminates in the stratum radiatum (Otmakhova et al., 2000). In addition to the excitatory glutamatergic synapse, the CA1 pyramidal neurons are richly innervated by different neuromodulatory systems. In particular, stratum lacunosum-moleculare appears to have a higher density of nicotinic, dopaminergic and alpha-adrenergic receptors (Swanson et al., 1987). The blockade of these receptors strongly inhibits plasticity in pyramidal neuron, for example the inhibition of dopaminergic D1/D5 receptors during induction prevents late-LTP in apical branches of hippocampal CA1 neurons (Frey et al., 1993). This indicates that the late-LTP in apical dendrites dependent on the synergistic activation of glutamatergic and other neuromodulatory inputs during induction i.e. LTP in apical dendrites are heterosynaptic in nature.

The hippocampal CA1 neuron is characterized by Ca2+ spikes that are originate largely in the dendrites where the local threshold may be lower (Wong et al., 1979; Benardo et al., 1982; Wong and Stewart, 1992; Andreasen and Lambert, 1995; Kamondi et al., 1998). The restriction of calcium spike initiation to the dendrites is likely the result of strong activation of potassium channels in the soma and proximal dendrites by sodium-dependent action potentials. Calcium spikes appear to detect specific spatial and temporal combinations of synaptic input and signal these events to the synaptic target of neuron through the generation of a distinctive burst of action potential out put (Lisman, 1997).

Calcium spikes may serve as a powerful regulator of synaptic plasticity, because they would likely mediate a substantial influx of calcium through voltage-gated calcium channels. Furthermore, the prolonged depolarizations mediated by calcium spikes would relieve the voltage-dependent block on NMDA receptors and induce additional calcium influx. Calcium spikes could thus serve as a robust cellular mechanism by which synaptic inputs conveying temporally correlated information

might be selectively reinforced. This mechanism would be expected to function effectively in distal dendritic regions in which the influence of back propagating action potentials is comparatively weak (Spruston et al., 1995; Kamondi et al., 1998).

1.3. Hippocampal synaptic plasticity

The hippocampus has been a major experimental system for the studies of synaptic plasticity in the context of putative information-storage mechanisms in the brain. Electrophysiological recordings and molecular imaging studies in animals as well as MRI imaging studies in humans provide correlative evidence that episodic or episodic-like learning and memory involves hippocampal activity (Berger et al., 1983; Duzel et al, 2008; Guzowski et al., 2004; Henke et al., 1997; Maguire., 2001). Its simple laminar pattern of neurons and neural pathways enables the use of extracellular recording techniques to record synaptic events for virtually unlimited periods in vivo (Andersen et al., 1969). Of all the properties of hippocampal synapses, the most important and well documented nature is their ability to respond to specific patterns of activation with long lasting increase or decrease in the synaptic efficacy. LTP, the much studied model of synaptic plasticity, was first identified in the hippocampus and has been extensively characterized using electrophysiological, molecular, biological and biochemical techniques (Bliss et al., 2007; Reymann and Frey, 2007). Recent studies have detected LTP-like changes in hippocampus following learning (Bear et al., 2006; Gruart et al., 2006). Other forms of activity-dependent plasticity have been found in hippocampus such as long-term depression (LTD), a persistant decrease in synaptic efficacy after a brief episode of low-frequency stimulation (Dunwiddie and Lynch, 1978), Spike-timing-dependent plasticity (plasticity in which pre and postsynaptic cells are stimulated independently and the timing with which spikes are evoked in the two types of cell determines the direction of plasticity) (STDP), depotentiaton (selective and time dependent reversal

of potentiated synapses by using low frequency stimulation) and de-depression (selective reversal of depression by high frequency stimulation) (Andersen et al., 1980; Abraham et al., 1985; Bear et al., 1992, 1993; Lynch et al., 1980; Madison et al., 2002; Kobayash et al., 2004).

Among the spectrum of experimental strategies used by neurobiologists to promote the understanding of brain function, the in vitro systems offer a number of opportunities. In in vitro studies a large number of well-defined independent variables can be readily introduced. The dependent variables are usually more accessible to measurement and can be monitored with a variety of techniques than in the case of in vivo. The interference from peripheral factors, which are more common in in vivo is greatly reduced (Lynch, 1980).

In our studies we are performing in vitro experiments with slices from hippocampus. The main reason is because it contains a considerable proportion of the major fibre projections and their attendant synaptic domains can be prepared. Most of the major intrinsic and extrinsic hippocampal fibre systems are organized according to a lamellar plan in which they travel at right angles to the longitudinal axis of the structure (Anderson et al., 1971, Blackstad et al., 1970).The hippocampal slice offers a variety of opportunities like visual control of electrode placement, possibility to direct electrodes to known parts of a given cell. For example 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 population of synapses. Furthermore in the slice preparation, the ability to change the concentration of interesting molecules at will provides a 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. Finally the transverse hippocampal slice enables

pharmacological agents to be rapidly washed in and washed off, allowing intracellular and patch-clamp recordings.

1.3.1. Properties of LTP

As a result of brief high frequency stimulation, the LTP expressed in CA3-CA1 synapse of hippocampal region show some basic properties such as 'input-specificity', 'co-operativity', 'associativity' and 'late-associativity' (Bear and Malenka, 1994; Bliss and Collingridge, 1993; Frey and Morris, 1997, 1998; Malenka and Bear, 2004). LTP is input-specific in general, which means those synapses who receive high frequency stimulation only will express LTP. This property of LTP is consistent with its involvement in memory formation. If the activation of one set of synapses leads to the simultaneous activation of all other synapses, even inactive ones, being potentiated, it would be difficult to activate selectively a particular sets of inputs, as is presumably required for learning and memory (Bliss and Collingridge, 1993). Another basic property of LTP is co-operativity, i.e. LTP can be induced either by strong tetanic stimulation of a single pathway, or cooperatively via a weaker stimulation of many of it, explained by the presence of a stimulus threshold that must be reached in order to induce LTP. Next property of LTP is 'associativity'. Here a weak stimulation of a pathway will not trigger LTP by itself. But, if one pathway is weakly activated and at the same time a neighbouring afferent on to the cell is strongly activated, both synapses undergo LTP. This selective enhancement of conjointly activated synaptic inputs is often considered as a cellular analog correlate of associative or classical conditioning. Otherways, associativity is expected in any network of neurons that links one set information with another. 'Late-associativity' is a novel property of LTP, which describes intersynaptic interventions within a time frame of few minutes to few hours (Frey and Morris, 1997, 1998a, 1998b). More clearly, a weak protein synthesis-independent early-LTP in one synaptic input can be

transformed into a late, protein synthesis-dependent form, if a protein synthesis-dependent late-LTP 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, 1998b; Frey , 2001 ; Frey and Frey, 2008; Kauderer and Kandel, 2000; Sajikumar and Frey, 2004a).

1.3.2. Distinct phases of LTP

Brief high-frequency stimulation (HFS) of CA3-CA1 synapses can result in LTP. This LTP can be divided into several temporal phases (Matthies et al., 1990; Frey and Frey, 2008). This synaptic potentiation uses different mechanisms for its induction, expression and maintenance. As shown in Fig.2 the initial induction of LTP results in an enhanced potential with duration of several seconds to minutes characterized by mainly presynaptic mechanisms and this phases is named as ‘posttetanic potentiation’ (PTP). PTP is followed by a ‘short-term potentiation’ (STP) with a duration of up to one hour, which is mainly maintained or carried by the postsynaptic activation of different kinases like CaMKII and tyrosine kinase (Dobrunz et al., Huang, 1998). STP is followed further by at least two phases, i.e. early-LTP and late-LTP (Frey et al., 1988; Frey et al., 2007; Huang et al 1998; Krug et al., 1984; Matthies et al., 1990). The early phase of LTP (early-LTP) is a transient form of LTP which lasts 4-6 h in the intact animal, while late-LTP lasts more than 6h or it can last even days or months (Abraham and Bear, 1996; Abraham 2003; Krug et al., 1984; Frey et al., 1988; Frey and Reymann 2007). A major difference between early-LTP and late-LTP is that early-LTP is protein synthesis-independent, while in contrast with early-LTP, late-LTP is protein synthesis dependent (Frey et al., 1988; Krug et al., 1984; Otani et al., 1989). The application of protein synthesis inhibitors at the time of induction of LTP resulted in a classical early-LTP, while the maintenance of late-LTP

was prevented (Otani and Abraham 1989; Frey et al., 1988, 1996; Krug et al., 1984; Mochida et al., 2001).

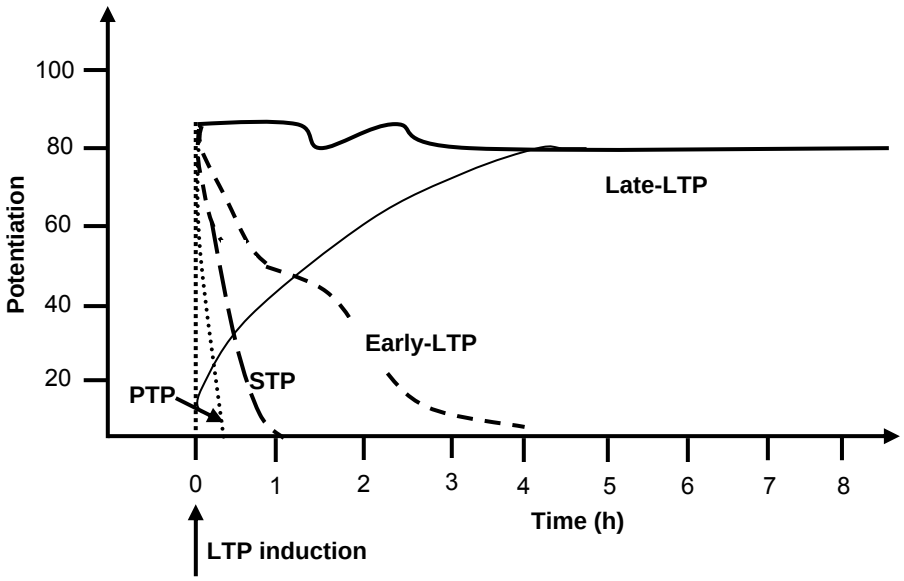


Fig. 2. The multiple phases of LTP such as post tetanic potentiation (PTP), short-term potentiation (STP), early-LTP and late-LTP (adapted from sajikumar, 2005) .

The different forms of LTP can be induced by distinct stimulus protocols in acute slices in vitro (Frey and Morris, 1997). A single, relatively weak high- frequency stimulus train of distinct stimulation strength can induce early-LTP, which is not sufficient to induce late-LTP. On the other hand, the induction of late-LTP requires repeated trains or stronger stimulus intensities of high-frequency stimulation. For example we can induce a protein synthesis- dependent late-LTP by repeated tetanization consisting of four spaced trains (Kandel et al., 1994) or by three spaced trains (Reymann et al., 1985; Frey et al., 1994) or even by a single tetanization consist of distinct stimulation strength (Sajikumar et al., 2008). Both the early and late form of LTP requires different cellular signalling mechanisms.

As mentioned above early-LTP is transient and protein synthesis-independent and is mainly maintained by second messenger systems activated by N-methyl-D-aspartate (NMDA) receptor-dependent Ca^{2+} influx, which activates kinases like CaMKII, PKC, tyrosine kinases (Malenka and Nicoll, 1999; Soderling and Derkach, 2000). Late-LTP starts gradually during the first 2-3 h and can last for 6-10 h in hippocampal slices in vitro and for days to even months *in vivo* (Abraham et al., 2002; Frey et al., 1988; Frey et al., 1995; Frey and Frey, 2008;; Kandel, 2001; Krug et al., 1989; Otani and Abraham, 1989).

LTP in the hippocampal CA1 region is dependent on N-methyl-D-aspartate (NMDA) receptor, which is a glutamate receptor subtype functioning in an activity dependent manner. NMDA receptor coupled channel is permeable to Ca^{2+} if activated, the critical trigger for the induction of LTP, and its permeability depends on both pre- and postsynaptic events. During the induction of LTP, the NMDA receptor must be activated by the neurotransmitter glutamate and sufficient depolarization of postsynaptic membrane occurs simultaneously. This relieves the magnesium block in the NMDA receptor-associated ion channel, which allow the entry of Ca^{2+} and Na^{+} in to the postsynaptic cell. The entry of Ca^{2+} activates a number of second-messenger processes. The depolarization of the postsynaptic cell is mainly mediated by the activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors, which are co-localized with NMDA receptors on postsynaptic sites and are also activated by the binding of presynaptically released glutamate. Because the NMDA receptors are sensitive to both presynaptic transmitter release and postsynaptic depolarization; they act as Hebbian coincidence detectors (Collingridge, 2003). NMDA-receptor-dependent LTP can be triggered experimentally either by delivering high-frequency trains to a critical number of presynaptic afferents, or by

pairing postsynaptic depolarization with pre-synaptic stimulation (Wigstrom and Gustafsson, 1986).

1.4 Requirements for maintaining synaptic plasticity in apical dendrites of CA1 neurons

1.4.1. Role of Dopamine during LTP in apical CA1 dendrites

Dopamine is a catecholamine neurotransmitter derived from the amino acid tyrosine. During dopamine synthesis tyrosine undergo a series of enzymatic reactions. First the tyrosine is converted into DOPA (dihydroxyphenylalanine) by doamine hydroxylase. Then the DOPA is converted into dopamine by DOPA decarboxylase. The hippocampus receives dopaminergic afferents from the ventral tegmental area (VTA) and substantia nigra of the midbrain neucleus (Nauta et al., 1978; Voorn et al., 1986; Descarries et al., 1996). It has been well established that the strength of the synaptic transmission can be modified on a long-term basis by specific patterns of activation such as high frequency trains that produce LTP and also by the action of endogenous modulators such as dopamine (Jay, 2003). Two types of dopamine receptors (D1 and D2) are identified on biochemical and pharmacological grounds, based on their ability to activate (D1) and inhibit (D2) adenylcyclase. But later studies with gene cloning revealed that five subgroups (D1-D5) of dopamine receptors while, D1 and D5 activate adenylcyclase but D2, D3 and D4 inhibit them.

LTP in hippocampal CA1 region is dependent on heterosynaptic requirements, in addition to the activation of glutamatergic synapses (Frey et al., 1988, 1990; Frey and Morris, 1998; Huang and Kandel 1995). Thus in in vitro hippocampal slice the induction of LTP at the CA1 region by the stimulation of Schaffer collaterals also stimulates other, neuromodulatory afferents. The apical dendrites of the hippocampal

neurons of CA1 region are innervated by dopaminergic fibres that course through the mesolimbic pathway (Baulac et al., 1986).

Dopamine plays a major role in learning as well as synaptic plasticity, especially in the hippocampal CA1 region (Wise, 2004). It has been shown that, the D1/D5 receptor subtype of dopaminergic receptors are majorly expressed in hippocampal CA1 pyramidal neurons (Ciliax et al., 2000). The D1/D5 receptor is positively coupled to adenyl cyclase which induces subsequent cAMP-dependent processes including activation of protein kinase A and the synthesis of plasticity-related proteins (PRPs) that are pre-requisites for late-LTP induction and its maintenance (Frey et al., 1993; Frey, 2001). The inhibition of dopamine receptors using the D1/D5 receptor antagonist SCH 23390 at the time of induction inhibits the maintenance of late-LTP in hippocampal slices (Frey et al., 1989a, 1990, 1991). Studies from other laboratories also showed that D1/D5 receptor activation is involved during LTP induction, especially in apical CA1 dendrites (Kandel et al., 1995; O'Carroll et al., 2006). In their findings they used the same D1/D5 receptor antagonists SCH 23390 and it depressed the expression of the late phase of LTP induced by three trains of tetanization. Some of the studies show that dopamine receptor activation in area CA1 initiates processes directly related to the synthesis of plasticity-related proteins and the coincident dopaminergic and glutamatergic activity is involved in the setting and stabilization of synaptic tag (Sajikumar and Frey, 2004).

Furthermore, a synergistic role of D1/D5 with NMDA-receptor function is essential for mediating the processes required for the maintenance of hippocampal CA1-LTP (Frey et al., 2003; Navakkode et al., 2007). All these data supports the fact that dopamine plays a major role in regulating synaptic plasticity in various regions of the hippocampus.

1.5. Protein synthesis during LTP and input specificity

As already mentioned LTP consist of distinct stages or phases like memory formation and requires protein synthesis for its long-lasting maintenance (Frey et al,. 1988; Krug et al., 1984; Matthies et al., 1990b; Reymann et al., 1988a, c). One question in protein synthesis-dependent LTP was whether the crucial molecular signals for initiating translation are strictly localized at dendrites or distributed between dendrites and cell bodies. Experiments using hippocampal slices have shown that pharmacological inhibitors of translation block long-lasting forms of LTP without affecting early stages of LTP expression (Frey et al., 1988; Tang et al., 2002). The intraventricular application of anisomycin, a reversible translation inhibitor, also prevents late-LTP maintenance in hippocampus *in vivo* (Krug et al., 1984; Otani and Abraham, 1989). The evidence for the involvement of somatic signaling cascades in long lasting LTP comes from the work of Frey et al., in 1989 in hippocampal CA1 slices. LTP that can be induced in dendritic stumps of CA1 pyramidal neurons, whose cell-body layer, the major site of transcription and translation, was surgically removed from the apical dendrites. The potentiation of the LTP induced, however, was prevented after 3-4 h. This time course was similar to that obtained in experiments with protein synthesis inhibitor, anisomycin. These results indicate that the mechanisms responsible for late-LTP are located, at least partially, in postsynaptic compartments and somatic factors are needed for NMDA-receptor dependent late-LTP in the CA1 region (Frey et al., 2007). But in contrast to somatic involvement during LTP, later studies has shown that high frequency stimulation of Schaffer collateral/commissural pathway in isolated CA1 dendrites maintained protein synthesis dependent late-LTP lasting >5h (Vickers et al., 2005). This LTP was NMDA-receptor dependent and was inhibited by translation inhibitors such as cycloheximide or rapamycin but not by transcription inhibitor actinomycin D (Vickers

et al., 2005; Sacktor et al., 2005).These findings also support the view of local dendritic translation of mRNAs is involved in the maintenance of protein synthesis-dependent late-LTP (Bradshaw et al., 2003, Cracco et al., 2005, Tsokas et al., 2005).

However, during the last few years, the question with respect to the locus of protein synthesis became more complicated. The local application of protein synthesis inhibitors in dendritic compartments revealed its contribution in protein synthesis, irrespective of somatic protein synthesis (Cracco et al., 2003). Although, there seems to be evidence that late-LTP depends on de-novo protein synthesis. Preliminary results revealed that in freely behaving animals a distinct level of PRPs are always present in neurons and this level of pre-existing PRPs will be rather fine tuned than newly synthesized, during plastic events by modulatory neurotransmitters and resulting in a long lasting change of synaptic efficacy (Frey et al., 2007).

It has been reported that the synthesis of mRNA is necessary for the establishment of mammalian long-term memory (Alkon et al., 1991; Matthies et al., 1974, 1990a). It could be possible that protein synthesis-dependent earlier stages of LTP (<6h) are carried by pre-existing mRNA and only a prolonged maintenance of LTP (>6h) requires a de novo synthesis of mRNA. Earlier studies have shown that mRNA inhibitors do not affect late-LTP (Otani et al., 1989). The failure of mRNA blockade to inhibit LTP may also be due to the methodological approach. Later works demonstrated that synaptic LTP in hippocampal slices was abolished after 3h when the RNA synthesis inhibitors were applied during tetanization (Nguyen et al., 1994). But a side effect of the drugs on mechanisms involved in LTP generation could not be excluded (Frey et al., 1996) Later investigations have shown that in intact animals as well as in slices, late-LTP can be maintained at least for the first 8 h under distinct circumstances, that means, with inhibited mRNA synthesis but an intact protein synthesis machinery (Sajikumar et al., 2005) but later stages may require additional

regulation of PRPs by gene expression (Frey and Frey., 2007; Sajikumar et al., 2007).

1.6. Synaptic tagging

Like memory, long-lasting forms of synaptic plasticity have been shown to require mRNA and protein synthesis, which takes place in the cell body or in the dendritic compartment respectively but not in synapses because in adult brain protein synthesis takes place extrasynaptically (Ostroff et al., 2002). Generally the long-lasting forms of plasticity can occur in a synapse-specific manner and any given neuron receives more than thousands of synaptic connections, each of them can be modified independently for longer period of time. Then, the mechanism behind the targeting of gene transcription and translation products to few activated synapses in a vast dendritic tree in the neuron has been not yet fully understood (Martin et al., 2002). To address this problem, in general there are considered to be four hypothesis about how the synapse specificity of late-LTP could be achieved (Frey and Morris, 1998a); the mail hypothesis; the local hypothesis; the sensitization hypothesis and the synaptic tag hypothesis. The mail hypothesis involves intracellular protein trafficking, in which the proteins will get a synaptic address during their synthesis to which they are targeted. The mail hypothesis is intrinsically unlikely because the proteins are required to travel from the soma to a specific synapse in the vast dendritic tree of the pyramidal cells, which might have more than 10,000 synapses (Frey and Morris, 1998a). The local synthesis hypothesis asserts that the relevant protein synthetic machinery is activated by the stimulation of nearby synapses. The local synthesis idea is supported by the presence of spine associated polyribosomes. The sensitization hypothesis describes the diffused distribution of plasticity related macromolecules to every synapse of the cell. These would have the effect of altering the threshold at which synaptic activation (or calcium influx) gives rise to lasting

synaptic changes. When few of these macromolecules are available, a high threshold prevails, and tetanization usually induces early LTP only, when many macromolecules are available, it is much easier for late LTP to be induced (Malinow et al., 2000). The sensitization hypothesis is supported by recent findings of a *de novo* protein synthesis dependent formation of protein kinase M Zeta (Ling et al., 2002; Hernandez et al., 2003; Muslimov et al., 2004). Synaptic tagging hypothesis has been put forward from a beautiful set of studies done by Frey and Morris in 1997. According to them the persistence of LTP is mediated by the intersection of two dissociable events, first the generation of a local, synapse specific tag due to synaptic activation and second it involves the production and diffuse distribution of plasticity-related proteins (PRPs) that are captured only by those synapses that have been **"**tagged**"** by previous synaptic activity.

The synaptic tagging hypothesis describes a mechanism; how input specificity is achieved during a protein synthesis-dependent stage (Frey and Morris, 1997, 1998a, 1998b; Martin and Kosik, 2002). In these experiments, it was possible to induce late-LTP in one pathway (S1) and the protein synthesis inhibitor, anisomycin was then bath applied just before the induction of late-LTP in an independent synaptic input S2. Normally, the protein synthesis inhibitor prevents late-LTP, but the LTP induced on S2 remained potentiated for up to 8 h post tetanus because the tag sets in synaptic input S2 could capture the PRPs synthesized in input S2 as a result of strong synaptic activation (Frey and Morris, 1997).

In addition to input-specificity, synaptic tagging is characterized by late-associative interactions in hippocampal slices. In follow-up experiments the authors showed that when stimuli that produce a protein synthesis-dependent, late-LTP were applied to one synaptic input (S1) to CA1 neurons in the Schaffer collateral pathway of the hippocampus, and then stimuli that normally would produce only early-LTP

were applied to a second input (S2), late-LTP was observed at both S1 and S2, as long as the stimuli to S1 and S2 were delivered within a discrete time window. Specifically, the weaker stimulus to S2 had to be delivered 1-2 h before, or less than 2.5-3 h after, the stronger stimulus was delivered to S1 (Frey and Morris, 1997, 1998; Frey and Frey, 2008). These results indicate that the weaker stimulus given to S2 created a synaptic tag that could “hijack” the products of gene expression, resulting in persistent synaptic strengthening at S2. Similar results showing synaptic capture, but at the single cell level, have been reported in Aplysia neurons in culture (Martin et al., 1997, 2002).

It has been reported that synaptic tagging also occurs during LTD with a similar time course as in LTP (Sajikumar and Frey, 2004a). The synaptic tagging hypothesis shows greater flexibility and more intracellular co-operativity than any of the other ideas and could help us to explain why inconsequent events, or events often remembered transiently, are better remembered when they occur temporally near to someone with a strong motivation/ emotional content (Frey and Morris, 1998a). Synaptic tagging has been reproduced by other laboratories and is now widely considered as a model for processes involved in the associative interaction of neurons in neuronal nets during memory formation (Frey and Morris, 1997, 1998a; Martin, 2002; Marin and Kosik, 2002; Sajikumar and Frey, 2004, Sajikumar et al., 2007).

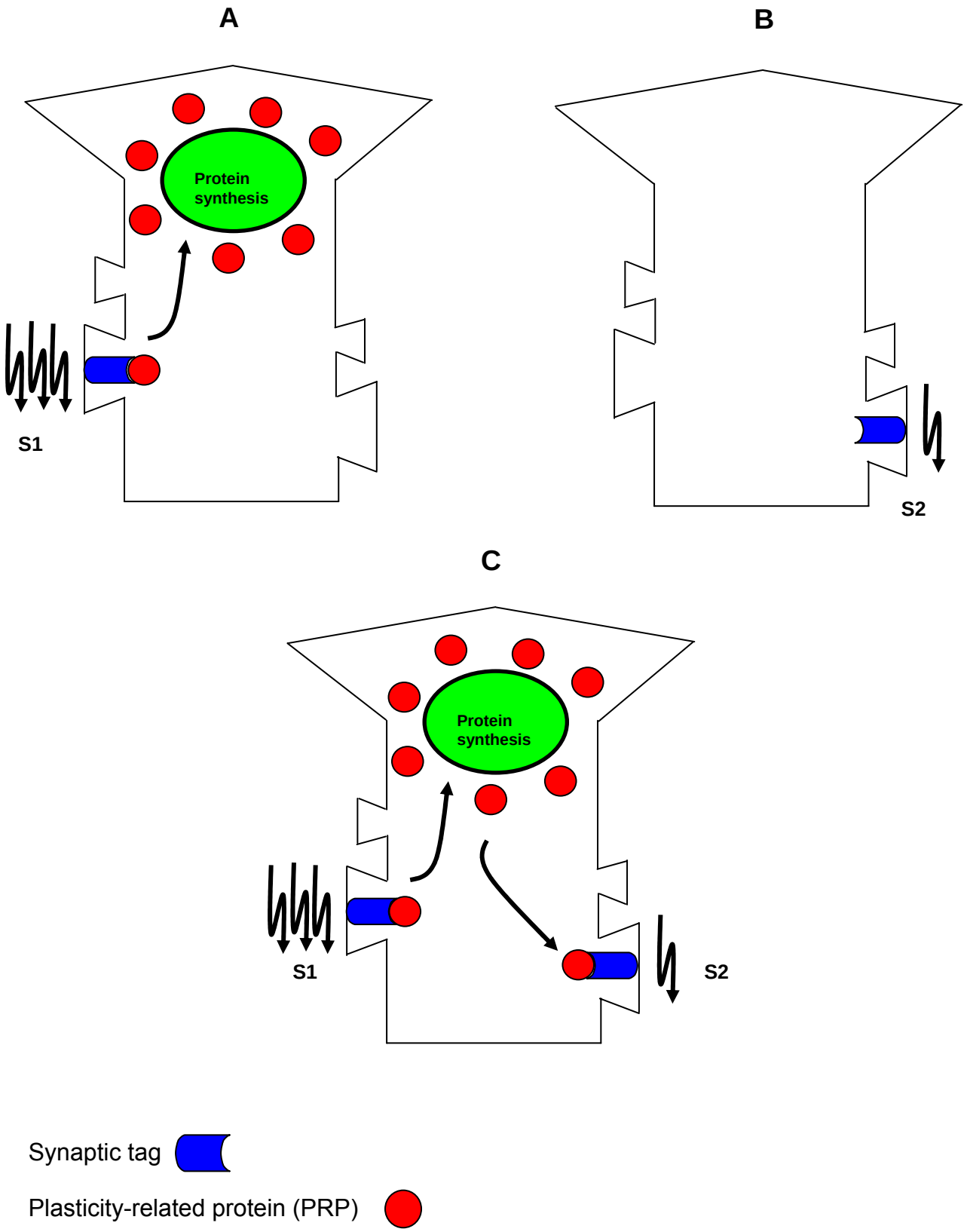


Fig.3: Schematic representation of synaptic tagging during LTP.

Figure A shows the schematic representation of late-LTP, induction of late-LTP leads to the setting of the tag molecules and synthesis of a pool of plasticity-related proteins (PRPs). Fig.B shows the schematic representation of early-LTP. Induction of early LTP leads to the setting of the tag only and there will not be any protein synthesis. Fig.C shows the schematic

representation of synaptic tagging during LTP. Here the early-LTP induced in synaptic input S2 can capture the PRPs from the late-LTP induced in S1 so the early form of LTP is reinforced in to a late form of LTP.

1.7. The actin network in synaptic plasticity

The actin cytoskeleton plays a major role in different cellular processes like cell motility, cell division, cell morphogenesis, intracellular trafficking of proteins etc. In the nervous system cytoskeleton plays an important part in axon and dendrite formation, which allows neurons to establish their exquisite and complex morphology (Dillon et al., 2005). The diversity of actin function is attributable to the dynamic turnover and remodeling of actin filaments, which are regulated by a group of proteins and signaling machinery (Dillon et al., 2005; Goda et al, 2008). Recent studies support that learning and memory involves the reorganization of neuronal cytoskeleton (Lynch et al., 2008; Khoutorsky et al., 2009; Yamaguchi et al., 2008).

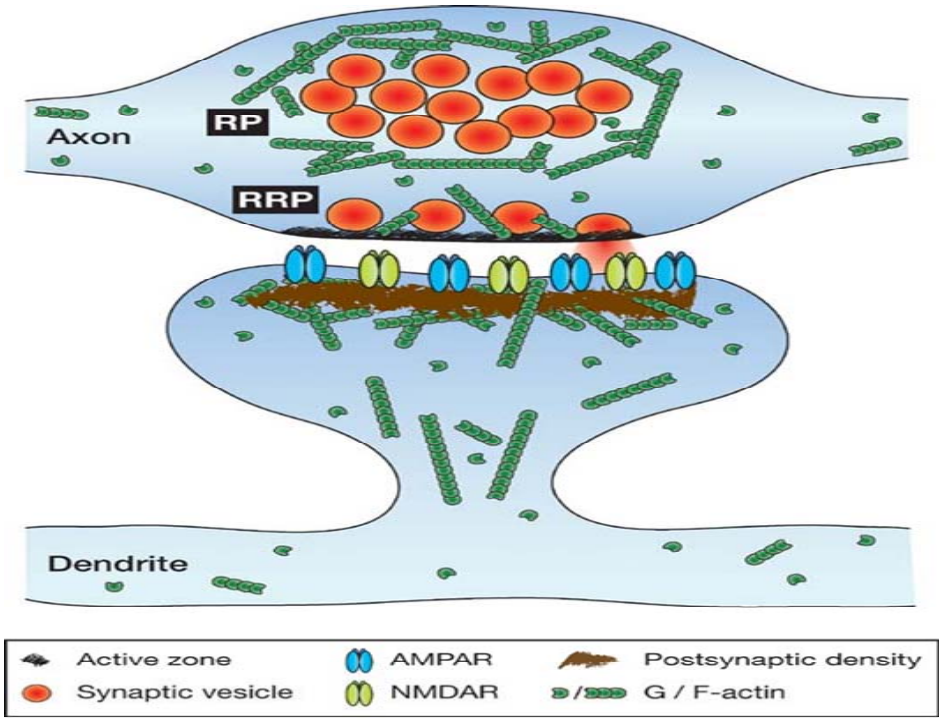
In cells, actin exists in two states, one is the monomeric G-actin and the asymmetric two-stranded helical filament (F-actin) composed of monomeric G-actin. The assembly and disassembly of F-actin can be rapid due to the weak non covalent interactions of G-actin. At steady state F-actin polymerize at the barbed end of the filament while G-actin monomers are lost at the pointed end. The difference in polymerization rates between the two ends results in a net turnover of the filaments. Some actin binding proteins (ABPs) can alter actin-filament dynamics whereas others can interlink F-actin into a variety of cytoskeletal networks (dos Remedios et al., 2003; Pollard et al., 2003; Revenu et al, 2004). Cellular signaling machineries exploit these properties to adapt and shape the synaptic cytoarchitecture in response to changes in synaptic activity (Goda et al, 2008).

The dendritic spines are small protrusions along the dendritic branch, which form the postsynaptic contact site for majority of excitatory synapse in the central

nervous system. Dendritic spines have attracted a lot of interest because it act as a potential mediator of plasticity that is believed to underlie learning and memory formation (Ecclas, 1979; Crick, 1982; Carlin and Siekevitz, 1983; Lisman and Harris, 1993). The spine exists in different form and the changes in spine shape are correlated with alterations in behaviour occurring in a variety of circumstances especially during learning (Ruiz Marcos and Valverde, 1969; Fifkova and Van Harreveld, 1977; Gould et al., 1990; Moser et al., 1994).

A typical dendritic spine contain an expanded head which is extremely rich in actin (Fifkova et al., 1982; Matus et al., 1982; Cohen et al, 1985) connected to the dendritic shaft through a narrow neck. Fig.4 shows the distribution of actin in dendritic spine. The changes in spine shape were actin based (Fukazawa et al., 2003; Poo et al., 2004) and if interfere with actin polymerization shows no major changes in spine number or morphology during learning paradigms.

Fig.4. Schematic model showing the distribution of the actin cytoskeleton in a prototypical glutamatergic synapse (adapted from Dillon and Goda, 2005)



It has been reported that the actin network plays an important role in hippocampal synaptic plasticity (Hayashi et al., 2000; Krucker et al., 2000; Lisman et al., 1999; Sacktor et al., 2007). Several studies have shown that structural changes occur in the hippocampus as a result of learning or LTP that are mediated by the actin dynamics. Actin plays a major role in the activity dependent forms of synaptic plasticity such as LTP and LTD. Long term plasticity is associated with a rapid and persistent reorganization of the spine actin cytoskeleton (Bozdagi et al., 2000; Engert and Bonhoeffer., 1999; Goda et al., 2008). As shown in Fig. 5 induction of LTP shifts the G-actin/F-actin ratio towards F-actin within 40 sec after tetanic stimulation and increases in spine volumes, but in LTD induction it shifts the ratio towards G-actin and results in spine shrinkage (Fukazawa et al., 2003; Hayashi et al., 2004; Lin et al., 2005; Okamoto et al., 2004). This reorganization of actin cytoskeleton is essential for the expression of synaptic plasticity, because actin-depolymerizing agents block both structural and functional pasticity (Fukazawa et al., 2003; Kasai et al., 2004; Krucker et al., 2000: Lisman et al., 1999). During induction of LTP, actin polymerization results in enlarged dendritic spines with a stable scaffold that has an increased capacity for anchoring structural and signaling molecules, such as neurotransmitter receptors (Craig et al., 1998; Krucker et al., 2000; Westbrook et al., 1993), Ca^{2+}/ Calmodulin-dependent protein kinase II (CaMKII) (Hayashi et al., 2004, 2007), and the immediate-early-gene product activity-regulated cytoskeleton-associated protein (Steward et al, 2007), which are required for stably enhancing the synaptic connection. Collectively, these findings establish the actin as a molecular regulator of bidirectional synaptic plasticity. Learning and memory are associated with long-term structural changes in the synaptic connections (Kleim et al., 2002; Lamprecht et al., 2004; Lendvai et al., 2000) and the actin dynamics play a central role in synapse

remodeling that contributes to learning and memory. A number of recent studies have tested the role of actin in behavioral learning paradigms. Intra-hippocampal injection

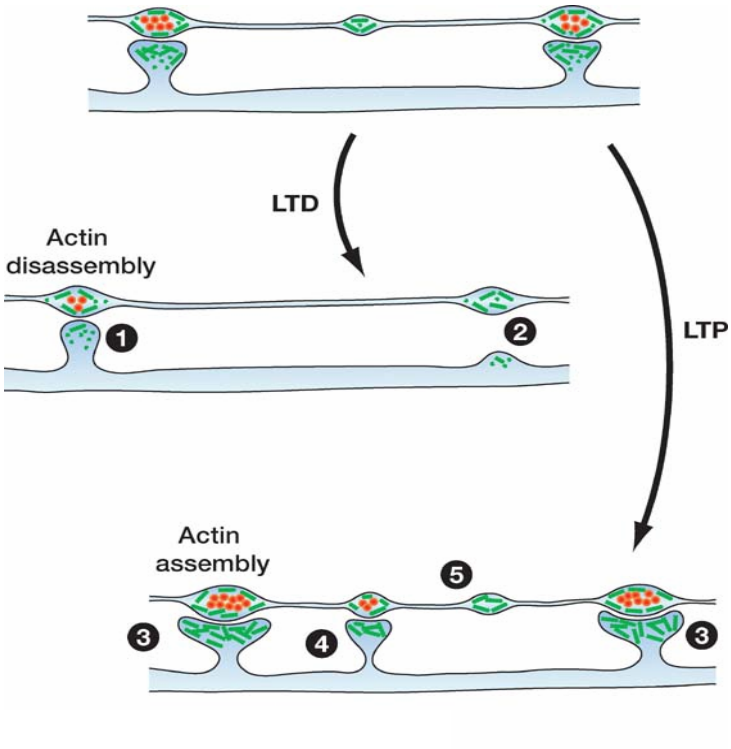


Fig.5.Model illustrating how actin remodeling mediates structural plasticity associated with LTD and LTP (adapted from Dillon and Goda, 2005). LTD induces a shift in the F/G-actin ration toward G-actin (1) and eventual regression of spines (2). LTP induction increases F-actin particularly dendritic spines and causes them to widen, which is a hall mark feature of stimulus-induced reversible change (3). An enlarged synapse subsequently splits to give rise to a new synapse (4). LTP induction also promotes the formation of new presynaptic actin puncta (5) which eventually become associated with an actively recycling vesicle pool and spines.

of actin assembly inhibitors such as latrunculin A/ cytochalasin D completely prevented both the acquisition and extinction of context-dependent fear responses for example, microinjection of cytochalasin D during posttraining impaired fear conditioning memory consolidation in lateral amygdale (Lamprecht et al., 2009). All

these studies demonstrate that actin rearrangements are required for the consolidation of memory at cellular level during learning process (Fischer et al., 2004).

1.8. Action of actin inhibitors on synaptic plasticity

Actin dynamics generally implies changes in length of actin filaments due to polymerization and depolymerization and is regulated by a wide range of proteins. During elongation or polymerization of actin filament G-actin in a complex with Mg2+-adenosine triphosphate (ATP) and an actin-binding protein profilin, which associates with the barbed end of the filament. The dissociation of profilin from the complex results in the hydrolysis of actin bound ATP in to adenosine diphosphate (ADP) by actins intrinsic ATPase activity. This is followed by the slow release of the resulting phosphate tends to destabilize the filament, which leads to the addition of actin monomer at the barbed end and dissociation at the pointed end. An actin filament will neither grow nor shrink unless the ends are first uncapped or the filament is severed (Fenteany et al., 2003).

Inhibitors that target actin have effects on cell shape, cell migration, cell division, endocytosis, exocytosis and other actin mediated processes in all kind of cells by affecting the function and organization of the actin cytoskeleton (Fenteany et al., 2003). It has been previously reported that the actin network plays an important role in hippocampal synaptic plasticity (Krucker et al., 2000; Lisman et al., 1999; Sacktor, 2007). The actin rearrangement is essential for extinction of hippocampal context-dependent fear (Fisher et al., 2004). The inhibitors that can primarily disrupt actin filament assembly by a variety of mechanisms and effectively destabilize the filament. So in this study we used structurally different actin polymerization inhibitors such as latrunculin A and cytochalasin D, both of these prevent synaptic plasticity in hippocamapal CA1 area through different mechanism. Generally these inhibitors acts

on actin network by shifting the equilibrium between monomeric G-actin and F-actin present in the cell (Fenteany et al., 2003).

Latrunculin A

Latrunculins are organic macrolides first isolated from a red sea sponge, *Latrunculia magnificans that* exudes a noxious, red fluid that kills fish within minutes (Spector, 1983, 1989). Fig.6 shows the chemical structure of latrunculin A. They have a simpler and more definable form of action on actin molecule. Latrunculin A is the most potent member of this family, which inhibits actin polymerization through binding G-actin molecule in a 1:1 complex. Latrunculin A will binds to actin in the cleft between subdomains 2 and 4, a site adjacent to the adenine nucleotide binding site. This binding reduces the rate of nucleotide exchange on actin to limit the flexibility of the cleft and trap nucleptide there by resulting in relative inhibition of nucleotide exchange (Ayscough et al., 1997; Bubb et al., 2000; Coue et al., 1980; Morton et al., 2000).

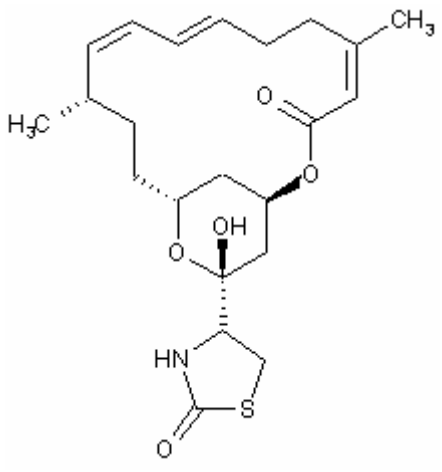


Fig.6. Structure of Latrunculin A

Cytochalasin D

Cytochalasins are the best-known actin targeted small molecules and been extensively studied elsewhere (Cooper,1987). The cytochalasins are naturally occurring organic compounds produced as a result of metabolism of a fungal species *Zygosporium mansonii,* which bind to actin and alter its polymerization. Among the members of this group cytochalasin D is the best studied one because of their greater selectivity for actin inhibition at the barbed end. Fig.7 shows the chemical structure of cytochalasin D. They have been widely used to study the role of actin in biological processes. Functionally, cytochalasins resemble capping proteins, which block an end of actin filament and block actin nucleation (Cooper et al., 1987).

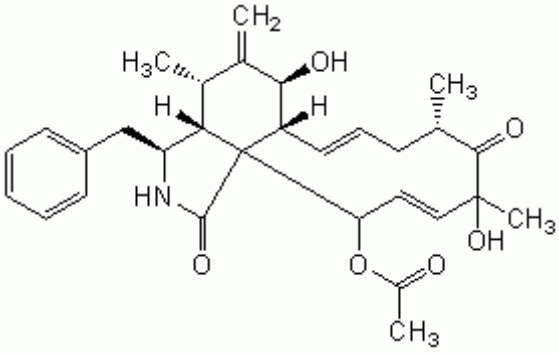


Fig.7. Structure of Cytochalasin D

Cytochalasins bind to the barbed end of actin filaments, which inhibits both the association and dissociation of subunits at that end. It has been shown that the cytochalasins may compete with cellular capping proteins for barbed ends and the barbed end of all actin filaments in cells must be capped, otherwise the ends will constantly undergo depolymerization (Kirschner, 1980). The competitive binding of cytochalasins resulted in the loss of binding of capping proteins to the barbed end, which specifies the location or function of the filament.

1.9. Aims of this dissertation

The main aim of my dissertation was to study the role of the actin network in long-term potentiation and its possible involvement in late associative processes such as synaptic tagging in hippocampal CA1 slices in vitro. In the first part of my dissertation I reproduced some basic experiments regarding late-LTP in apical CA1 dendrites. Then, I studied the role of the actin network during the induction of distinct phases of LTP such as an early and a late form. I used structurally different actin inhibitors at the time of the induction of LTP. In the further series of experiments I investigated whether the actin network inhibitors have any role in the maintenance phase of LTP.

The second part of my dissertation deals with the possible involvement of actin in synaptic tagging and therefore, I specifically investigated, whether the actin network interferes with tagging-processes. It has been proposed that a number of molecules act as synaptic tag or whether it mediates the setting of the tag in LTP. Here, I studied whether the actin network functions as a tag candidate or it mediating the setting of synaptic tag machinery during LTP. From my studies I could show that the actin network plays an important role in mediating the setting of tag.

2. Materials and Methods

2.1. Preparation of hippocampal slice and incubation

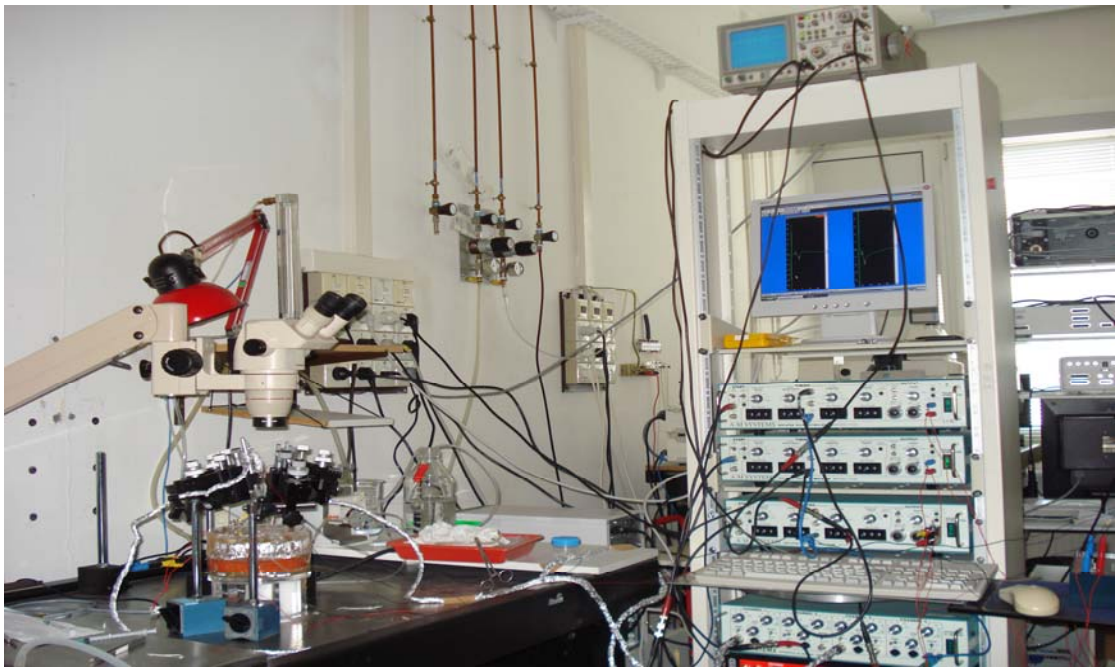
In all of our experiments we prepared the slices (400μm thick) from the right hippocampus of 7 weeks old male Wistar (strain: Schoenwalde) rats (total number of animals: 192). At first the animal was stunned by a blow behind the foramen magnum (cervical dislocation) and decapitated immediately (Frey et al., 1988; Frey and Morris, 1997; Sajikumar and Frey, 2003; Sajikumar et al., 2005). 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 the dura was removed before transferring the brain into cooled and carbogenated (carbogen: gas consisting of 95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) (temperature 4^0C) (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). Cooling the petridish and tissue slicer support on ice may help to reduce tissue deterioration. Brain is placed in a petridish on filter paper and the cerebellum and frontal cortex is dissected away. Then the remaining part of the brain is divided 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 a manuel chopper (Cambden, UK). The hippocampus was chopped into 400μm thick slices at 70^0 angle 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 glass vessel containing the cooled and carbogenated ACSF, and immediately transferred to the nylon net in the experimental chamber maintained at 32^0C by a wide mouthed pipette. One of the critical point of this stage is the removal of the brain and placing of slices in the incubation chamber that should be performed with in 3 min and preferably at a

temperature of 4^0C to minimize the cellular metabolism and avoid irreversible intracellular phase changes (Sajikumar et al., 2005). It is well known that ischemic or hypoxic conditions influence brain function with duration of more 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 (Djuricic et al., 1994; Djuricic et al., 1995; Erdogdu et al., 1993). Further more to obtain these physiological characteristics we use a new, cleaned razor blade for each preparation to get 3-4 slices from the right hippocampus of a single animal. When slices are prepared with proper care we can observe the stimulation responses similar to those seen in intact animals. Slices were incubated within an interface chamber at 32^0C (the carbogenated incubation medium contained 124mM NaCl, 4.9 mM KCl, 1.2 mM KH_2PO_4, 2.0 mM $MgSO_4$, 2.0mM $CaCl_2$, 24.6mM $NaHCO_3$, 10 mM D-glucose). The carbogen flow rate was 32l/h, which was maintained both in chamber and solution for oxygenation and buffering the pH and also prevents the drying out of the slices (Sajikumar et al., 2005).

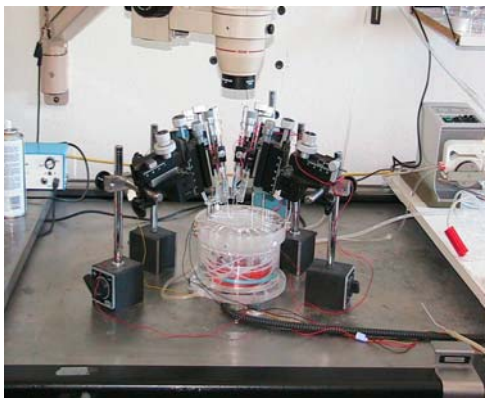
The hippocampal slices were incubated in an interface chamber for at least 4 h, which is an unusual protocol because of some specific reasons that are precious for maintaining a stable long term recording as well as for the study of long term functional plasticity up to 16 h. From our experiment it has been shown that more reliable and stable recordings were achieved if a preincubation period of 4 is allowed (Sajikumar et al., 2005).

After preparation, hippocampal slices in vitro are characterized by very low levels of spontaneous activity which may result from an almost ‘absolute rest’ during preincubation. Biochemical studies have shown that in slices the metabolic stability is reached after 2-4 h, which is maintained for at least 8 h of incubation (Whittingham et al., 1984).

(A)



(B)



(C)

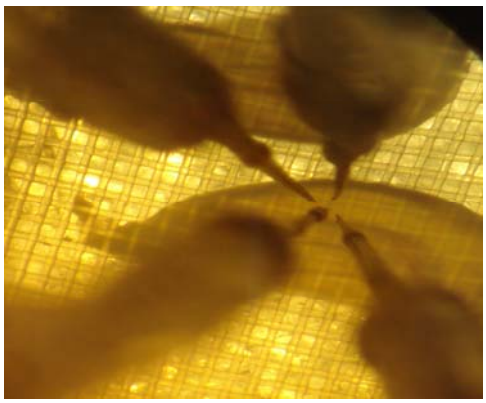


Fig. 8. Interface chamber and electrical set-up for long-term extra cellular recordings

(A) An overview of interface chamber and its electrical set-up

(B) Interface chamber with manipulators

(C) Microscopic view of hippocampal slice with electrodes located

This metabolic stability includes parameters for the activity of the enzymes, second messenger systems, pH and others (Ho et al., 2004; Whittingham et al., 1984) and these parameters will stabilize at very low concentration, if strong electrical stimulation is not delivered to the tissue. In addition to the process of acute slice preparation, low electrical activity may result in the delayed but prolonged metabolic stability at low level after 4 h, if no stimulation is applied to the tissue. This procedure may leads to a reduction in the amount of plasticity-related proteins (PRPs) near zero if the half life of the protein is considered as 2h (Frey and Morris, 1997, 1998; Sajikumar et al., 2004).Thus starting the functional experiments after 4-5 h of preincubation results in a low but comparable, basal metabolic and plasticity level in the slice preparation. Tetanization of slices activates the process from low level for example, from zero (a situation which never happens in behaving animals) which is mechanistically more useful to determine time constants during plastic events, than using freely behaving untreated animals. In intact animals the protein synthesis is blocked by pharmacological reversible inhibitors, which is similar as in slices revealing similar time constants for early form of potentiation in vitro. The reversible protein synthesis inhibitors, unfortunately reduce the synthesis of macromolecules in intact animals for several hours making this preparation nonavailable for studying the process of synaptic tagging in vivo (Frey and Morris, 1997). Thus experiments in hippocampal slices in vitro provide an ideal however, partially artificial model to study the processes of synaptic tagging and late-associativity. One of the major problem regarding the brain slice incubation are known since long time was most of the laboratories starts their experiments after a very short peincubation period, even less than one hour. Regarding the metabolic instability during low incubation period we maintained the slices at least 4 hours of preincubation to obtain comparable and more physiological results describing functional processes in vitro. This methodology

is supported by additional findings such as measuring the pattern of basal endogenous protein phosphorylation (Ho et al., 2004) and the translocation of different protein kinase C isoforms (α, β,γ) to the membrane as markers of their activation in tissue obtained from hippocampal slices in vitro or from intact animals. It has been shown that the slices that are incubated quite long as described here showed comparable pattern of phophorylation and enzyme translocation as detected in intact animals (Angenstein et al., 1997).

In addition to this several other external factors also influence the viability of the slice during long lasting experiments, such as temperature, p^H etc (Hsu et al., 2000; Masino et al., 2000; Masino et al., 2001). The minimal changes in pH and temperature can result in the induction of unintentional plastic events. It has been shown that temperature conditions are crucial for mammalian slice experiments in vitro and suggest that, ideally it should be done at near physiological temperatures. Recent studies have shown that sub physiological temperatures might dramatically affect functional plasticity in mammalian presynaptic terminal (Micheva et al., 2005).

After the preincubation period, the test stimulation strength was determined for each input to elicit a population spike of 40% of its maximal amplitude for control input and 25% for LTP-inducing input, which was determined by a slice specific input-output relationship. The baseline was recorded for a minimum period of 1 h before LTP 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 and then every 15 min up to 6 h (Sajikumar and Frey, 2004; Sajikumar et al; 2005).

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 our institution/local government

which can be provided upon request. All efforts were made to minimize the number of animals used and their suffering.

2.2. Field potential recording

Field potentials are extracellular potentials of a field generated by a group of nerve cells in response to synaptic or antidromic stimulation. So in our study we recorded the potential extracellularly from a population of neurons. In extracellular recording the potential difference is measured between two electrodes, one of which is placed with in the tissue of interest and the other which is outside the tissue and act as a 'reference electrode'. The voltage at the reference electrode is supposed to be zero. During neuronal 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´ produce a potential difference ´V´. The change of the potentials against time can be measured (Stevens, 1966; Rall and Shepherd, 1968; Nicolson and Freeman, 1975). The potential difference that can be recorded extracellularly due to the activity of a single 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 higher than in submerged chambers. Since field potentials are recorded from a population of neurons, changes can reflect not only in the amplitude of the responses in the individual cells but also the number of neurons involved and their synchronous activity (Richardson et al., 1987).

From hippocampus we can record mainly two types of field potentials: the field excitatory post synaptic potential (fEPSP) and the population spike (PS). At low stimulation intensities (below spike threshold), the fEPSP is a reflection of the individual EPSPs of the neurons as well as IPSPs due to feed forward inhibition. Above spike 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 the activity at excitatory synapses, although the amplitude of the fEPSP shows similar changes. However, the developing spike of above threshold stimulation can falsify the amplitude because of the interference of the spike-related dipole. The fEPSP measured from the dendritic area is considered as negative (sink), while the potential recorded from cell body layer is considered to be positive (source) (Andersen et al., 1966a; Bliss and Gardner-Medwin, 1971; Bliss and Richards, 1971). The PS which is usually measured 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 measured between the negative peak of the potential and the positive peak preceding it. Since the action potentials are "all-or-none" the population spike reflects the number of neurons involved and their synchronous firing activity (Andersen et al, 1969).

2.3. Experimental protocol

In all experiments, two monopolar lacquer- coated, electrolytically sharpened stain less steel electrodes (input resistance: 5MΩ; AM-Systems, USA) were positioned within the stratum radiatum of the CA1 region (as shown in Fig.8) for stimulating two separate independent synaptic inputs S1and S2.

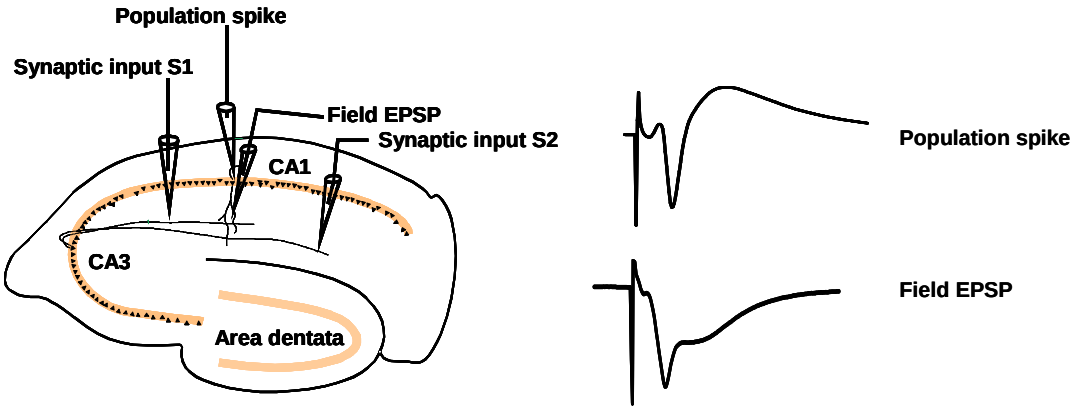


Figure.9.Transverse hippocampal slice showing the positioning of electrodes

Two independent synaptic input S1 and S2 to a single neuronal population and two recording electrodes in the dendritic and cell body layer to record field EPSP and population spike respectively and their analog traces are shown.

For recording, two electrodes (5MΩ; AM-Systems) were placed in the CA1 dendritic and cell body layer of a single neuronal population. Recorded potentials were amplified by a custom made amplifier (INH, Magdeburg, Germany). The analog signals were then digitized using a CED 1401 A/D converter and analyzed with custom-made software (PWIN, Magdeburg, Germany).

At first the preparation of slices, it takes three minutes. In the first minute the rat was killed by a blow to the back of the neck and the right hippocampus is removed. In the second and third minutes the hippocampus was cooled in carbogenated artificial cerebrospinal fluid (ACSF) at 4^0C, and three to four slices are cut and gently placed in the incubation chamber at 32^0C. After this the slices are incubated in an interface chamber at 32^0C for 4h. 30 min after the electrodes were gently positioned in the slice to filter the signal. We used electrolytically sharpened stainless steel electrode that are cleaned with alcohol and ACSF shortly before they

are gently and slowly positioned in the middle of the hippocampal slice (about 200µm from the surface) to reach the neuronal area that has been best preserved.

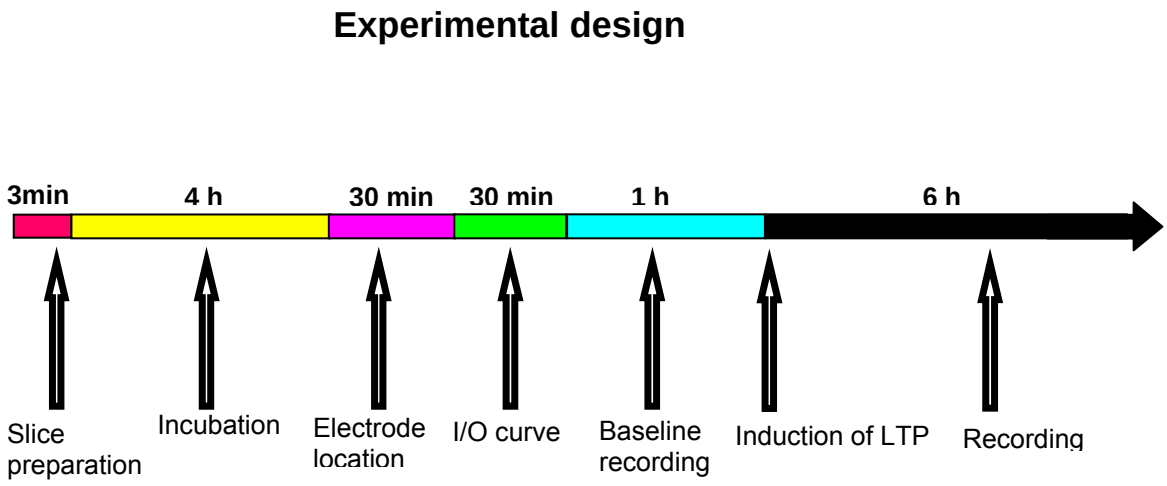


Fig.10. The schematic representation of the protocol for doing long-lasting functional plasticity experiments in hippocampal slice *in vitro* (adapted from Sajikumar et al., 2005)

We used biphasic constant current pulse for stimulation. The stimulation strength for the LTP inducing and control pathways was determined according to an input-output relationship and from this point the baseline recordings begins. The field potentials were amplified by a custom made amplifier. Then we recorded a stable base line for at least one hour and after this the LTP is induced. The potentials were recorded for the next 6 hour (see figure 10) (Sajikumar and Frey, 2004; Sajikumar et al; 2005).

2.4. Stimulation protocols: late-LTP and early-LTP

For inducing late-LTP we used three stimulus trains of 100 pulses (strong tetanus (STET): f=100 Hz, stimulus duration 0.2 ms per polarity with 10 min inter train-intervals) (Frey and Morris, 1997, 1998b). In experiments with induction of early-

LTP, a single tetanus with 21 pulses was used (weak tetanus (WTET): f=100 Hz, stimulus duration 0.2 ms per polarity, population spike threshold stimulus intensity for tetanization) (Frey and Morris, 1997, 1998b).

2.5. Pharmacology

For studying the role of the actin network in LTP and synaptic tagging we used Latrunculin A (Calbiochem, UK), dissolved in ACSF and 0.1% dimethyl sulfoxide (DMSO) to prevent actin polymerization, Latrunculin A was used at a concentration of 0.1μM (Kim and Lisman, 1999). A structurally different actin polymerization inhibitor, Cytochalasin D (Calbiochem, UK) was used at a concentration of 0.1μM (Krucker et al., 2000). Cytochalasin D was dissolved in ACSF and 0.1% DMSO. D-2-amino-5-phosophonopentanoic acid (AP-5; Sigma) was used at a concentration of 50 μM (dissolved in ACSF) to block the NMDA-receptor to study its role in LTP. Anisomycin (Sigma), a reversible protein synthesis-inhibitor, was used at a concentration of 25 μM (a concentration that blocked at least 85% of 3H-leucine incorporation in to hippocampal slices; (Frey et al., 1991a)). A structurally different irreversible protein synthesis inhibitor, emetine (Tocris) was used at a concentration of 20 μM (dissolved in ACSF) to study the late-LTP is dependent on protein synthesis. The selective dopaminergic D1/D5 receptor antagonist SCH-23390 was used at a concentration of 0.1 μM (Tocris; dissolved in ACSF) to study the dopaminergic requirement during LTP induction.

2.6. Statistics

In our experiments the datas were analysed by using nonparametric tests. The tests that do not make assumptions about the population distributions are referred to as nonparametric tests and include the Wilcoxon, Mann-Whitney test and Kruskal-Wallis tests. These tests are also called distribution-free tests. The nonparametric tests have some advantages (Siegel and Castellan; 1988) such as:

a. Nonparametric tests typically make fewer assumptions about the data and may be more relevant to a particular situation. In addition, the hypothesis tested by the non parametric tests may be more appropriate for the research investigation.

b. Nonparametric tests are available to analyze data which are inherently in ranks as well as data whose seemingly numerical scores have the strength of ranks.

c. Nonparametric statistical tests are typically much easier to learn and to apply. In addition their interpretation is more direct.

In experimental data analysis the average values of the population spike (in millivolts) and slope function of the field EPSP (millivolts / milli second) were calculated as percentage per time point were subjected to statistical analysis by the 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. Protein synthesis and NMDA-receptor-dependent late-LTP

Like long-term memory, late-LTP in hippocampal CA1 region shows distinct phases such as an early phase and a late phase dependent on protein synthesis independent or dependent respectively. Here we investigated whether the late-LTP in hippocampal slices in vitro is dependent on protein synthesis and NMDA-receptor activation during its induction. First , late-LTP was induced in a synaptic input S1 using a strong tetanization (STET) protocol which resulted in a stable, long lasting late-LTP which is maintained up to a duration of 6 h (Fig.11 A; filled circles). A control stimulation of an independent synaptic input S2, revealed that the potentials remain stable at baseline level throughout the experimental session (Fig.11. A; open circles). Then, we investigated whether the late-LTP is dependent on protein synthesis. Application of protein synthesis inhibitor anisomycin (25μM) 30 min before and until 30 after the strong tetanization (STET) of synaptic input S1 (Fig.11.B; filled circles) resulted in the prevention of late phase of LTP and converted the potentials to the baseline like a transient early form (early -LTP) (statistically significant difference is shown from 255 min when we compare the value between S1 and S2; $p>0.05$, U test). This same experiment was repeated using another protein synthesis inhibitor; emetine (20μM), that also prevented the late-LTP maintenance (Fig.11.C). These experimental results show that late-LTP maintenance in CA1 region was dependent on protein synthesis. The control input S2 was not influenced by the drug application (Fig.11.B&C; open circles). In the next experimental session we applied the NMDA-receptor antagonist AP5 (50μM) (D-2-Aimno-5-phosphonopentanoic acid) 30 min before and until 30 min after the induction of late-LTP in synaptic input S1 (Fig.11.D.filled circles). Here AP5 prevented the induction of LTP, at all suggesting that NMDA-receptor activation is an essential component for LTP induction. The

control input S2 (Fig.11.D; open circles) was not influenced by tetanization or drug application.

Then, we induced a transient form of LTP (early-LTP) by using a weak tetanization protocol (WTET) in synaptic input S1 (Fig.11.E; filled circles). The potentials decayed to the baseline after 3h of induction (early-LTP potentials are statistically significant up to 255 min when compared with its own base line (Fig.11.E; open circles) U test $p > 0.05$) and statistically significant up to 215 min when compared with the baseline prior to weak tetanization, Wilcoxon test, $p<0.05$).

In general, protein synthesis- and NMDA-receptor activation-dependant late-LTP and a transient, protein synthesis-independent early form of LTP can be induced in the stratum radiatum of hippocampal slices in vitro by using different stimulation protocols.

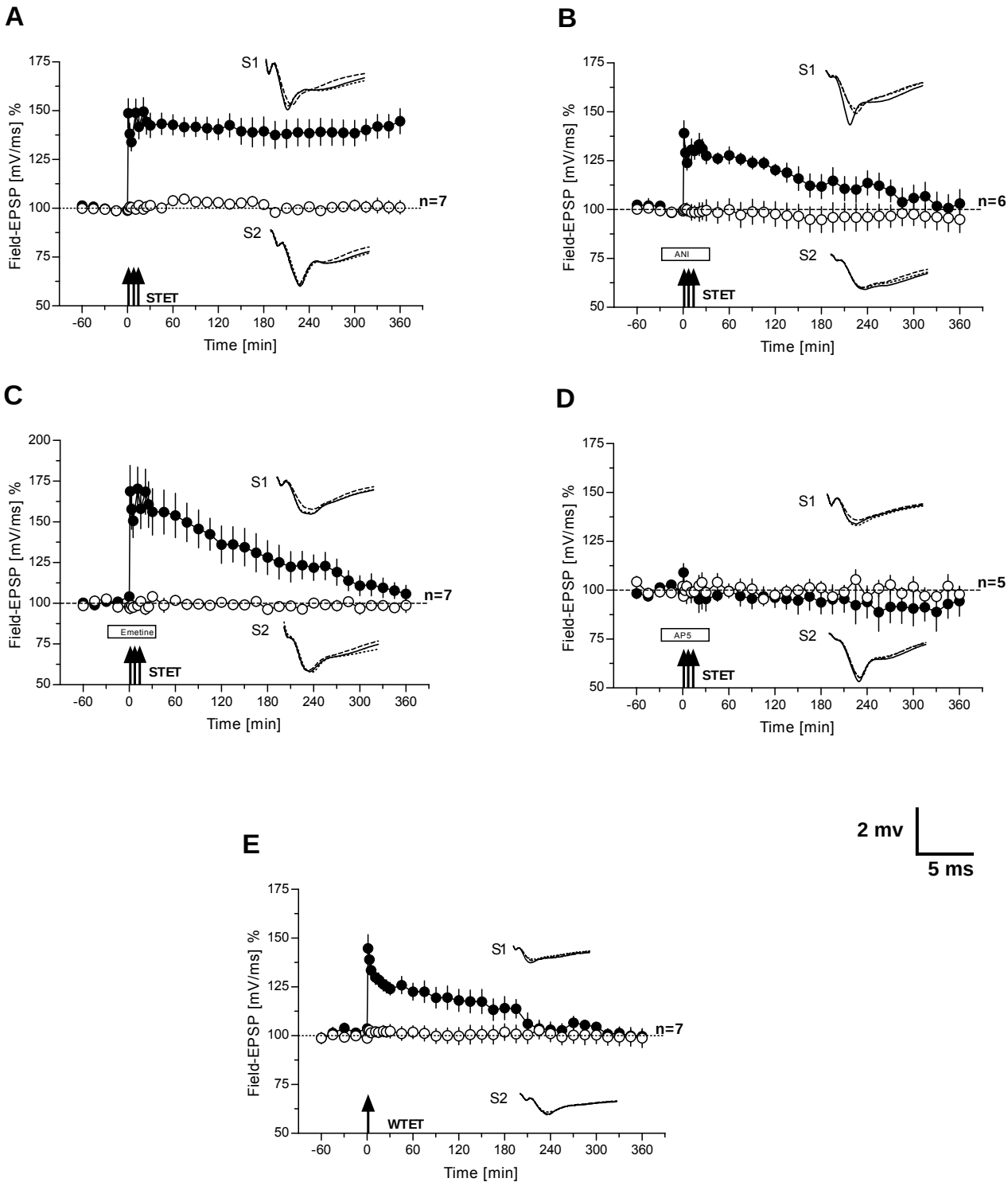


Fig.11. Protein synthesis and NMDA-receptor-dependent long-term potentiation (LTP) (time courses of field-EPSP recordings)

A) Induction of late-LTP in S1 (filled circles) by using a strong tetanization protocol (STET) resulted in a statistically significant ($p<0.05$) form of LTP through out the experiment (6 h). The control potential remained stable (open circles). (B and C). The application of protein synthesis inhibitors anisomycin (25μM) or emetine (20μM), 30 min before and until 30 min after STET in S1 (filled circles) resulted in a

transient early-LTP. The control input S2 (open circles) was not influenced by the drug application. Statistically significant difference between the potentials of S1 and S2 was detected for the first 210 min and 315 min after the application of anisomycin and emetine respectively. (D) Application of NMDA-receptor antagonist AP-5 30 min before and until 30 min after STET in S1 (filled circles) prevented induction of late-LTP. The control potential in S2 (open circles) remained stable through out the experiment. E) A weak tetanization protocol (WTET) was used to induce a transient early-LTP in input (S1) with a statistically significant duration of 215-230 min when compared with its own baseline before tetanization (Wilcoxon test, $p<0.05$).

Analog traces represent typical field-EPSPs 30 min before the normal time point of tetanization (dashed line); 30 min (solid line) and 6 h (dotted line) after tetanization of input S1. The analog traces for S2 recorded at the same time points however, without tetanization. Calibration bar for all analog traces: 2 mv / 5 ms. Arrows indicate the time point of tetanization of synaptic input S1 and three arrows represent strong tetanization (STET) to induce late-LTP while, single arrow represent weak tetanization (WTET) to induce early-LTP. Filled boxes represent drug application.

3.2. Dopaminergic requirements during LTP in apical CA1 dendrites

High frequency stimulation (HFS) of afferents within the field of the hippocampus also activates different modulatory inputs other than the glutamatergic input. So, here we investigated whether the induction of late-LTP in apical dendrites of CA1 area required the heterosynaptic activation of glutamatergic and dopaminergic D1/D5-receptors. To investigate that, we used a D1/D5-receptor-specific antagonist SCH23390 (0.1μM) which was applied 30 min before and until 30min after the induction of late-LTP in synaptic input S1 (Fig.12. C; filled circles). Induction of late-LTP in presence of SCH23390 resulted in the decay of potentials towards the baseline within 6h of the experimental session. The control input S2 (Fig.12.C; open circles) remained stable at the baseline and was not affected by drug application. The normal late-LTP and early-LTP has been replicated here as control in Fig.12A and B to distinguish the effect of drug application during LTP induction (Fig.12C). The result

shows that activation of dopaminergic D1/D5 receptors are directly involved maintaining the late-LTP in apical dendrites of hippocampal CA1 pyramidal neurons.

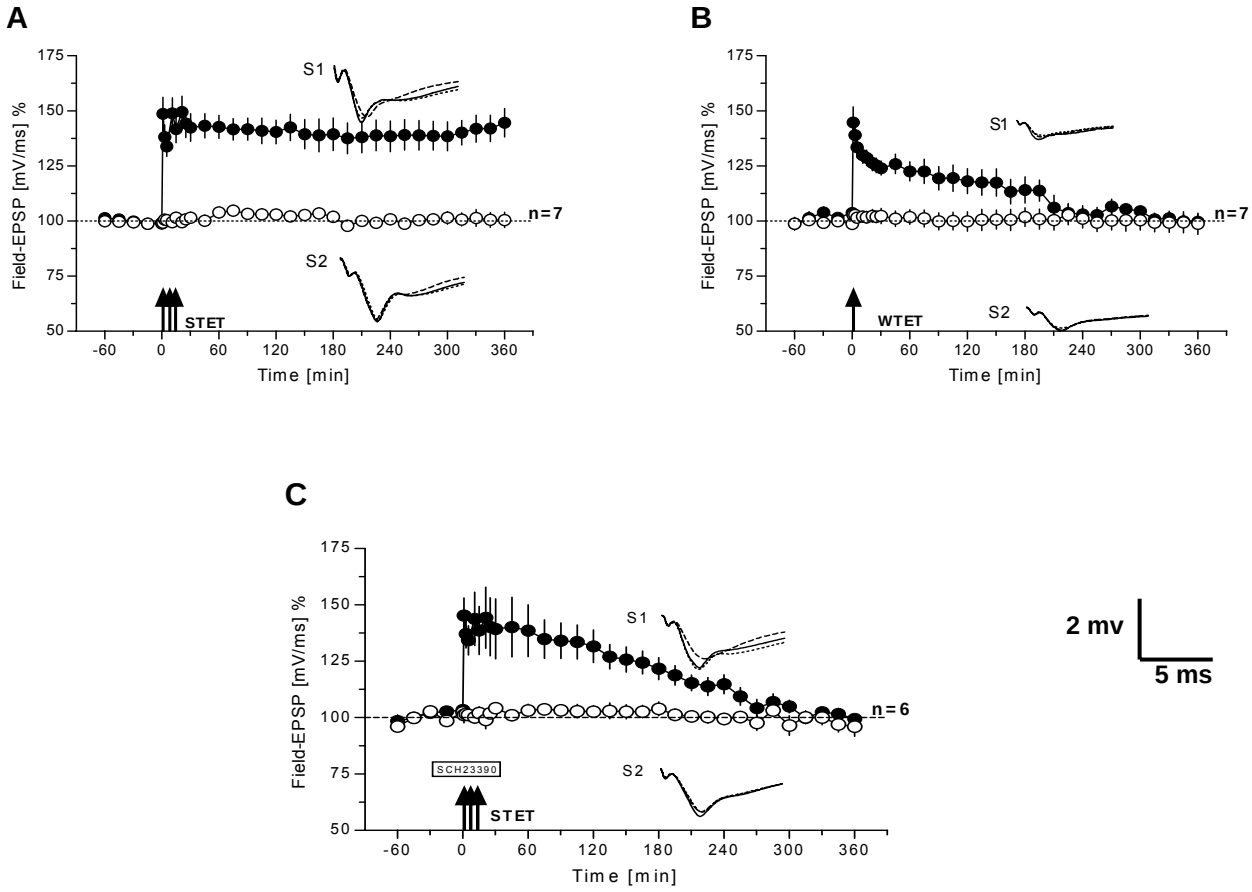


Fig.12. Dopaminergic requirement during long-term potentiation (late-LTP) (time courses of field-EPSP recordings)

The time course of the slope of field EPSP after induction of late-LTP in S1 by strong tetanization (STET) (filled circles). Open circles represents control pathway in synaptic input S2 (n=7). B) Time course of the slope of Field-EPSEP after induction of early-LTP by weak tetanization (WTET) in S1 (filled circles). The control pathway S2 (open circles) remained stable throughout the experiment (n=7). C) Induction of late-LTP in the presence of dopamine D1/D5 receptor antagonist SCH23390 (0.1μM). The SCH 23390 was applied 30 min before the stimulation of S1 (filled circles) with a strong tetanization (STET) protocol (wash out of the drug, 30 min after the first tetanization; open circles control input, n= 6). Analog traces represent typical field-EPSPs 30 min before the normal time point of tetanization (dashed line); 30 min (solid line) and 6 h (dotted line) after tetanization of input S1. The analog traces for S2 recorded at the same time points however, without tetanization. Calibration bar for all analog traces: 2 mv / 5 ms. Arrows indicate the time point of tetanization of synaptic input S1

and three arrows represent strong tetanization (STET) to induce late-LTP while, single arrow represent weak tetanization (WTET) to induce early-LTP. Filled boxes represent drug application.

3.3. Synaptic tagging during LTP

Initially, I had shown that late-LTP in hippocampal CA1 region is dependent on NMDA-receptor-activation and protein synthesis. Now I investigated whether synaptic tagging, the late-associative property of LTP (Frey and Morris) can be reproduced, as a pre-requisite for subsequent studies.

To investigate `synaptic tagging´ during LTP, we used a protocol of “strong before weak” that means we induced a late form of LTP in synaptic input S1 (Fig.13. C, filled circles), which resulted in a statistically significant form of LTP up to 6 h (Wilcoxon test, $p>0.05$). 30 min after the induction of late-LTP in input S1 we induced an early form of LTP in synaptic input S2 (Fig.13.C. open circles). Normally the early form of LTP decays to the baseline within 2-3 h (Fig.13.B) but here it was reinforced into a statistically significant form with duration of up to 6 h (Wilcoxon test, $p> 0.05$, when compared with its own baseline before Tetanization). This result suggests that the induction of late-LTP in S1 initiated protein synthesis which will synthesize a pool of plasticity-related proteins that are available for the establishment of late-LTP in S2. The normal late-LTP and early-LTP has been replicated here as control in Fig.13A and B to show the tagging interaction during LTP induction.

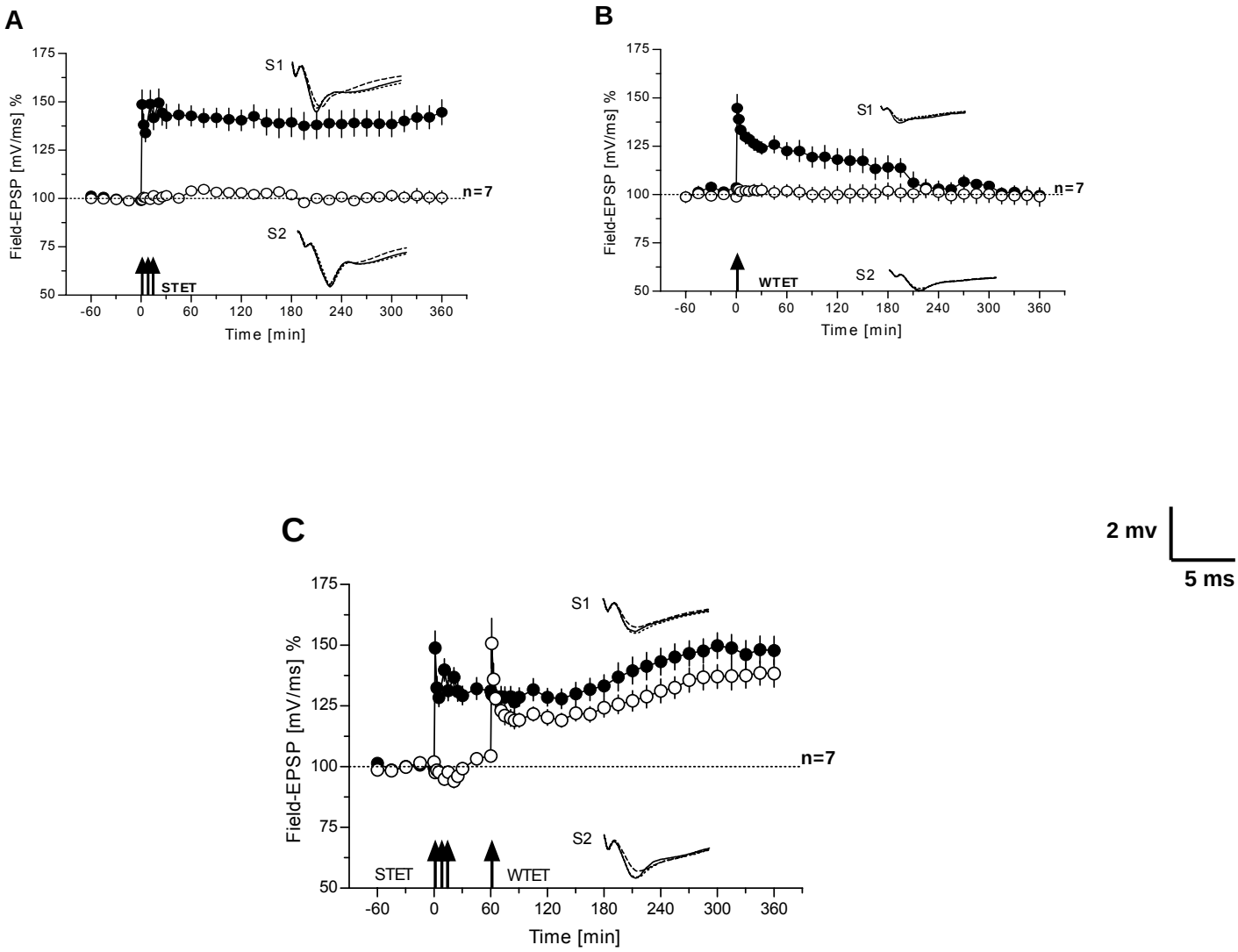


Fig.13. Synaptic tagging during long-term potentiation, i.e. late-LTP (time courses of field-EPSP recordings

A) The time course of the slope of field EPSP after induction of late-LTP by STET in S1 (filled circles). Open circles represents control pathway in synaptic input, S2 (n=8). B) Time course of the slope of field EPSP after the induction of early-LTP by WTET in S1 (filled circles). The control pathway S2 (open circles) remained stable through out the experiment (n=7). C) Synaptic tagging. Strong tetanization (STET) of input S1 (Fig.12.C: filled circles) resulted in late-LTP and was statistically significant through out the experiment (6h). 30 min after an early-LTP was induced by WTET in S2

(Fig.13.C: open circles). Normally the early form will decays to the baseline with in 2-3 h, but here the tag sets in the second synaptic input capture the proteins synthesized in S1 so the early-LTP is reinforced in to a late-form (n=7). Analog traces represent typical field-EPSPs 30 min before the normal time point of tetanization (dashed line); 30 min (solid line) and 6 h (dotted line) after tetanization of input S1. The analog traces for S2 recorded at the same time points. Calibration bar for all analog traces: 2 mv / 5 ms. Arrows indicate the time point of tetanization of corresponding synaptic inputs and three arrows represent strong tetanization (STET) to induce late-LTP while, single arrow represent weak tetanization (WTET) to induce early-LTP

3.4. Actin network and LTP

Role of the actin network in early decremental forms of long-term potentiation was reported previously (Lisman et al., 1999; Krucker et al., 2000; Sacktor et al., 2007). So in my studies we first reproduced these data under our experimental conditions and investigated actins involvement in distinct phases of LTP, before investigating its role in the late associative property of LTP, i.e. during synaptic tagging.

Intially we were interested to investigate the role of actin network for the long lasting maintenance of late-LTP. To study the effect of actin inhibitors on late form of LTP we replicated a late form of LTP to show as a control to distinguish from the effect of the drug application during the induction of late-LTP. The late-LTP was induced by a strong tetanization (STET) to synaptic input S1 (Fig.14 A, filled circles) resulted in a long lasting form of LTP which is statistically significant with a duration of at least 6h, when compared with the values of control baseline in an independent pathway S2 (Fig.14 A, open circles) or its own baseline values before TET (Wilcoxon, $p<0.05$). The potentials in the control input S2, without strong tetanization (STET), remained stable at baseline values for the entire experimental session (Fig.14.A, open circles). In the following set of experiments (Fig.14.B) we were investigated the effect of actin polymerization inhibitor latrunculin A on the maintenance of late-LTP. So we have applied the drug during the induction of late-LTP. As shown in figure 14B

the application of latrunculin A (0.1μM) 30 min before and 30 min after the induction of LTP in input S1 (filled circles) non-significantly affected the level of potentiation immediately after tetanization and prevented the expression of late form of LTP. The potentiation was statistically significant different from potentials of the control pathway until 4h and 15 min (U-test, $p<0.05$). Control responses obtained from S2 (Fig.14.B, open circles) remained relatively stable at baseline values. To confirm this result we used another structurally different actin inhibitor, cytochalasin D. The application of cytochalasin D (0.1μM) during late-LTP induction in input S1 (Fig.14.C, filled circles) again non-significantly affected the level of the initial potentiation and also prevented the late form of LTP. The potential values were reached to the baseline after 2h and 45 min (U-test). Control responses in S2 (Fig.14.C, open circles) again remained relatively stable at baseline levels. Here the actin inhibitors can use different mechanisms to block the maintenance of long lasting LTP. They can either inhibit the the synthesis of plasticity-related proteins (PRPs) or its translocation in to the activated synapses during LTP induction or it can disrupt the setting of the tag, which will be discussed more in later sections.

Subsequently, we were interested to investigate whether the actin network has any role in maintaining the transient form of early-LTP. The Induction of early-LTP in synaptic input S1 by a weak tetanization (WTET) (Fig.15.A, filled circles) resulted in a transient form of LTP which was statistically significant different from control values measured in input S2 (open circles in Fig.15.A) for up to 90 min (U-test, $p<0.05$) or for 2h when compared to its own pre-tetanization levels (Wilcoxon, $p<0.05$). The control input S2 without tetanization remained stable at baseline levels for the entire experimental session (Fig.15.A, open circles). Then we were interested to investigate whether the early maintenance of LTP dependent on actin polymerization. The induction of early LTP in synaptic input S1 (Fig.15.B, S1 filled circles). In

presence of latrunculin A affected the immediate maintenance of early-LTP when compared to untreated early-LTP (Fig.15.A, filled circles) 60 min after tetanization suggesting that the actin network is partially involved in maintaining the transient level of potentiation, during early-LTP (U-test, $p<0.05$). The control input S2, remained relatively stable at baseline values for the entire experimental session (Fig.15.B, open circles). Later to confirm this result we replicated the same experiment with cytochalasin D. The application of cytochalasin D (0.1μM) during early-LTP induction in input S1 (Fig.15C, filled circles) affected the level of the initial potentiation and also prevented the early form of LTP. The potentiation was statistically significant different from potentials of the control pathway until 25 min (U-test, $p<0.05$). Control responses in input S2 (Fig.15.C open circles) remained relatively stable at baseline levels.

Finally we were interested to know whether the action of actin inhibitors has a specific time window to disrupt the late-LTP consolidation. The application of latrunculin A 30 min after strong tetanization (STET) in input S1 (Fig.15.D, filled circles) for a duration of 1h couldn't showed any effect on the maintenance of LTP. The control input (open circles, Fig.15.D) remained relatively stable at baseline values, although a small statistically significant but transient "wash-in" effect was seen from 45-120 min after STET (Wilcoxon, $p<0.05$). Then we applied the structurally different actin inhibitor cytochalasin D 30 min after STET instead of latrunculin A (Fig.15.E), in this case also the late-LTP was not prevented in S1, although a statistically non-significant reduction of late-LTP after the application of cytochalasin D was observed (filled circles) and a similar transient "wash-in" effect was observed in input S2 (Statistically different form 45-75 min after STET, Wilcoxon, $p<0.05$). This result shows that the actin inhibitor has a distinct time window to inhibit the actin polymerization during LTP and if we apply after late-LTP-induction the

maintenance of late-LTP was not influenced anymore. The normal late-LTP and early-LTP has been replicated here as control in Fig.14A and 15A to distinguish the effect of drug application during LTP induction.

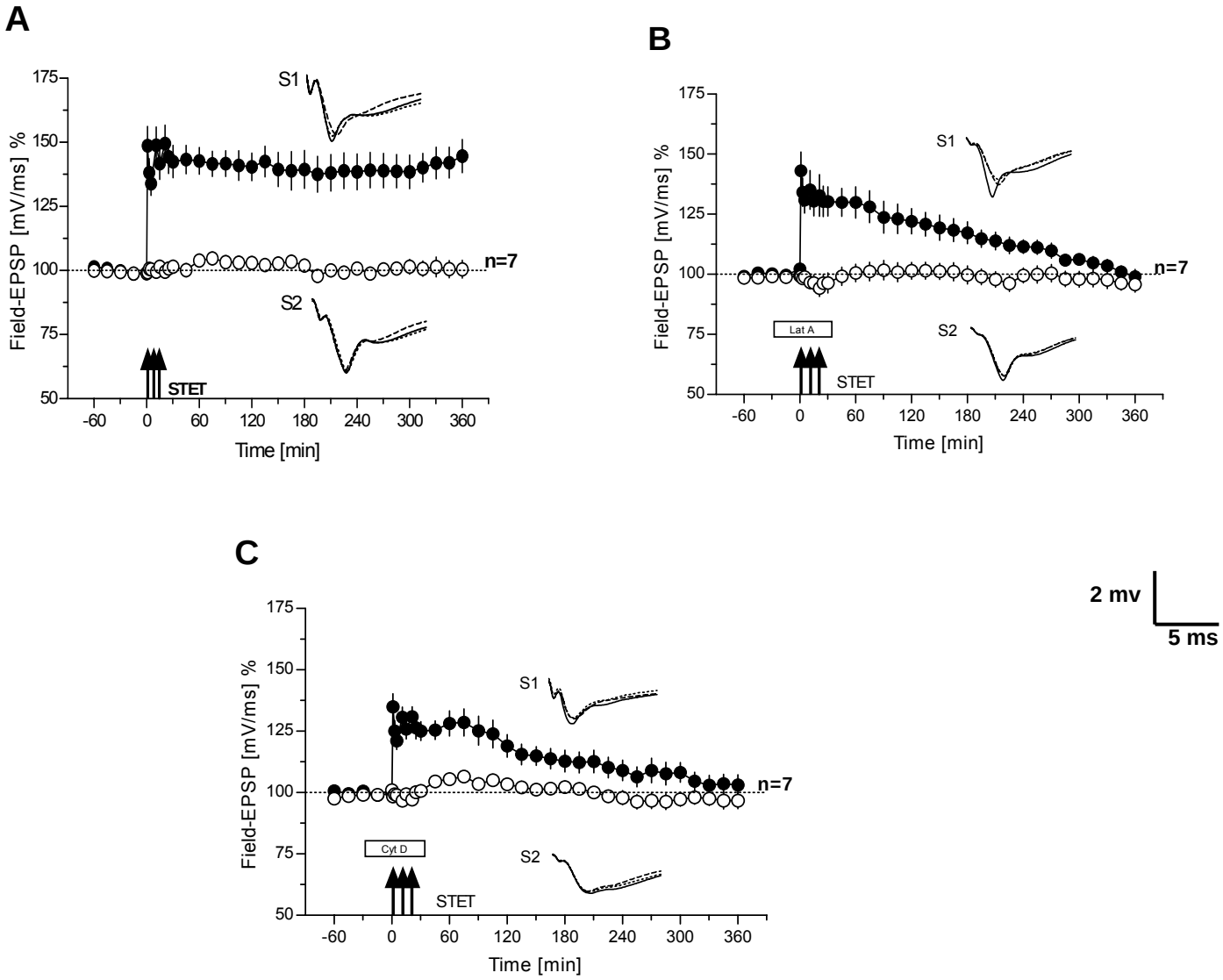


Fig.14. Role of the actin network in late long-term potentiation (late-LTP) (time course of field-EPSP recording)

A) The time course of the slope of the Field-EPSP after induction of late-LTP in S1 by STET (filled circles) is shown. Open circles represent recordings from the control synaptic pathway S2 (n=7). B) Induction of late-LTP in the presence of actin polymerization inhibitor latrunculin A. Latrunculin A was

applied 30 min before STET of S1 (filled circles; wash out of the drug: 30 min after the first tetanization; open circles control input, n=7). C) Induction of late-LTP in synaptic input S1 (filled circles) in the presence of actin polymerization inhibitor cytochalasin D (n=7). Cytochalasin D was applied 30 min before STET and it was washed out 30 min after the first tetanization (open circles: control input S2). Analog traces represent typical Field-EPSPs 30 min before the normal time point of tetanization (dashed line); 30 min (solid line) and 6 h (dotted line) after tetanization of input S1. Analog traces for S2 recorded at the same time points however, without tetanization. Calibration bar for all analog traces: 2 mv / 5 ms. Arrows indicate the time point of tetanization of synaptic input S1 and three arrows represent strong tetanization (STET) to induce late-LTP. Filled boxes represent drug application, Lat A: latrunculin A, Cyt D: cytochalasin D.

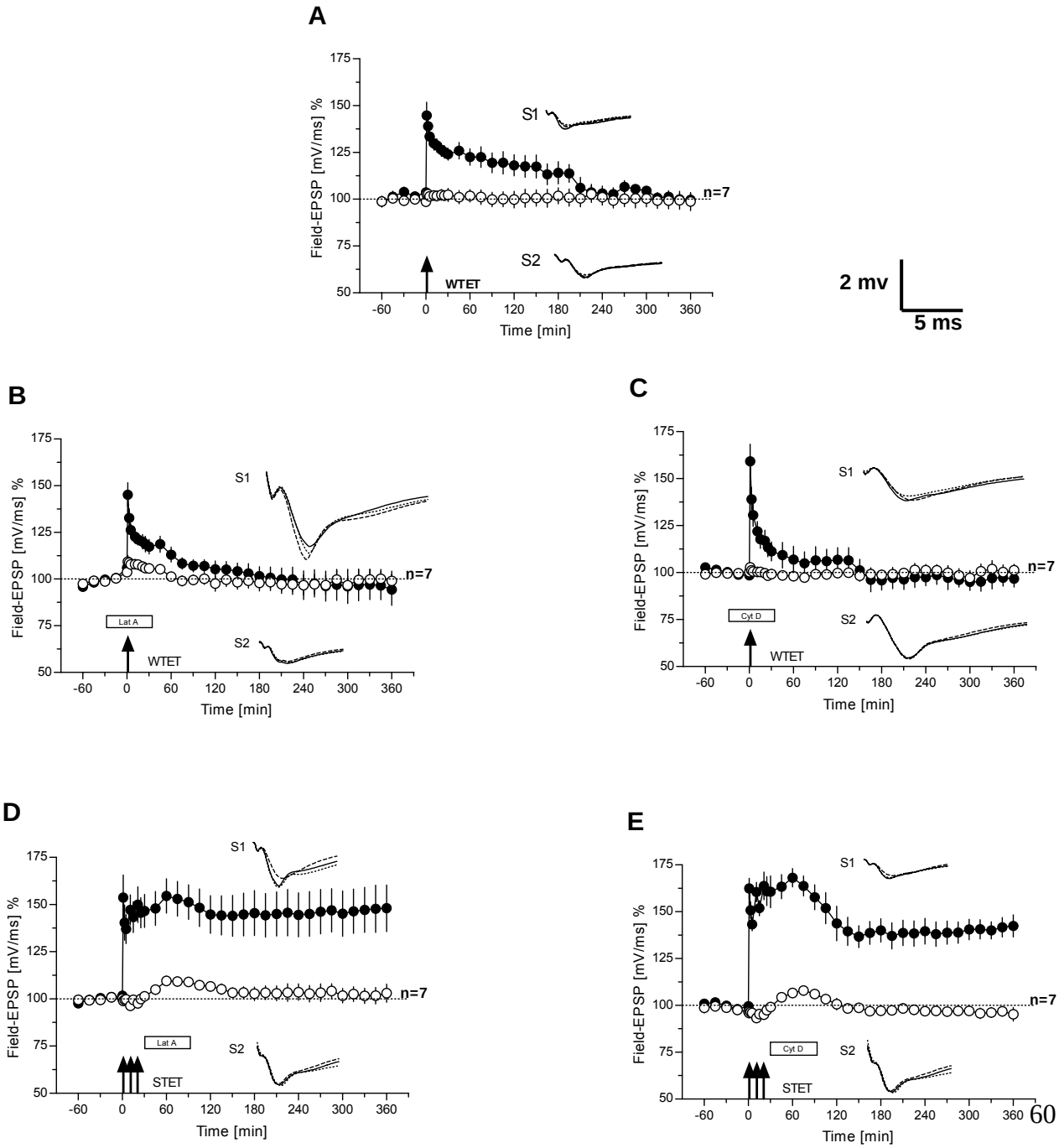


Fig.15. The role of the actin network for early-LTP and late-LTP maintenance (time course of field-EPSP recordings)

A) Time course of the slope of Field-EPSP after the induction of early-LTP by WTET in S1 (filled circles). The control pathway S2 (open circles) remained stable throughout the experiment (n=7). B) The influence of latrunculin A on early-LTP: the drug was applied 30 min before WTET of S1 (filled circles; open circles represent the time course of the control input S2) (n=7). C) Similar experiment like B but instead of latrunculin A, cytochalasin D was applied 30 min before WTET of S1 (filled circles; open circles represent the time course of the control input S2; (n=7). D) The effect of latrunculin A on late-LTP if applied 30 min after STET in S1 (filled circles). Open circles represent the time course of the control input S2 (n=6). E) Similar to D, but instead of latrunculin A cytochalasin D was applied. Analog traces represent typical Field-EPSPs 30 min before the normal time point of tetanization (dashed line); 30 min (solid line) and 6 h (dotted line) after tetanization of input S1. The analog traces for S2 recorded at the same time points however, without tetanization. Calibration bar for all analog traces: 2 mv / 5 ms. Arrows indicate the time point of tetanization of synaptic input S1 and three arrows represent strong tetanization (STET) to induce late-LTP while, single arrow represent weak tetanization (WTET) to induce early-LTP. Filled boxes represent drug application, Lat A: latrunculin A, Cyt D: cytochalasin D.

5. Effect of actin inhibition on processes of synaptic tagging.

In our studies it has been showed that actin polymerization inhibition by latrunculin A (0.1μM) and cytochalasin D (0.1μM) prevented the early and late phase of LTP, if applied during their induction. But the actin network function remains intact when the inhibitors were applied after the induction of LTP. This showed that the actin network function must be intact and do not have any role in its maintenance. Therefore, it is possible to study the tagging interactions if we apply an early form of LTP in another synaptic input in presence of latrunculin A and cytochalasin D. This enables to study the possible role of actin in mediating the setting of synaptic tagging during long-term potentiation.

In the first series of experiments we have reproduced the normal synaptic tagging process during LTP by using a strong before weak protocol (Fig.16A) to show as a cotrol to distinguish from the rest of the synaptic tagging experiments which is treated with structurally different actin inhibitors. So as an initial step here we induced a late form of LTP in synaptic input S1 by using a strong tetanization protocol (STET) (Fig.16A; filled circles) and 30 min after the tetanization protocol we induced an early form of LTP in synaptic input S2 by using a weak tetanization protocol (WTET) (Fig.16A, open circles). As shown in Fig.16A, the transient form of early LTP in input S2 was transformed in to a late form by the prior induction of late-LTP in S1. Here the strong tetanization in input S1 activates the synapse specific tag complex and the synthesis of synapse-unspecific plasticity-related proteins (PRPs). But the induction of early-LTP in independent synaptic input S2 by using a weak tetanization protocol can only sets the tag but unable to synthesize its own PRPs. So if we induce the early-LTP within a specific time window the tag sets in input S2 can capture the PRPs synthesized in input S1 as a result of strong tetanization (STET). So it can reinforce the early form of LTP in to a late, long lasting form (Frey and Morris, 1997; Frey and Morris, 1998a; Frey and Morris, 1998b). So here we were interested to investigate whether the actin network has any role in synaptic tagging process during LTP. Here we induced a late form of LTP in synaptic input S1 (Fig.16B, filled circles) which was statistically significant up to 6 h (wilcocon, $p<0.05$) and 60 min after the first strong tetanization (STET) in the S1 an early form of LTP was induced in S2 by using a weak tetanization (WTET) in presence of the actin inhibitor latrunculin A (Fig.16 B, open circles). The latrunculin A was applied 30 min before and 30 min after the induction of early LTP in input S2. But surprisingly in contrast to normal tagging interaction as shown in Fig.16A latrunculin A prevented the reinforcement of early into a late-LTP. The potentiation in S2 was statistically significant from 1 min to 2h

and 45 min after weak tetanization to S2 (wilcoxon, $p<0.05$). Then we confirmed our result by using a structurally different actin polymerization inhibitor cytochalasin D (0.1μM) which has also shown the blockade of the transformation of early-LTP in to a long lasting form (Fig.16C, open circles). The potentiation in S2 was statistically significant from 1 min to 2h and 45 min after the weak tetanization to S2 (wilcoxon, $p<0.05$). From this result we can speculate that the actin inhibitors can either prevents the translocation of the PRPs synthesized as result of the strong stimulation in the synaptic input S1 or it may disrupt the setting of the synaptic tag complex.

Next series of experiments we further investigated, whether the inhibition of the actin network prevents the setting of the tag complex or the synthesis of PRPs or is it inhibit both of these process. So for this study we used a strong before strong protocol (Fig.16D). First we induced a late form of LTP in syanptic input S1 by a strong tetanization (STET) (Fig.16D, filled circles), which sets its synapse specific tag and in addition to this it will synthesize a pool of PRPs. Then 30 min after the strong tetanization in S1 the actin inhibitor latrunculin A was applied for another 1h. During this time window another late-LTP was induced in synaptic input S2 (Fig.16D, open circles). But the normal long lasting LTP was observed only in input S1 but the late-LTP induced in S2 in presence of actin inhibitor latrunculin A was blocked. The potentials in the S2 was statistically significant for 1 min to 5 h after strong tetanization (STET) to S2 (wilcoxon,$p<0.05$). This result clearly shows that the actin network inhibition directly disrupt the setting of the tag rather than the synthesis of PRPs, because if it was the later case it can capture the PRPs from the first synaptic input and can be reinforced in to a late form of LTP. To confirm this result we conducted similar series of experiments but here instead of latrunculin A we have used a structurally different actin inhibitor cytochalasin D (Fig.16E). Here also the cytochalasin D prevented the late-LTP induced in input S2 (Fig.16E, open circles).

The potentiation was statistically significant from 1 min to 5 h and 30 min after strong tetanization to input S2 (wilcoxon, $p<0.05$). These results could suggest that the inhibition of the actin network might be more related to the inactivation of the tag complex rather than interfering with the macromolecular synthesis.

Then we were conducted experiments to confirm our result that the inhibition of actin network is directly involed in the setting of the synaptic tag complex only, rather than macromolecular synthesis. Here we induced an early form of LTP in synaptic input S1 by using a weak tetanization protocol (WTET) (Fig.17A, filled circles). This will leads to the setting of the synapse specific tag complex but there will not be any PRPs synthesis (Frey and Morris, 1997). Thirty min after the weak tetanization the actin inhibitor latrunculin A was applied for another 1h. During this time window, but 30 min after weak tetanization in input S1, a strong tetanization (STET) was applied to synaptic input S1 (Fig.17A, open circles). The strong tetanization in the presence of latrunculin A blocked the late maintenance of LTP in input S2. The potentiation was statistically significant from1 min to 2h and 45 min after strong tetanization (wilcoxon, $p<0.05$). Surprisingly the early form of LTP induced in synaptic input S1 was reinforced on to a late-LTP during this procedure. This result support our speculation that the inhibiton of the actin network directly interfers with the setting of the synaptic tag machinery rather than the synthesis of PRPs and this PRPs synthesized in the second synaptic input even in the presence of actin inhibitors can be captured by the tag sets in the S1 input, as a result of weak tetanization so it has been reinforced in to a late form of LTP. But later we confirmed this result by using a structuarally different actin network inhibitor cytochalasin D (Fig.17B). In this series of experiments also the early-LTP induced in synaptic input S1 (filled circles) was transformed in to a late form of LTP even when the late-LTP was induced in S2 under actin inhibitors treatment (Fig.17B, filled circles). The Late-

LTP induced in synaptic input S2 was statistically significant from 1 min to 4h and 30 min after the induction of late-LTP (wilcoxon, $p<0.05$).

Finally we conducted experiments to varify whether the reinforcement of early-LTP in to a long lasting form of LTP was as a result of the capture of PRPs synthesized from the strong tetanization in the S2 even in the presence of actin inhibitors. Here we induced an early form of LTP in the first synaptic input by using a weak tetanization protocol (WTET). Then for another 1 h we coapplied the actin inhibitor latrunculin A and the protein synthesis inhibitor anisomycin, but in between that is 60 min after the weak tetanization in input S1, we induced a late form of LTP in the second synaptic input S2 (Fig.17C, open circles). Here the actin inhibitors prevent setting of the tag and anisomycin prevents the synthesis of PRPs in synaptic input S2. As a result we found that there will not be any reinforcement of early-LTP in to a late-LTP in the first synaptic input, because of the unavailability of PRPs for the capture process. So we repeated the same experiment with another actin polymerization inhibitor cytochalasin D along with anisomycin which also prevented the transformation of early-LTP in to a late, long lasting form of LTP induced in synaptic input S1. This result also support our hypothesis that the actin network mediates the setting of the tag molecule or as it act as apart of the synaptic tag complex in apical dendrites of hippocampal CA1 neurons.

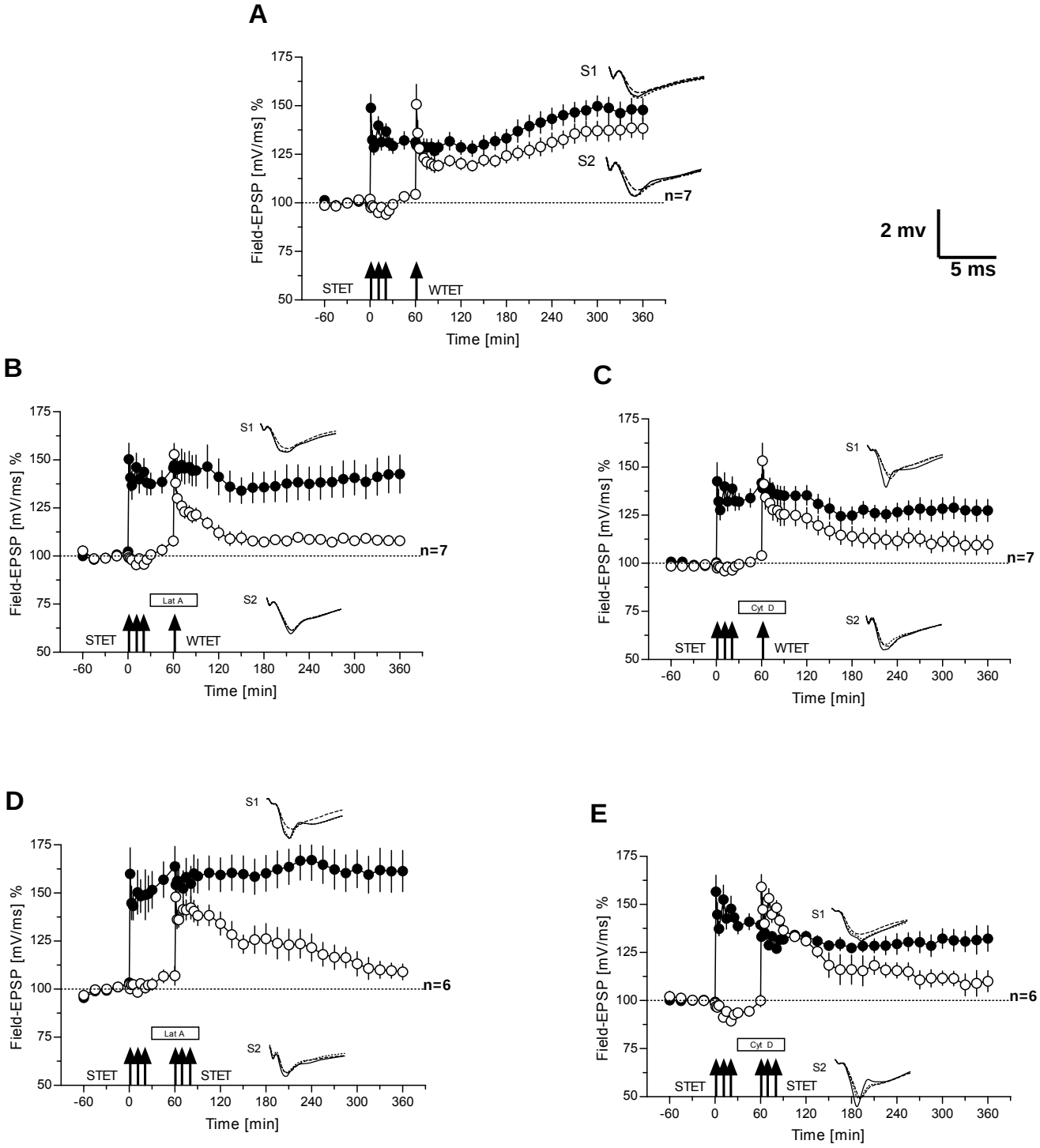


Fig.16. The role of the actin network for synaptic tagging (time courses of field-EPSP recordings) - "strong before weak-tetanization" and "strong before strong tetanization.

A) This panel represents a control experiment verifying synaptic tagging in our preparation. The time course of the slope of the Field-EPSP after STET of input S1 (filled circles) is presented. One hour after STET to S1, a WTET to S2 (open circles) was applied. The normally induced early-LTP in S2

was transformed in to late-LTP by the prior induction of late-LTP in S1 (n=7). B) The time course of the same tagging experiment as in A) is presented however, with the application of latrunculin A during the time of WTET to S2 (open circles, box represents drug application). Latrunculin A was applied 30 min before and until 30 min after WTET of S2 (n=7). Synaptic tagging in S2 was thus prevented. C) A similar experiment as in B, however instead of latrunculin A now cytochalasin D was applied (n=7). Again, synaptic tagging in S2 was prevented by the drug. D) The same procedure as in B, however, now STET was also applied to S2 (open circles) in the presence of latrunculin A (n=6). Late-LTP in S2 was also prevented by the drug irrespective of the prior STET and induction of late-LTP in S1 (filled circles). E) The same procedure as in D, with the exception that instead of latrunculin A now cytochalasin D was applied (n=6). Again, late-LTP in S2 was prevented. The time point of the analog was same like S1 but the S2 was also tetanized. So the traces of S1 and S2 are representative examples of 30 min before (dashed line), 30 min (solid line) and 6h (dotted line) after tetanization of the adequate input. Calibration bar for all analog traces: 2 mv / 5 ms. Arrows indicate the time point of tetanization of corresponding synaptic inputs and three arrows represent strong tetanization (STET) to induce late-LTP while, single arrow represent weak tetanization (WTET) to induce early-LTP. Filled boxes represent drug application, Lat A: latrunculin A, Cyt D: cytochalasin D.

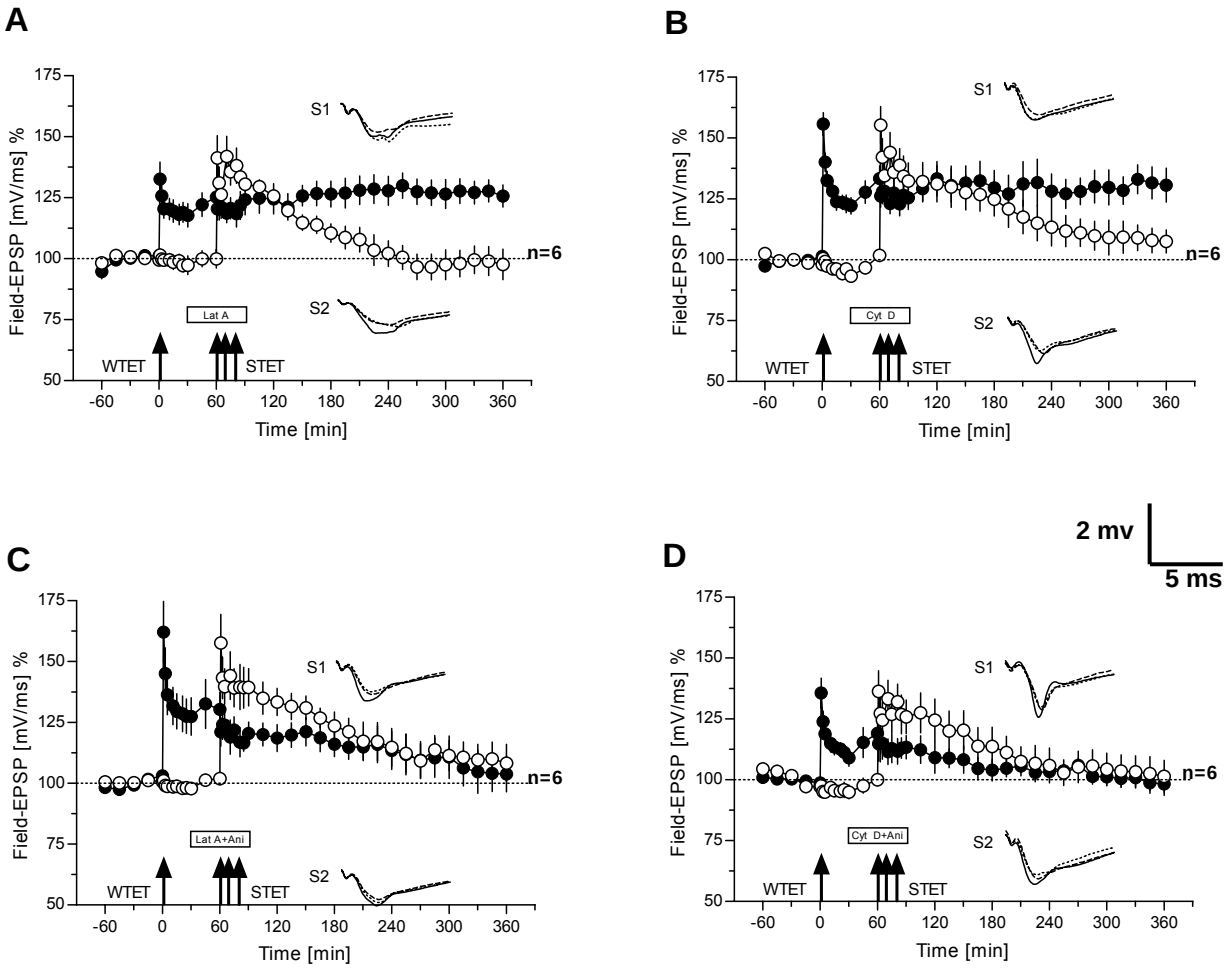


Fig.17. The role of the actin network for synaptic tagging (time courses of field-EPSP recordings) - "weak-tetanization before strong-tetanization"

A) A "weak before strong protocol" was used (Frey and Morris, 1998b). In S1 (filled circles) WTET was applied at the time point indicated by the single arrow. One hour later a STET was applied to input S2 (open circles). However, now latrunculin A was applied (30 min before STET to S2 until 30 min after STET to S2). Although, late-LTP in S2 was prevented, the tagging process was observed in S1, i.e. the normal early-LTP was transformed into late-LTP in S1 by the STET of S2 even under inhibition of actin polymerization (n=6). B) A similar "weak before strong" experiment as in A) with the exception that instead of latrunculin A the structurally different actin polymerization inhibitor cytochalasin D was applied. Again late-LTP in S2 was blocked, however tagging in S1 took place (n=6). C) A similar experimental design as in A) with the exception that together with latrunculin A, the reversible protein synthesis inhibitor anisomycin was applied n=6). All late forms or LTP were prevented. D) The same

as in C) with the exception that instead of latrunculin A, cytochalasin D was applied together with anisomycin. Again, all late forms of LTP were blocked (n=6). The time point of the analog was same like S1 but the S2 was also tetanized. So the traces of S1 and S2 are representative examples of 30 min before (dashed line), 30 min (solid line) and 6h (dotted line) after tetanization of the adequate input. Calibration bar for all analog traces: 2 mv / 5 ms. Arrows indicate the time point of tetanization of corresponding synaptic inputs and three arrows represent strong tetanization (STET) to induce late-LTP while, single arrow represent weak tetanization (WTET) to induce early-LTP. Filled boxes represent drug application, Lat A: latrunculin A, Cyt D: cytochalasin D, Ani: anisomycin.

4. Discussion

My initial studies were conducted to reproduce a series of control experiments on long lasting forms of LTP in rat hippocampal slices in vitro as a basis for the subsequent studies. All my studies were performed within the CA1 region of the hippocampus and could reproduce all forms of LTP by using different induction protocols. LTP in CA1 region consists of a transient, protein synthesis-independent early form (early-LTP: Fig.11.E) and a long lasting, late form (late-LTP: Fig.11.A) which requires protein synthesis.

The LTP in CA1 region is dependent on NMDA-receptor activation and the LTP was blocked when an NMDA-receptor antagonist (AP5, 50μM, Fig.11.D) when applied during its induction. Experiments with inhibitors of protein synthesis such as anisomycin (25μM; Fig.11.B) or emetine (20μM; Fig.11.C) also confirmed that the late-LTP was dependent on protein synthesis.

Then I investigated the role of heterosynaptic requirements during late-LTP induction by using an antagonist of the dopamine D1/D5 receptor, SCH 23390 (0.1μM; Fig.12.C) and found that the late-LTP in CA1 region is dependent on the synergistic activation of glutamatergic and dopaminergic function (as innervating inputs).

In further experiments i investigated the late-associative processes during LTP such as synaptic tagging (Fig.13.C). This series of experiments clearly showed the input specificity of LTP and how it interacts with differentially activated synapses during a specific time window. Finally my experiments were conducted to investigate the dynamic of actin cytoskeleton during different stages of LTP and synaptic tagging.

4.1. The actin network in long-term potentiation

In my studies, in contrast to already existing work, we were interested to study the role of the actin network in distinct forms of LTP in CA1 neurons of hippocampal slice in vitro. It has been shown that LTP is decreased or impaired when actin polymerization inhibitors are applied at the time of tetanization (Kim and Lisman, 1999; Krucker et al., 2000; Meng et al., 2002; Soldering et al., 2003; Fisher et al., 2004; Chen et al., 2007; Sacktor et al., 2007).

During this study, I used two structurally different inhibitors of actin polymerization such as latrunculin A and cytochalasin D, whose mode of action is different from each other but they showed the same function. First I investigated the role of the actin network during the induction of late-LTP, i.e. the actin inhibitors were applied during its induction. The second series of the experiments investigated role of the actin network in the maintenance of early-LTP. Finally I studied the role of actin network in the maintenance phase of late-LTP. During this series of experiments the actin network inhibitors were applied after the induction of late-LTP.

The morphological changes in the dendritic spine that supports late-LTP maintenance and the actin cytoskeleton is the main target that regulate spine morphology, which is directly correlated with the distribution of AMPARs within the spines (Matsuzaki et al., 2001). The changes in synaptic efficacy are accompanied by the structural remodeling of the dendritic spines, including the formation of multiple synaptic boutons and the sprouting of postsynaptic architecture (Buchs and Muller., 1996; Colicos et al., 2001; Geinisman et al., 1996; Toni et al., 1999; Trommald et al., 1996; Weeks et al., 1998). The pharmacological manipulation of actin dynamics affects the morphogenetic regulation of dendritic spines, which is critical for the increase in synaptic efficacy following multiple tetanic stimuli during late-LTP (Colicose et al., 2001). Thus the actin network mediates the trafficking/ insertion of

AMPARs in to the synaptic membrane and its internalization from the membrane during LTP and LTD respectively (Carroll et al., 2001; Hayashi et al., 2000; Lledo et al., 1998; Sheng et al., 2001; Shi et a., 2001). The inhibition of actin network prevents the insertion or internalization of AMPARs in to or from the membrane. Recent studies also support that the inhibition of actin polymerization during LTP regulates the recruitment of AMPARs to the postsynaptic density (Sacktor et al., 2007). In our late-LTP experiments with latrunculin A and cytochalasin D (Fig.14.B & 14.C) was shown, that it also prevented late-LTP which again supports the role of actin network in LTP.

It has been already reported that the late-LTP requires protein synthesis for its long lasting maintenance (Frey et al,. 1988; Krug et al., 1984; Matthies et al., 1990b; Reymann et al., 1988a, c). The application of actin network inhibitors block the accumulation of F-actin content during LTP, which is not dependent on protein synthesis but it is important for the functioning of newly synthesized proteins that are necessary for the late phase of LTP (Fukazawa et al., 2003). The actin filament in the dendritic spine mediates the local trafficking of proteins that are necessary for late-LTP and this filament constitute a track for delivering the proteins synthesized locally from the dendritic shaft to the postsynaptic site through the spine cytoplasm. Disruption of actin elongation prevents the fusion of membrane proteins with postsynaptic membrane there by changing the synaptic efficacy.

Moreover, the actin filaments act as a scaffold on which the other post synaptic proteins are anchored (Allison et al., 1998; Halpain, 2000; van Rossum and Hanisch, 1999, Ziff, 1997). Plasticity-dependent changes in actin dynamics could alter the arrangement and functional state of postsynaptic proteins, including neurotransmitter receptors, signaling molecules and scaffold proteins. During LTP the

actin filament plays a critical role for PSD by anchoring the AMPARs at its postsynaptic sites (Lisman and Zhabotinsky, 2001).

Recent works had shown that there is a direct relationship between dynamic actin polymerization and PKM Zeta (the only identified process specific plasticity-related protein (PRP) (Sajikumar et al., 2005). They suggest that actin polymerization is essential for the synthesis of PKM Zeta (Sacktor et al., 2007). In addition to this an intact actin cytoskeleton is critical for efficient protein synthesis (Stapulionis et al., 1997), acting as a platform for the function of translation factors, in particular elongation factor 1A (Kandel et al., 2002). The translocation of elongation factor1A to stimulated regions of dendrites during LTP is dependent upon actin dynamics (Huang et al., 2005) and thus the factor may be a critical component of LTP-induced protein synthesis (Tsokas et al., 2005). But in our tagging experiments with latrunculin A and cytochalasin D did not block LTP-induced protein synthesis and could reinforce an early-LTP in to a late form induced in an independent synaptic input (see discussion actin network and synaptic tagging: Fig.17.A & 17.B).

We found that the application of different actin polymerization inhibitors prevents even the maintenance of early-LTP, which normally decays within 2 -3 h. It has been reported that a relatively quick reassembly of the actin network is partially involved in the immediate maintenance of LTP (Chen et al., 2007). The early phase of LTP is mainly carried/ maintained by the activation of different kinases including CamKII (Matthies et al., 1990; Huang et al 1998; Frey et al., 2007). The binding of CamKII to the actin filament is essential for its targeting to the activated synapses (Shen et al; 1998) and also the insertion of new AMPA receptors at existing synapses has also been identified as an important step during early LTP maintenance. Inhibition of actin polymerization blocks both of these events (Allison et al; 1998; Shi et al; 1999).

The application of actin inhibitor latrunculin blocked LTP even when applied immediately after tetanization (Krucker et al., 2000). But in our studies we found that the application of actin inhibitors 30 min after the induction of late-LTP had no effect (Fig.15.D & 15.E) and also did not significantly change the baseline recordings. This result supports the idea that memory like consolidation process begins within seconds of LTP induction but the pharmacological manipulations become progressively less effective over following 30 min. This indicates the existence of a critical time window in which the inhibitors can prevent actin polymerization and the actin inhibitors are synapse and activity specific.

Finally from my study, all these mentioned parallels between synaptic plasticity and actin polymerization status strongly suggest that actin dynamics is an important determinant of synaptic architecture, making it an ideal substrate for the long-term modification underlying long-term synaptic plasticity.

4.2. Synaptic tagging and actin network inhibition

The main goal of our synaptic tagging experiments during LTP was, whether the actin micro filament, one among several speculated molecules meets the criteria for acting as a synaptic tag/tag complex. The validity of the synaptic tagging hypothesis resets on the identification of the tag mole. But a number of studies showed that the tag shows some distinct criteria where it does not necessarily have to be a single molecule. Any candidate should fulfill the following criteria function as a tag such as (a) the tag is induced in a protein synthesis-independent manner, (b) the tag posses a life time of 1-2 h, (c) the tag is induced in an input-specific and physically immobile manner, (d) the tag should be able to interact with cell-wide molecular events that occur after strong stimulation to produce long-term, synapse specific strengthening. A number of possible postsynaptic modifications have been enumerated as candidates for the synaptic tag (Frey and Morris, 1998a; Martin and Kosik, 2002).

It has been speculated that a number of molecules may act as potential candidates for synaptic tag molecules (Martin et al, 2002). Persistently active kinases meet several criteria for the tag, as they allow a synapse to remember previous activity in a spatially restricted and reversible manner. Calcium/calmodulin-dependent protein kinase II (CaMKII), which becomes autonomously and persistently active by autophosphorylation, has been activated by synaptic stimulation, meets an identity of the tag. The CaMKII holoenzymes were shown to be capable of associating with one another in response to $Ca2^{+}$ .Therefore CaMKII may form a scaffold that, in combination with other synaptic proteins, recruits and localizes additional proteins to the postsynaptic density (Hudmon et al., 2005). The atypical protein kinase C known as protein kinase M Zeta (PKMζ), the persistent activity of which requires protein synthesis, has been shown to be another possible candidate. But recently it has

been found that PKMζ is the first LTP specific plasticity-related protein (PRP) and not a tag molecule in apical CA1 branches (Sajikumar et al., 2005) but in basal dendrites the coactivation of PKA or PKM Zeta is required for synaptic tagging (Sajikumar et al., 2007). The local activation of PKA and local regulation of ubiquitin-proteosome pathway can serve as synaptic tag that combine with transcriptional events to produce persistent and local synaptic strengthening (Chain et al., 1999; Hegde et al., 1997; Schwartz et al., 1999).

Changes in adhesion molecule are likely to underlie the morphological changes that are associated with synaptic strengthening (Kandel et al., 1993). In consistent with the idea that cell adhesion molecule dynamics could serve as synaptic tags, alterations in cell adhesion molecule at the synapse have been found to occur during many form of the synaptic plasticity. For example, the cadherins are synaptic adhesion proteins that have been implicated in synapse formation and targeting. They have been shown to dimerize and alter their conformation during depolarization (Tanaka et al., 2000).

Another potential candidate for a synaptic tag that has recently received significant attention is the actin micro filament network at the synapse. The actin network in neurons is extremely dynamic, and these dynamics have been shown to change with activity (Matus et al., 2000; Murthy et al., 2001). Changes in the actin cytoskeleton probably accompany changes in cell-adhesion molecules, as most adhesion molecules are linked to the actin cytoskeleton. In addition, changes in the actin microfilament network are likely to underlie the growth of new synaptic structures that have been observed after repetitive stimulation of hippocampal synapses (Engert et al., 1999; Muller et al., 1999; Svoboda et al., 1999).

The stimuli that can produce a synaptic tag are not necessarily sufficient to activate protein synthesis, and are therefore frequently considered to be sub-

threshold for long-term plasticity. Such stimuli induce a host of changes at the synapse and any number of these changes could potentially serve as a tag. Many of these changes are related to the activity and strength of the synapse, such as rapid addition of AMPA receptors to ionotropic glutamate receptor clusters (Shi et al., 1999), the lateral mobility of NMDA receptors between synaptic and extra-synaptic sites (Westbrook et al., 2002) etc. Such events could serve as localized traces of previous synaptic activity that are able to produce synaptic strengthening on their own with in a limited time period. However, to function as synaptic tags, they would need to be able to interact with cell wide events to produce local and persistent increase in synaptic efficacy.

The major goal of my experiments was to investigate the possible involvement of actin network on the processes of synaptic tagging during LTP. From my studies it has been revealed that actin mediated processes are required for distinct phases of LTP in apical branches of hippocampal CA1 region. The main question regarding the involvement of actin network in synaptic tagging was whether it was related to the tagging machinery or the process involved in the activation and synthesis of plasticity-related proteins (PRPs). It has been reported that in hippocampal CA1 neurons LTP in either the apical or basal dendrites, different molecules mediate the setting of the tag complex/machinery. In CA1 apical dendrites Cam Kinase II identified as a process specific tag molecule but PKM zeta act as a LTP specific PRP (Sajikumar et al., 2005) but in basal dendrites (stratum oriens) the setting of the tag is mediated by either protein kinase A or protein kinase M zeta (Sajikumar et.al. 2007).

During the initial experiments of synaptic tagging with actin inhibitors (Fig.16.D & 16.E) a strong tetanization (STET) was induced in synaptic input S1. Subsequently, after a short time window another STET was applied to synaptic input S2 in presence of actin inhibitors. Here the late-LTP induced in S1 was maintained and S2 blocked.

This result was interesting because it was more correlated with our expected idea, i.e. actin is directly involved in synaptic tagging process rather than the synthesis of macromolecule. If the blockade of late-LTP in S2 was directly related to the inhibition of PRP synthesis, it could capture the synapse unspecific PRPs synthesized in S1 and transformed in to a late form (Frey and Morris., 1998). Instead of this the late-LTP in S2 was blocked, which shows that the inhibition of actin network directly related to the setting of the tag.

Recently, it has shown that the inhibition of actin polymerization regulates the synthesis of PKM Zeta (Sacktor et al., 2007). Our tagging experiments (Fig.17.A&17.B) revealed that the blockade of actin network does not directly interfere with the synthesis of plasticity-related proteins (PRPs). If it would have been the case, then the weak tetanization (WTET) of synaptic input S1 (Fig.17.A and 16.B) which would normally result only in early-LTP should not have been transformed in to late-LTP by subsequent strong tetanization (STET) of an independent input S2, in presence of actin inhibitors. Normally the early-LTP induced in synaptic input S1 was sufficient to activate a synaptic tag. However, the tag as such is unable to convert early-LTP in to late-LTP because setting of tag is independent of protein synthesis (Frey and Morris, 1998). Subsequent induction of strong tetanization to S2 activates the synthesis of its own process-unspecific PRPs even in the presence of actin inhibitors. But here the setting of the tag was blocked by actin inhibitors. Therefore, the synapse unspecific PRPs synthesized in S2 could not be captured by the distorted S2-tags, but by the S1 tags. This blocked late-LTP in S2 and converted the early-LTP in to late-LTP in S1.

Earlier, it had been shown that the setting of LTP tags in apical dendrites of CA1 region does not require protein synthesis (Frey and Morris., 1997; Frey and Morris., 1998; Frey and Frey., 2008). Therefore, in our experiments Fig.16.C and

16.D clearly shows that actin network is directly related to the tagging machinery rather than the PRP-related processes. However, the application of protein synthesis inhibitor, anisomycin, in addition to the actin blocker prevented the transformation of early-LTP in S1 in to late-LTP (Fig.17.C & 17.D). Here the actin blockers prevented the setting of the S2 tags and at the same time the synthesis of PRPs are inhibited by protein synthesis inhibitor , anisomycin, which leads to the prevention of late-LTP in S2. The S1 input was only able to express early-LTP even with its tag, because there were no PRPs synthesized. Thus we have identified that actin network acts as a second tag-specific molecule, i.e. it involved in tagging machinery at least in apical CA1 dendrites. However, we don't know yet, whether the action of actin is specific for the LTP-tag complex, as it is the case of CamKII (Sajikumar et al., 2007) or it can also function as a more general tag machinery required for LTD tagging.

There is a strong possibility for the actin network to be involved in a general tagging process by guaranteeing the molecular/morphological basis. This process can be compared with a tag-related "housekeeping process". Moreover, the other key molecules e.g., the CamKII or MAPK which are only capable of exerting their action in making activated synapses specifically marked either for LTP or LTD processes. It is also to be noted that the actin network could also be involved in the PRP capture process. This network provides a distinct geometry for required interactions of kinases with the PRPs, as it changes the geometry of active synaptic zones. The whole synaptic spine may also be affected. It would then guarantee the activation of effector processes, e.g., the activation and/or transport into spines or transport of new AMPA receptors to the synaptic surface in order to maintain LTP.

In summary, our experimental data strongly support the hypothesis that the synaptic tag consists of not a single molecule but it contains a complex of different components which are activated synergistically during synaptic stimulation (Frey and

Morris, 1998; Frey and Frey 2008). In addition to already identified process specific tag molecules such as CamKII for LTP and MAPK for LTD in apical dendrites of CA1 region, the actin network is also actively involved in LTP-tag-machinery in the apical dendritic branches..

Future studies:

In our studies we have shown the involvement of actin network in synaptic tagging process in apical dendrites (stratum radiatum) of CA1 region only. But future studies should investigate whether the actin network is also involved in the setting of the tag in basal dendrites (stratum oriens) during LTP or is it restricted to specific functional compartment of the pyramidal neurons only. In addition to the role of actin network during LTP, its role in LTD has to be investigated. Apart form these the possible involvement of microtubule system as a potential tag molecule during bidirectional plasticity in the CA1 region of the hippocampus has to be investigated.

5. Conclusion

The principle findings of this dissertation are the following:

a) A strong high frequency stimulation (HFS) can reliably induce input specific late-LTP in hippocampal CA1 area, lasting at least 6h and a weak HFS can induce an early-LTP lasting 2-3h-in vitro.

b) Late-LTP induced by HFS is dependent on protein synthesis and requires the activation of the NMDA-receptor, while early-LTP is independent of protein synthesis.

c) Late-LTP is characterized by a late-associative property, i.e. synaptic tagging and heterosynaptic induction processes.

d) The activation of D1/D5 receptor is essential for the maintenance of late-LTP in apical dendrites of hippocampal CA1 neurons.

e) The polymerization of the actin network is essential for the maintenance of late-LTP in hippocampal CA1 area.

f) Inhibition of actin polymerization prevents late-LTP at the time of induction only. But after a critical time window it has no effect on LTP maintenance.

g) Actin inhibitors prevent the maintenance of early-LTP.

h) Inhibition of actin polymerization prevents synaptic tagging in hippocampal CA1 area.

i) Inhibition of actin polymerization does not block LTP-induced protein synthesis.

j) The actin network functions as a part of the synaptic tag complex or it mediates the tag machinery.

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Appendices

I. Zusammenfassung der dissertation

Die bedeutenden Formen von synaptischer Plastizität im Hippocampus wie „long-term potentiation“ (LTP) und „long-term depression“ (LTD) werden als zelluläre Korrelate von Lernprozessen und Gedächtnisbildung angesehen. In den letzten Jahren wurden beeindruckende Forschungsanstrengungen unternommen, um die zellulären und molekularen Mechanismen von synaptischer Plastizität im Hippocampus, insbesondere LTP, zu verstehen.

Während meiner anfänglichen Studien konnte ich elektrisch induzierte LTP in apikalen Dendriten von pyramidalen Neuronen in der CA1-Region von Hippocampus-Schnitten in vitro reproduzieren. In Abhängigkeit von verschiedenen Induktionsprotokollen konnten eindeutige Formen von LTP wie eine transiente, Proteinsynthese-unabhängige frühe LTP (mit einer Dauer von 3 – 4 h) oder eine von einer de novo-Proteinsynthese abhängige späte LTP induziert werden. Beide Formen von LTP erforderten die Aktivierung von NMDA-Rezeptoren und insbesondere die späte LTP setzte die synergistische Aktivierung von glutamatergen und dopaminergen Afferenzen während ihrer Induktion voraus.

Es ist berichtet worden, dass die LTP in der CA1-Region durch Prozesse der „synaptischen Etikettierung“ (synaptic tagging) charakterisiert ist. Während der Induktion von LTP werden die aktivierten Synapsen durch einen „synaptic tag / tag complex“ markiert, der synapsenunspezifische, plastizitätsbezogene Proteine (plasticity-related proteins = PRPs) zu erfassen vermag. Eine frühe, in einer synaptischen Afferenz induzierte LTP wurde während des „synaptic tagging“ in eine späte LTP transformiert, vorausgesetzt, die späte LTP wurde in einer unabhängigen synaptischen Afferenz derselben neuronalen Population innerhalb eines ganz

bestimmten Zeitfensters induziert. Die Synthese von prozessunspezifischen PRPs durch Induktion einer späten LTP reicht aus, um die frühe LTP in eine späte LTP, die durch einen „synaptic tag / tag complex“ markiert ist, zu transformieren/zu verstärken.

Weiterhin interessierte mich die Untersuchung der Fragestellung, ob die Funktion des Aktinnetzwerks für die Aufrechterhaltung von LTP in der CA1-Region des Hippocampus erforderlich ist. Es ist berichtet worden, dass die Dynamik des Aktinzytoskeletts entscheidend für die Aufrechterhaltung von LTP ist. Hierzu fanden wir heraus, dass die Inhibierung der Aktinpolymerisierung die Proteinsynthese-unabhängige frühe LTP und die Proteinsynthese-abhängige späte LTP beeinträchtigt. Interessanterweise vermochte die Verabreichung von Aktin inhibitoren nach der Induktion von späten LTP die LTP jedoch überhaupt nicht zu blockieren, was einen frühen, für die Induktion und Aufrechterhaltung von LTP notwendigen Mechanismus nahelegt.

In der letzten Serie von Experimenten untersuchte ich, ob die Inhibierung des Aktinnetzwerks mit Prozessen des „synaptic tagging“ interferiert. Die Transformierung von frühen in späte LTP wurde durch die Verabreichung von strukturell verschiedenen Inhibitoren der Aktinpolymerisierung, Latrunculin A und Cytochalasin D, blockiert. Wir schließen daraus, dass das Aktinnetzwerk für frühe „house keeping“-Prozesse zur Induktion und Aufrechterhaltung von frühen LTP erforderlich ist. Darüber hinaus interagiert die Inhibierung der Aktindynamik negativ mit dem Setzen des „synaptic tag complex“. Wir sehen Aktin als ein markierungsspezifisches Molekül in apikalen CA1-Dendriten an, wo es direkt an der Markierungs-/Erfassungsmaschinerie und der Inhibierung von Aktinnetzwerken beteiligt ist und demzufolge die Interaktion mit PRPs unterbindet. Dies resultiert in der Verhinderung von späten LTP durch die Inhibierung des Aktinnetzwerks während der Induktion von LTP.

II. Curriculum Vitae

Name: Binu Ramachandran

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Education:

2005-2009	**: Doctoral student, Department of Neurophysiology, Leibniz Institute for Neurobiology, Magdeburg under Otto-von-Guericke University.**
2001-2002	**: Bachelor of Education in Natural Science Mahatma Gandhi University, Kottayam Kerala, India.**
1999-2001	**: Master of Science in Zoology University of Kerala Kerala, India.**
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III. Scientific publications:

Ramachandran B and Frey JU (2009) Interfering with the actin network and its effect on long-term potentiation and synaptic tagging in hippocampal CA1 neurons in slices in vitro. Journal of Neuroscience 29:12167-12173.

Parvez S, **Ramachandran B** and Frey JU (2010) Functional differences between and across different regions of the apical branch of hippocampal CA1 dendrites with respect to long-term depression induction and synaptic cross-tagging. Journal of Neuroscience 30:5118-5123.

IV. Erklärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zum dem Thema

“Late-LTP in apical CA1 dendrites of hippocampal slices in vitro”

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.

Magdeburg, 26.11.2009 Unterschrift