# Remembering makes memories fragile:

# the cellular basis of reconsolidation.

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# CHAPTER I

## INTRODUCTION

It is widely believed that changes in synaptic strength of neuronal connections underlie the formation of memory. The idea that neuronal connections are plastic and hence their coupling strength modifiable by synaptic activity was introduced by Donald Hebb more than 50 years ago: "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 changes takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased" (Sejnowski, 1999;Brown and Milner, 2003). This sentence stated for the first time the concept of synaptic plasticity, but the first demonstration of activity-dependent neuronal plasticity came only in 1973, by the work of Bliss and Lømo with the discovery of Long-Term Potentiation (LTP) (Bliss and Lomo, 1973). Since then, motivated by the hypothesis that LTP might represent a cellular model of learning and memory, the mechanisms underlying LTP induction and expression have been intensively studied (Malenka and Nicoll, 1999;Lisman, 2003b). The discovery of LTP provided an experimental analogue of the learning-induced changes in synaptic connectivity postulated by Hebb (Tsien, 2000). However, a direct demonstration linking learning with altered patterns of neural activity representing the occurrence of events is still lacking (Martin et al., 2000b). Later on, Long-Term synaptic Depression (or LTD) was also found in the brain, demonstrating that synaptic strength could be decreased in an activity-dependent manner and providing an additional cellular mechanism for memory storage (Artola and Singer, 1993;Bear and Malenka, 1994;Stanton, 1996). Initially, this long-lasting synaptic depression was discovered as a "by-product" of LTP induction, representing what is now called heterosynaptic LTD (Lynch et

al., 1977). Afterwards, the two phenomena were dissected and it is now well established that the molecular mechanisms underlying the induction of LTP and LTD are distinct. LTD refers to an activity-dependent, pathway-specific and long-lasting depression in synaptic efficacy inducible by a low-frequency stimulus (typically 900 pulses at 1 Hz). Although LTD induction results in a decrease in synaptic efficacy in previously potentiated synapses it does not represent a reversal of potentiation. Depotentiation, the reversal of LTP, is restricted to previously potentiated synapses and does not lead to changes in synaptic efficacy in naïve synapses (Wagner and Alger, 1996). LTP and LTD can exist as homo and heterosynaptic forms and it is not clear how these different forms are induced. The induction of heterosynaptic LTD or LTP concomitant with the induction of homosynaptic LTP or LTD, respectively, is a very attractive cellular mechanism to increase "contrast" between synapses. Although poorly understood, these positive and negative interactions between synapses might be important to keep overall neuronal excitability constant and might represent a mechanism to select particular input pathways over others.

A great deal of knowledge has been gathered in the last few years concerning the biochemical events underlying the induction and expression of LTP and LTD (Malinow et al., 2000;Nicoll, 2003). Increases in the postsynaptic calcium concentration after the synaptic activation of NMDA receptor or voltage-gated calcium channels, are believed to be the general triggering mechanism for LTP and LTD induction. This rise in calcium concentration triggers a variety of enzymatic reactions that lead to direct and indirect phosphorylation of protein resulting in a change in receptor sensitivity and number (Malinow and Malenka, 2002). Among the molecules that can serve as a calcium sensor are several protein kinases, including protein kinase C (PKC), cyclic adenosine 3',5'- monophosphate (cAMP) – dependent protein kinase (PKA), the tyrosine kinase Src, the mitogen-activated protein kinase (MAPK) and the α-calcium-calmodulin-dependent protein kinase II (CaMKII), one of the

most prominent molecules in the molecular machinery of LTP. CaMKII is highly enriched in the post-synaptic density, a sub-membrane component of synapses and dendritic spines (Soderling and Derkach, 2000). An important property of this kinase is that autophosphorylation renders its activity independent of Ca-calmodulin activation, increasing several-fold the time during which the kinase is active. CaMKII is involved in phosphorylating and activating several key molecules, such as AMPA and NMDA receptors. Calcium rises also result in the activation of protein phosphatases including protein phosphatases 1, 2A (PP1, PP2A) and 2B (PP2B or calcineurin), which are highly enriched in synapses. Generally, it is believed that the temporal and spatial dynamics of post-synaptic calcium concentration determines whether protein kinases or phosphatases are preferentially activated, and consequently whether synapses become potentiated or depressed. Ultimately, LTP and LTD induction are thought to depend on the relative, combined activity of the kinases and phosphatases (Mansuy, 2003;Munton et al., 2004).

Although the mechanisms underling LTP induction are by and large known, the mechanisms involved in the maintenance of LTP are still poorly understood. LTP can last for several hours *in vitro* and is considered to be at least a two-stage process (Malinow et al., 2000;Dudai, 2002;Abraham and Williams, 2003). The induction of LTP triggers several biochemical cascades that lead to post-translational modifications of pre-existing pre and post-synaptic proteins. Simultaneously, protein synthesis-dependent mechanisms take over and the enhanced synaptic strength is maintained and supported by processes involving gene transcription and translation (Malenka and Nicoll, 1999;Malinow et al., 2000;Dudai, 2002;Abraham and Williams, 2003). The observation that the later stages of LTP require *de novo* protein synthesis for its maintenance led to the classic distinction between an early-phase LTP (E-LTP), independent of protein synthesis and a late-phase LTP (L-LTP), requiring the *de novo* synthesis of proteins. Once L-LTP is established, blocking protein

synthesis does not lead to decay in LTP (Huang et al., 1996;Barco et al., 2002;Calixto et al., 2003;Cammalleri et al., 2003). This observation gave rise to the general idea that the maintenance of L-LTP does not require *de novo* protein synthesis (Huang et al., 1996).

Like LTP, the establishment of new memories is thought to proceed by stages or phases (McGaugh, 2000). According to this model, input to the brain is processed into short-term memory before it is transformed through one or more stages into a more permanent long-term memory (Nadel and Moscovitch, 1997;Abel and Lattal, 2001;Dudai, 2002). Studies using, for example, electroconvulsive shocks have shown that until memories have been converted into a long-term form, retrieval or recall of this memory is easily disrupted. In addition, it is now an established fact that in order for long-term memory to be consolidated, new proteins must be produced, since the presence of inhibitors applied during the learning phase can induce amnesia of a newly learned memory (Nadel and Moscovitch, 1997;Schafe et al., 2001;Abel and Lattal, 2001;Dudai, 2002). Once long-term memory is established it becomes insensitive to these treatments. This process of memory consolidation, which is also dependent on gene transcription, bears several striking similarities with the establishment of late-phase LTP, linking synaptic plasticity to memory formation (Nadel and Moscovitch, 1997;Schafe et al., 2001;Abel and Lattal, 2001;Dudai, 2002).

However, early studies had already reported that electroconvulsive shocks could disrupt a previously consolidated memory and thereby induce retrograde amnesia, if the electroconvulsive shock was presented shortly after the reactivation or retrieval of the memory (Misanin et al., 1968). This indicates that memories become labile when retrieved and may require a second phase of consolidation or "reconsolidation". The relationship between consolidation and reconsolidation was recently addressed using the amygdala-dependent learning model of auditory fear conditioning (Nader et al., 2000), demonstrating that reactivation in the presence of a protein synthesis inhibitor is sufficient to induce

retrograde amnesia. This result suggests that retrieved memories may have to go through a process of reconsolidation, which is protein synthesis-dependent (Nader, 2003). This finding was then extended to hippocampus-dependent spatial learning paradigms (Debiec et al., 2002;Nader, 2003), and other systems (Eisenberg et al., 2003), suggesting that reconsolidation may be a general property of memories. Although the process of memory consolidation and reconsolidation shares the requirement of *de novo* protein synthesis, the initial hypothesis that reconsolidation might represent a recapitulation of the consolidation process appears not to hold. Two recent reports have pointed out that the molecular mechanisms involved in the two processes are distinct (Lee et al., 2004;Suzuki et al., 2004). Irrespective of the molecular mechanisms, these results reveal that the neural representation of memory is highly dynamic and plastic.

The "reconsolidation" theory added a labile and unstable property to consolidated memories and raised questions about how this may be implemented at the cellular level. The fact that L-LTP shares the protein synthesis dependence with long-term memory raises the question whether it is possible to re-sensitize L-LTP to protein synthesis inhibition by an appropriate form of synaptic reactivation. If, however, changes in synaptic efficacy result from a selective localization or stabilization of receptors in the neuronal plasma membrane, how is this dynamic state achieved and how is it compatible with a long-lasting maintenance of synaptic enhancement? Since a highly coordinated synthesis and trafficking of receptors and channels is probably required (Barry and Ziff, 2002;Inoue and Okabe, 2003;Bredt and Nicoll, 2003), a significant effort has been undertaken to understand how synaptic activation can be translated into gene transcription and specific synthesis and insertion of proteins into the membrane (Huang, 1999). Several cellular pathways have been identified that can be triggered by intracellular calcium rises and that lead to the translocation of transcription factors (West et al., 2002;Deisseroth et al., 2003). The best-studied example of information

transfer from the plasma membrane and the nucleus is the $Ca^{2+}$/cAMP responsive element binding protein (CREB). Synaptic activity can lead to intracellular calcium rise initiating a fast CaM-dependent kinase cascade and/or a slow MAP kinase cascade that result in CREB phosphorylation (Deisseroth et al., 1996;Deisseroth et al., 1998;Deisseroth et al., 2003). The nuclear translocation of CREB and triggered transcription of several genes, such as c-fos, Homer1b, zif/268, Arc and BDNF among others, have been shown to be necessary for L-LTP and long-term memory establishment, an additional similarity between these two processes (Silva et al., 1998;Lamprecht, 1999;Leil et al., 2003).

Most proteins are translated in the cell nucleus, but there is mounting evidence that protein translation also occurs in dendrites and spines, i.e. close to synaptic sites (Martin et al., 2000a). mRNA localization to and translation in specific sub-cellular compartments allows the molecular composition to change locally and very rapidly (Martin et al., 2000a). The original indication that translation might occur at synapses came from the discovery of synapses-associated polyribosomes complexes (SPRCs) (Martin et al., 2000a). Later, *in situ* hybridisation studies revealed a variety of mRNAs that were present in the dendritic compartment (Martin et al., 2000a). A number of recent reports addressed the question whether local protein synthesis is required for synapse plasticity. For example, studies of long-lasting facilitation in *Aplysia* sensory-motor synapses have shown that local protein synthesis is important for the retrograde signalling to the nucleus and the stabilization of synaptic strength (Martin et al., 2000a). Local protein synthesis has been implicated in changes in synaptic plasticity in the hippocampus. *De novo* protein synthesis upon LTP induction was demonstrated by monitoring changes in fluorescence of tagged proteins in dendrites that have been severed from the cell bodies. L-LTP could still be induced in theses dendrites suggesting that nuclear translation is not required (Martin et al., 2000a;Aakalu et al., 2001). A recent study from Bliss an colleagues (Bradshaw et al., 2003) showed that

protein synthesis inhibitors infused to either apical or basal dendrites affected the expression of LTP locally, whereas the application of the inhibitors to the cell bodies yielded no effect, demonstrating that local protein synthesis is sufficient for L-LTP to be established.

The local synthesis of proteins depends on a very tight localization of the mRNA or of the translation machinery to dendrites. Several examples from non-neuronal cells have identified *cis*-acting sequences in the 3' and 5' untranslated regions (UTR) of some mRNAs, which determines dendritic or axonal localization. Moreover, *trans*-acting regulatory proteins can bind to these sequences promoting localized translation. In neurons such a mechanism was found to regulate CamKIIα mRNA dendritic localization and translation (Martin et al., 2000a;Miller et al., 2002). Interestingly, a link between neuronal activity and regulated translation has been reported. The mRNA 3' UTR of CamKIIα contains a regulatory sequence (CPE) where CPEB (CPE binding protein) can bind, resulting in the translation of CamKIIα mRNA (Martin et al., 2000a;Mendez and Richter, 2001). CPEB is present in the dendritic compartment, and its activation can be regulated by activity (Wells et al., 2000;Wells et al., 2001).

In light of the link between plasticity and activity-dependent protein synthesis, it is conceivable that the maintenance of LTP, which requires *de novo* protein synthesis, may be affected by different rates of synaptic activation. To test this, I recorded LTP using different frequencies of synaptic activation while protein synthesis was blocked and measured the decay of LTP. In the Chapter II of this thesis, I show that LTP requires *de novo* protein synthesis in an activity-dependent fashion. Moreover, I show that for certain patterns of synaptic activation, protein synthesis inhibition applied after the induction of LTP can still be effective at destabilizing L-LTP, in contrast to the widely held assumption in the field that once LTP has entered the late phase it becomes insensitive to protein synthesis inhibition (Huang et al., 1996;Barco et al., 2002;Calixto et al., 2003;Cammalleri et al., 2003). This

result reveals an important role of synaptic activation after the induction of LTP for determining the protein synthesis-dependent stability of LTP.

The initial picture that protein synthesis is required for L-LTP expression became more complex with the discovery that activity-dependent protein degradation affects intracellular trafficking, selective localization and stabilization of receptors in the neuronal plasma membrane (Ehlers, 2003;Steward and Schuman, 2003;Pak and Sheng, 2003). In as much as *de novo* protein synthesis is required for L-LTP, mechanisms that inactivate, degrade or otherwise decrease the availability of these proteins should also play an important role for the stability of LTP. Recent evidence has shown that the process of protein ubiquitination, via the proteosome-dependent degradation pathway, can rapidly and reversibly diminish the pool of biological active proteins inside neurons (Hegde and DiAntonio, 2002;Cline, 2003). Several recent reports have shown that ubiquitination may be important for synaptic plasticity (Hegde et al., 1993;Hegde et al., 1997;Lopez-Salon et al., 2001;Weeber et al., 2003), but a direct role has not been observed for LTP. In Chapter III I present preliminary data that shows that proteosome activity is required for the induction of LTP and that protein turn-over is an important cellular variable for synaptic plasticity.

The fact that most forms of LTP and LTD are by and large input-specific and dependent on *de novo* protein synthesis a cellular mechanism is necessary by which new proteins are properly targeted to activated synapses destined to undergo potentiation. Delivery and targeting of products from the cell nucleus to specific synapses in a complex dendritic tree is a daunting task. Synthesized proteins need to be provided selectively to those synapses that undergo changes in strength (Steward and Schuman, 2001). If the source of new proteins is the soma, some protein transport and targeting mechanism must be involved in this process. The synaptic tagging hypothesis (Frey and Morris, 1997) proposes that synaptic activation creates a localized tag that then can redirect the proteins and/or mRNA

and promote the enhancement of the specific synapses. Although the nature of this tag is unknown the existence of this synaptic tag has been confirmed for several experimental preparations (Martin et al., 1997;Martin and Kosik, 2002). In the rat hippocampus, weak activation of the *Schaffer collaterals* can result in a short enhancement of synaptic potentials. By contrast, a stronger activation induces a long-lasting enhancement, which is dependent on translation and transcription. This short potentiation (E-LTP) can be transformed into a persistent one if a second input is strongly activated (see Figure 1). The hypothesis is that the weakly activated input can profit from potential "LTP"-molecules that had been synthesized in response to the previously activated strong input. According to the hypothesis, the tag acts as a sink for the diffusely distributed resources enabling the enhancement of weakly activated inputs (Frey and Morris, 1997;Frey and Morris, 1998a;Frey and Morris, 1998b).

Several specific requirements can be postulated for the tag: first, the setting of the tag has to be activity-dependent; second, the tag should be spatially restricted in nature; third, it has to be present for a limited time and show reversibility, and, fourth, it should be able to capture a potential "plasticity" molecule that is distributed throughout the cell triggered by LTP-type stimulation. In general anything that leaves an activity-dependent localized trace at synapses can be considered a tag. For example, synaptically activated protein kinases meet several of the criteria for a tag, since they can be activated in a reversible as well as spatially and temporally restricted manner (Martin and Kosik, 2002). CaMKII, PKC and PKA are some of the examples of such protein kinases. Other candidates, like adhesion molecules can provide the link to the cytoskeleton providing a structural component to synaptic changes (Martin and Kosik, 2002).

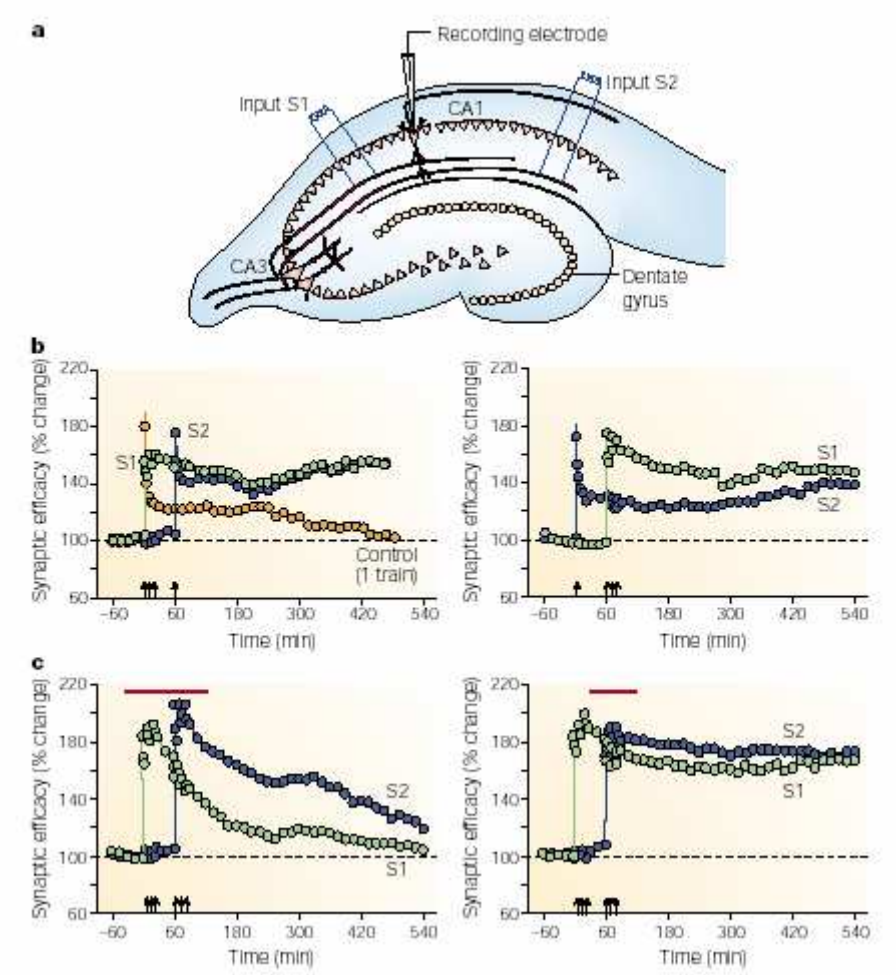



**Figure 1. Synaptic tagging in rodent hippocampus. a.** In a hippocampal slice, two stimulating electrodes are used to stimulate two independent pathways – S1 and S2 – that project to the same neuronal population in area CA1. **b.** A single train of high frequency stimulation to one pathway produces LTP that decays after 1.5 hours (E-LTP; control). By contrast, three trains produce L-LTP. If a single train is given to S2 either before (right panel) or after (left panel) and three titanic stimuli are applied to S1, persistent LTP occurs in both pathways. **c.** Blockade of protein synthesis by anisomycin (red bars) during the three trains of stimulation inhibits persistent LTP in both pathways (left panel). However, once L-LTP have been induced in S1, subsequent blockade of protein synthesis does not inhibited L-LTP in S2. *Adapted from: Synaptic tagging – Who's it?, Kelsey C. Martin and Kenneth S. Kosik, Nature Review Neuroscience 3 (2002), 813-820.*

The activation of local translation may also provide a powerful mechanism to integrate activity in both temporal and spatial domains. Synaptic activity can induce a controlled spatial and temporal release of dendritic mRNAs into a translationally active pool. mRNAs are present in dendrites as granules in a translational silent state and can be recruited in an activity-dependent manner by CPEB (see above). Simultaneously, activity-driven

increase in the availability of polyribosomes located near synaptic sites and/or specific translation factors (Martin and Kosik, 2002) can result in a localized increase in translation of a subset of mRNA rather than a global increase in protein synthesis. One very important feature of the synapse-associated polyribosome complexes (SPRCs) is the selectivity of their localization. The SPRCs are most often localized at the base of the spines, positioned beneath the postsynaptic sites and absent from other parts of the dendrites. A translational control together with this specific localization of the translational machinery may provide the means to tag a synapse (Steward and Schuman, 2001). The mechanisms that determine this synaptic localization of the SPRCs are unknown. Moreover, the constraint of a localized and regulated translation obviates the need for targeting the mRNA to specific synapses, requiring only a localization signal that targets them to the dendrites as opposed to axonal domains. BDNF, Arc and the α subunit of CaMKII mRNAs are good examples of dendritic localization. A link between synaptic activity, mRNA synthesis and trafficking to the dendrites has been demonstrated. For example, patterned synaptic activation of the entorhinal projections was shown to induce *Arc* expression in a layer-specific fashion along the dendrites of the dentate granule cells (Steward and Schuman, 2001).

The cytoskeleton is perhaps the major candidate if we are looking for mechanisms that can provide specific localization and transport of macromolecules. The actin network in neurons is extremely dynamic and its polymerization can be changed by activity (Matus et al., 2000;Martin and Kosik, 2002). For example, tetanic stimulation can lead to a remodeling of the actin cytoskeleton at the synapse both pre and post-synaptically and these changes are persistent, lasting for at least 18 hours after stimulation (Martin and Kosik, 2002). A dynamic actin cytoskeleton is required for LTP induction since drugs that lead to polymerization or depolymerization of actin can disrupt LTP (Kim and Lisman, 1999;Krucker et al., 2000). Moreover, LTP induction leads to an accumulation of F-actin in spines and leads to the

inactivation of cofilin, a protein involved in the depolimerization of the actin network (Fukazawa et al., 2003;Lisman, 2003a). Since actin dynamics required for LTP might be related to changes in protein composition of the potentiated or depressed synapses.

The requirement for "tags" have also been demonstrated for LTD, which like LTP also displays input specificity (Sajikumar and Frey, 2004). The study by Sajikumar, et al. showed that proteins can be "shared" between depressed pathways and between depressed and potentiated pathways, a phenomenon described by the authors as "cross-tagging – LTP and LTD tagging interactions" (Sajikumar and Frey, 2004). In other words, a set of synapses "tagged by potentiation" can benefit from proteins whose synthesis was induced by a previous episode of LTD induction. This result suggests that the "plasticity" proteins are common to both processes. However, the specificity of the tag is unclear. If the tag would be merely a docking site for these newly synthesized proteins then it may not show specificity for LTP or LTD "plasticity" proteins. That is to say that neither the "plasticity" proteins nor the tag are specific to potentiation or depression. In this view, the state of the synapse is maintained irrespective of the signalling cascades activated at the time of induction.

Theses reports indicate that proteins can be shared between synapses resulting in positive interactions. In Chapter IV, I provide evidence for negative interactions between multiple potentiated pathways. I discuss these findings in the context of a synaptic competition model, in which "plasticity" proteins are available in limiting amounts and are recruited to synapses in an activity-dependent way. The idea that synaptic connections can compete with one another has been widely demonstrated. During development competition between inputs is a powerful mechanism for the shaping of functionally mature circuits (Lichtman and Colman, 2000). Whereas competitive pruning and sprouting of synaptic connections during development appears to be an important and widespread process, it is unclear whether competition plays a significant role in the adult brain, for instance during

learning and memory formation. Therefore, it is conceivable that fine tuning of connections between cells proceeds in a competitive fashion. On the other hand, the cellular mechanisms that are involved in setting up the developmental competition can still be used to fine tune the properties of the post-natal circuitry.

Throughout my thesis I attempt to elucidate the role of synaptic activity for the dependence of LTP on protein synthesis and degradation. At first I show that under certain patterns of synaptic activation the stability of L-LTP requires sustained synthesis of proteins. I then show that these "plasticity" proteins, which are required for the maintenance of LTP, can be shared between two potentiated inputs in a competitive fashion. The results presented here show that, depending on the recent history of activity that the potentiated synapses have experienced, sustained *de novo* protein synthesis extending beyond the LTP induction phase is required for LTP to remain stable. This suggests that synaptic activation can re-sensitize L-LTP to protein synthesis inhibition, in contrast to the dogma in the field. Similarly, memory reactivation also re-sensitizes hitherto consolidated memory to protein synthesis inhibition, forming an intriguing analogy to the results presented here.

## CHAPTER II

# The interplay of protein synthesis and neuronal activity for the maintenance of late-phase LTP


## SUMMARY

Although protein synthesis inhibitors applied during LTP induction can block the establishment of late-phase LTP (L-LTP), it is widely held that application of protein synthesis inhibitors after the induction phase of LTP does not disrupt its expression. We show here that synaptic activation changes the sensitivity of LTP to protein synthesis inhibition. We found that the decay of LTP depends heavily on the test pulse stimulation frequency if protein synthesis is inhibited during LTP induction. We then designed a further stimulus protocol in which synaptic activation was omitted after the initial LTP induction. We found that protein synthesis inhibition while suspending synaptic activation, i.e. test pulse stimulation, did not have any effect on the maintenance of LTP. If, however, synapses were activated concurrent to the application of protein synthesis inhibitor a marked reduction in L-LTP was observed, suggesting that the requirement of protein synthesis during the maintenance phase is dependent on the pattern of synaptic activation following LTP induction.

## INTRODUCTION

Long-term potentiation (LTP) in the hippocampus is characterized by input specificity, associativity and persistence (Malinow et al., 2000;Bliss et al., 2003;Lisman, 2003b). The fact that brief bursts of synaptic activity can induce changes in synaptic transmission that persist for several hours or even days has made LTP the leading cellular model for memory formation. Analogous to memory formation, LTP can be divided into at least two stages or phases. In the first hours after induction of LTP, synaptic potentiation is largely achieved by direct and local modification of pre- and postsynaptic parameters, which affect synaptic efficacy (Soderling and Derkach, 2000;Nicoll, 2003). Simultaneously, *de novo* protein synthesis-dependent mechanisms, involving gene transcription and translation, develop and are responsible for maintaining the enhanced synaptic strength (Frey et al., 1988;Huang et al., 1996). The establishment of late-phase LTP (L-LTP) can be blocked by applying protein synthesis inhibitors during LTP induction (Frey et al., 1988), but later application of protein synthesis inhibitors does not affect L-LTP maintenance (Huang et al., 1996;Barco et al., 2002;Calixto et al., 2003;Cammalleri et al., 2003). This observation has given rise to the view that L-LTP, once it is established, is stable and insensitive to protein synthesis inhibition, since it depends only on a transient and not sustained up-regulation of protein synthesis after LTP induction.

Whereas it is well documented that stimuli that induce synaptic plasticity regulate gene expression (Deisseroth et al., 1996;Bito et al., 1996), recent evidence suggests that also baseline synaptic activation, stimuli that do not affect synaptic efficacy, can affect protein synthesis (Sutton et al., 2004). Moreover, it was recently shown that these types of stimuli can affect the maintenance of L-LTP (Villarreal et al., 2002;Zhou et al., 2003). In both of these reports synaptic activation associated with spontaneous activity or the test pulse

stimulation is effective in reducing LTP, suggesting that LTP decrement may involve an active mechanism (Zhou and Poo, 2004). Furthermore, it has recently been shown that synaptic activity can increase the turn-over of specific proteins that constitute the post synaptic fraction (PSD) (Ehlers, 2003). All this suggests that activity might have a fundamental role in modulating the availability of proteins involved in LTP expression and maintenance and raises the possibility that the sensitivity of L-LTP to post-induction application of protein synthesis inhibitors might be affected by the extent of baseline synaptic activation that potentiated synapses experience after LTP induction. We therefore reasoned that it might be possible to disrupt L-LTP by restricting the test-pulse stimulation after LTP induction, to the time window of protein synthesis inhibitor application.

Initially, we set out to test whether extent of baseline synaptic activation, i.e. the frequency of test-pulse stimulation, affects the decay of LTP when protein synthesis is blocked during induction. We observed that the decay of LTP depends strongly on the test-pulse stimulation frequency, suggesting that the requirement of LTP on protein synthesis is regulated by baseline synaptic activation. This prompted us to devise a protocol of discontinuous test pulse stimulation to unmask a requirement of sustained protein synthesis for L-LTP maintenance. We found that suspending synaptic activation after LTP induction and resuming it during protein synthesis inhibition applied during the maintenance phase of LTP, led to a pronounced decay of LTP, demonstrating a link between synaptic activation and the synthesis of proteins required for LTP maintenance.

## MATERIAL AND METHODS

*Slice preparation*

Male Wistar rats (3-4 weeks old) were decapitated under halothane anesthesia; the brains were quickly removed and immersed in ice-cold artificial cerebro-spinal fluid (ACSF). The ACSF was saturated with 95%$O_2$/5%$CO_2$ and contained (in mM): NaCl 124, KCl 3, $KH_2PO_4$ 1.25, $NaHCO_3$ 26, $MgSO_4$ 2, $CaCl_2$ 2.5, Glucose 10. The hippocampi were isolated and cut into 400 μm-thick transverse slices by a custom-made tissue slicer (Katz, 1987). Slices were maintained in ACSF at 20º C for at least one hour before recording. They were then transferred to a submersion chamber and perfused continuously (2 ml/min; medium recirculated) with ACSF at 32º C.

*Electrophysiological recordings*

Recordings started after a 20 min resting phase in the recording chamber. Schaffer collaterals were stimulated with 0.2 ms pulses using monopolar tungsten electrodes. Field excitatory postsynaptic potentials (fEPSP) were recorded extracellularly in the *stratum radiatum* of the CA1 region (~130 μm below slice surface) using glass microelectrodes filled with 3M NaCl (tip resistance 5-20MΩ). Stimulus intensities were set to evoke 50% of the maximal fEPSP slope. LTP was induced after recording a stable 20 min baseline of fEPSPs. The test pulse frequency was 0.1 Hz, unless stated otherwise.

*Induction of Long Term Potentiation*

Two stimulating electrodes were positioned in the *stratum radiatum* layer allowing us to activate two independent sets of Schaffer collaterals. Pathway independence was assessed by applying two pulses with 25 ms interpulse interval and confirming the absence of paired

pulse facilitation between the pathways. After recording a 20 minutes baseline, one of the pathways received a tetanus at a frequency of 100 Hz for 1 sec while the other one continued to be stimulated at 0.1 Hz. In some experiments, LTP was then recorded intermittently: test pulse stimulation is present for 20 minutes after the induction, during a second window of 20 min, 2 hours after induction and resumed continuously 2 hours after the end of this second window. The control pathway is monitored continuously (see schematic timeline of the experimental paradigms in the figures).

*Protein synthesis inhibitor and AP-5 treatment*

Anisomycin (Sigma) was dissolved in Dimethyl Sulfoxide (DMSO), and diluted down to achieve a final concentration of 25 μM (in 0.01% DMSO). Anisomycin at this concentration reliably blocks protein synthesis (Stanton and Sarvey, 1984;Frey et al., 1988). For the control experiments only DMSO (0.01%) was added to the Artificial Cerebral Spinal Fluid (ACSF). AP-5 (Sigma) was dissolved in water and diluted in ACSF to achieve a final concentration of 50μM.

*Data Analysis*

Electrophysiological data were collected using an Axoclamp 2B amplifier (Axon Instruments, Union City, CA) and band-pass filtered (low pass 1 kHz, high pass filter 1 Hz). Data were sampled at 5 kHz using a Lab-PC-1200 data acquisition board (National Instruments, Austin, TX) and stored on a PC. Offline data analysis was performed using a customized LabView-program (National Instruments). As a measure for synaptic strength the initial slope of the evoked fEPSPs was calculated and expressed as percent changes from the baseline mean. Error bars denote SEM values. To test for group differences between LTP values across conditions, a two-tailed Student's t-test was performed (green triangles in the

Figures indicate p values). For the t-test LTP values were averaged over 10 min data bins for the recordings up to 3.5 hours and over 20 min data bins for the recordings up to 10 hours.

## RESULTS

*Late-phase LTP is attenuated by protein synthesis blockers.*

In our first series of experiments we tested the effect of protein synthesis blockade on L-LTP establishment. We bath applied anisomycin from the beginning of the experiment and recorded a baseline by applying single test stimuli every minute to the Schaffer collaterals and recorded the compound population EPSP extracellularly in the CA1 region of hippocampal slices. After 40 minutes of baseline recording LTP was induced by a tetanic stimulation. Anisomycin was kept in the bath for a further hour and postsynaptic responses of test and control pathway were further recorded with a frequency of 0.017 Hz. Figure 2A shows that these experimental conditions resulted in reduced L-LTP (after approx. 2 hours) while the earlier phase of LTP (E-LTP) seems unaffected. This is in agreement with a large number of earlier studies, which have shown that anisomycin affects L-LTP while E-LTP is unaffected. Our experiments also confirmed earlier studies (Huang et al., 1996;Barco et al., 2002;Calixto et al., 2003;Cammalleri et al., 2003) in that they showed that ansiomycin had to be present during LTP induction to result in a reduction of L-LTP. Later application of anisomycin did not have any effect. If LTP was continuously recorded at a frequency of 0.1Hz, even a more prolonged application of protein synthesis inhibitor (3 hours) yields no effect o the maintenance of LTP (Figure 2B; green triangles denote p values).

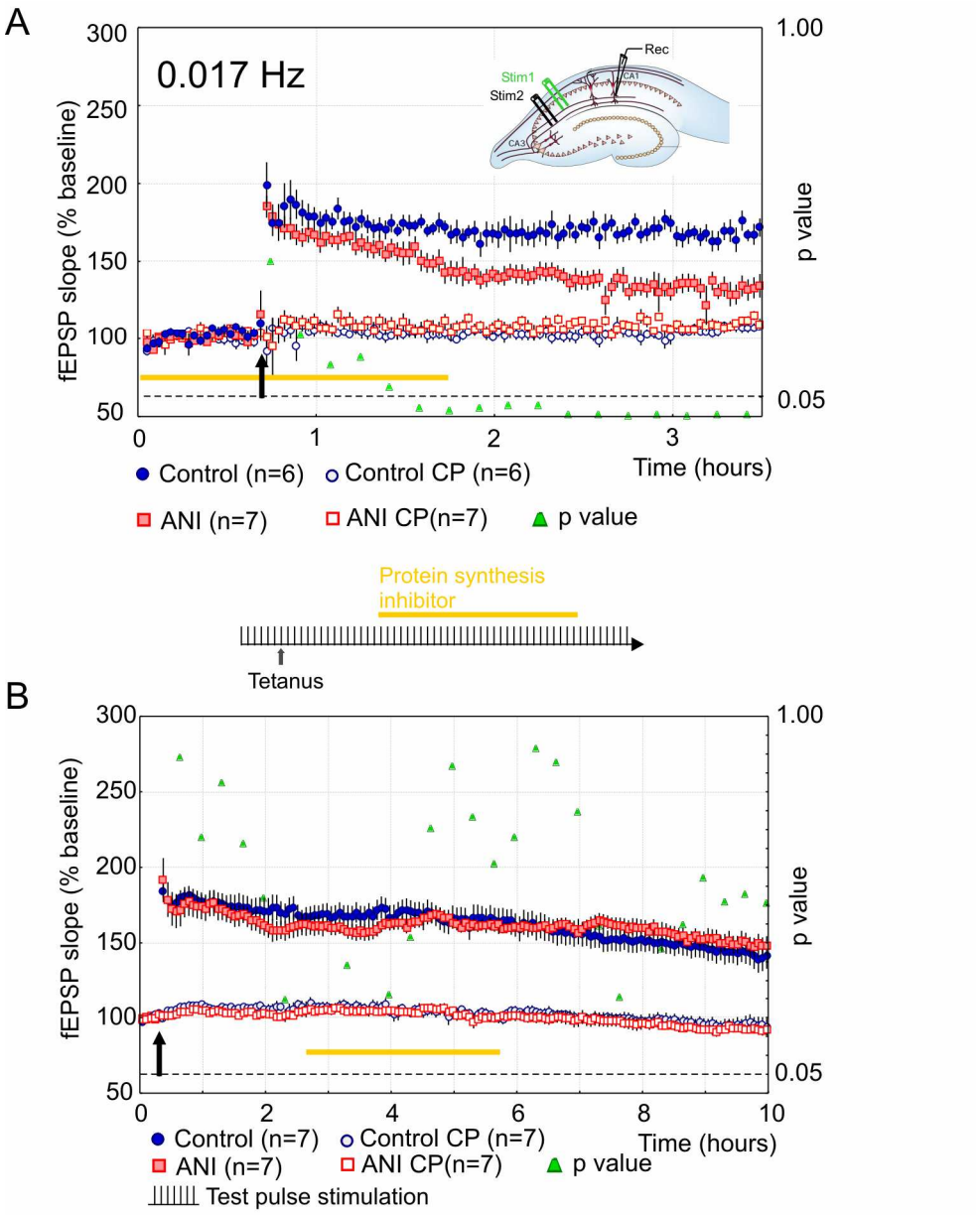

A
0.017 Hz
Rec
Stim1
Stim2
CA1
CA3
fEPSP slope (% baseline)
p value
300
250
200
150
100
50
1.00
0.05
0
1
2
3
Time (hours)
Control (n=6)
Control CP (n=6)
ANI (n=7)
ANI CP(n=7)
p value
Protein synthesis inhibitor
Tetanus
B
fEPSP slope (% baseline)
p value
300
250
200
150
100
50
1.00
0.05
0
2
4
6
8
10
Time (hours)
Control (n=7)
Control CP (n=7)
ANI (n=7)
ANI CP(n=7)
p value
Test pulse stimulation

Figure 2

**Figure 2. Late-phase LTP is blocked if protein synthesis is blocked during the induction but its maintenance is not affected by late applications of protein synthesis inhibitor.** **A.** Application of the protein synthesis inhibitor during the induction blocks L-LTP expression. LTP was induced by a single train of a tetanic stimulation, 100 Hz for 1 sec and recorded using a test pulse frequency of 0.017 Hz. Anisomycin (25 μM) was bath applied 40 minutes before and washed out 1 hour after the tetanus. Application of anisomycin does not alter basal synaptic transmission in a second independent pathway (open red symbols) but is efficient in blocking L-LTP induction (filled red symbols). LTP values for anisomycin treated slices are significantly lower than control slices (green triangles denote p values for statistical significance of mean differences). The inset illustrates the positioning of the two stimulating (Stim 1 and 2 for experimental and control pathway) and the recording electrodes. **B.** If LTP is recorded continuously at 0.1 Hz, application of anisomycin more than two hours after LTP induction does not impair L-LTP (see diagram for experimental timeline). n: number of slices.

*The test pulse frequency determines the decay of LTP when protein synthesis is blocked.*

We had noticed that virtually all earlier studies had worked at very low stimulation frequencies of 1/min or lower (Frey et al., 1988;Huang et al., 1996). Moreover recent studies have reported that baseline synaptic activation can have an effect in protein turn-over and L-LTP stability (Ehlers, 2003;Kelleher, III et al., 2004;Sutton et al., 2004). We therefore reasoned that it is not unlikely that baseline synaptic activation at higher frequencies might actually affect LTP decay when protein synthesis is inhibited by anisomycin or other protein synthesis blockers. We therefore tested this by recording LTP using different frequencies of test-pulse stimulation. We induced LTP in the presence of anisomycin and measured the decay of LTP with test pulse frequencies of 0.2, 0.1, and 0.033 Hz. Anisomycin was again bath applied 40 min before and was washed out 1 hour after LTP induction. In the control conditions without anisomycin robust LTP was induced and maintained irrespective of the test pulse frequency. In the presence of anisomycin however L-LTP expression was dramatically reduced and the decay was dependent on the frequency of the test pulse (Figure 3A). Interestingly however the effect of the varying test-pulse frequencies was not limited to late phase LTP but it was apparent also in the early phases of LTP, already 30 minutes after the initial induction by the tetanic stimulation. The percentage decay of LTP values within the first half-hour after induction depended strongly on the stimulation frequency (34 ± 4% for 0.2 Hz; 29 ± 2% for 0.1 Hz; 22 ± 4% for 0.033 Hz; 10 ± 3% for 0.017 Hz; Figure 3B). For control conditions the decay within the first half-hour after induction was small (10 ± 3% for 0.2 Hz; 6 ± 2% for 0.1 Hz; 9 ± 5% for 0.033 Hz; 5 ± 3% for 0.017 Hz) and it did not depend on the test pulse frequency (Figure 3B). Interestingly, the frequency of test-pulse stimulation did not influence the percentage decay in LTP values reached at the end of the recorded time, when protein synthesis was blocked but rather the rate at which this value was reached

(Figure 3C). In all experiments a second control pathway was monitored throughout the experiment. Neither in anisomycin nor in control experiments a change in synaptic transmission was observed (Figure 2A and 3A). To check for possible non-specific effects of anisomycin, we also made use of the protein synthesis blockers cycloheximide and emetine (Stanton and Sarvey, 1984). For both inhibitors we found the same effect as for anisomycin at a test pulse frequency of 0.1 Hz (data not shown).

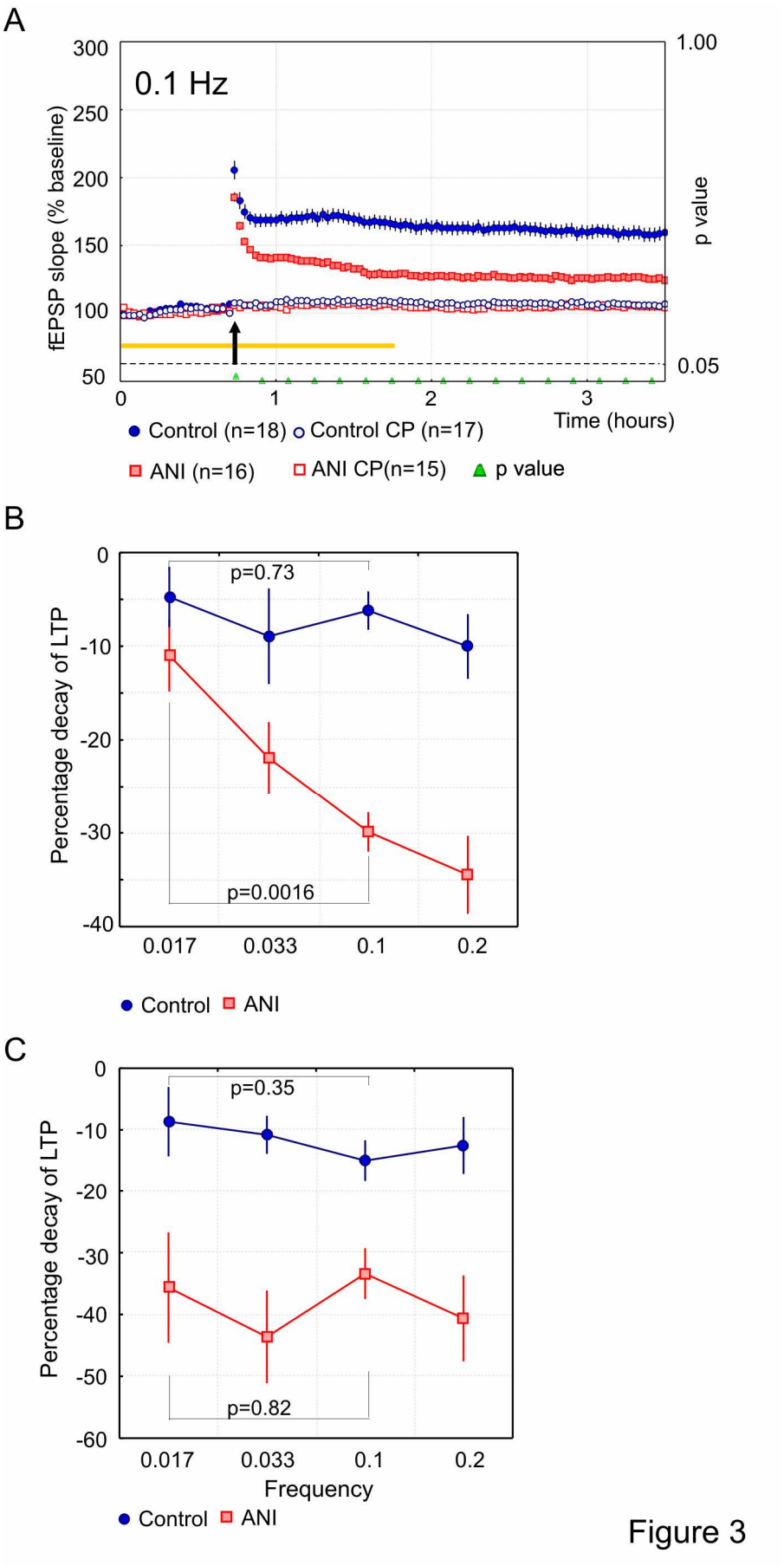

A
0.1 Hz
fEPSP slope (% baseline)
300
250
200
150
100
50
1.00
p value
0.05
0
1
2
3
Time (hours)
Control (n=18)
Control CP (n=17)
ANI (n=16)
ANI CP(n=15)
p value
B
Percentage decay of LTP
0
-10
-20
-30
-40
p=0.73
p=0.0016
0.017
0.033
0.1
0.2
Control
ANI
C
Percentage decay of LTP
0
-10
-20
-30
-40
-50
-60
p=0.35
p=0.82
0.017
0.033
0.1
0.2
Frequency
Control
ANI

Figure 3

**Figure 3. The test pulse frequency determines the decay of LTP when protein synthesis is blocked. A.** LTP was recorded using a test pulse frequency of 0.1 Hz. At this frequency protein synthesis inhibition results in a similar blockade of LTP, but with a faster decay of LTP than for 0.017 Hz (green triangles denote p values). **B/C.** The frequency of test pulse stimulation influences the decay of LTP when protein synthesis is blocked but does not change the percentage of inhibition. Summary plot for the effect of test pulse frequency on the decay of LTP when protein synthesis is blocked. Plotted is the percentage decay in LTP values for anisomycin and control slices within the first 30 minutes after LTP induction (B) and at the end of the recording (C), for the four stimulating frequencies used (0.2, 0.1, 0.033 and 0.017 Hz). n: number of slices.

*The sensitivity of L-LTP to protein synthesis inhibition is dependent on concurrent synaptic activation*

In light of reports in the literature that synaptic activation can result in an increase in protein turn-over, our explanation for the above results was that interference of anisomycin with the synthesis of new proteins due to the concurrent synaptic activation was causing the decay in potentiation. And this decay was stronger and affected earlier components for comparatively higher stimulation frequencies (such as 0.1 Hz) and less pronounced for very low frequencies of 0.017 Hz or below (Frey et al., 1988;Huang et al., 1996). This interpretation made us predict that if synaptic activation was ceased after the induction of LTP, anisomycin application during the maintenance phase of LTP, while stimulating the synapse for a brief period, might disrupt LTP maintenance. Figure 4A shows such an experiment where LTP was induced after 20 minutes of baseline stimulation and monitored for a short time thereafter (20 minutes). Following that period, stimulation was ceased altogether and anisomycin was applied for 100 minutes, starting 1 hour after synaptic activation has been suspended. Electrical test-pulse stimulation was recommenced, 2 hours afterwards, to ensure the washout of the drug, and the synaptic strength compared to the values measured before pausing the stimulation. Figure 4A shows that potentiation values after the long intermission in electrical stimulation are indistinguishable for anisomycin (red squares, p values between control and anisomycin-treated slices indicated as green triangles) and control cases (163 ± 8 % n = 8, compared with 156 ± 7%, n = 9; p = 0.63). In other words, application of anisomycin without concurrent stimulation did not have, as predicted, any effect in LTP maintenance.

Our hypothesis that synaptic activation during protein synthesis inhibition is the reason for the observed decay in LTP let us make the further prediction that an identical

experiment to the one shown in Figure 4A but with electrical stimulation during the time of anisomycin application should again result in a decease in LTP. Figure 4B shows that this is indeed what happens. If stimulation is resumed briefly (for 20 minutes) during the application of anisomycin (red squares, p values between control and anisomycin-treated slices indicated as green triangles), LTP as it is tested later is indeed weakened considerably (124 ± 5%, n = 13, compared to 161 ± 5%, n = 14; p = 0.00004).

The experiment described above shows an interesting parallel to the susceptibility of reconsolidation to protein synthesis inhibition first reported for fear conditioning in the amygdala (Nader et al., 2000). In these experiments a (fear) memory is induced in a behavioral paradigm (loosely equivalent to our induction of LTP). If this memory was later "reactivated" by the presentation of the conditioning stimulus while protein synthesis is inhibited, the memory "faded" whereas in the control situation the fear memory remained stable. Also in our case, if potentiated synapses were reactivated under protein synthesis inhibition the potentiation was abolished whereas in the control case the potentiated synapses remain unaffected. We will further elaborate on this resemblance of the effects in the discussion section.

To test whether NMDA receptor activation might play a role in this effect we performed the same experiment as the one shown in Figure 4B but in the presence of AP-5, a selective antagonist of NMDA glutamate receptors. We found that indeed activation of NMDA receptors is needed for the decay in LTP to occur (Figure 4C; red squares, p values between control and AP-5/anisomycin-treated slices indicated as green triangles). When anisomycin was added together with APV, LTP values at the end of the recording were not statistically different from those in control experiments (AP-5/ani: 171 ± 9%, n = 8; AP-5: 162 ± 5%, n = 6; p= 0.46).

As noted above, our working hypothesis was that interference with protein synthesis caused by the test-pulse stimulation is in part responsible for the decrease in LTP that is observed after the application of anisomycin, during or after LTP. We therefore asked ourselves whether synaptic activation after the induction of LTP might help to stabilize LTP. Indeed, Figure 5A shows that if synaptic stimulation was continued for 1 hour after induction of LTP this rendered synapses insensitive to later disruption due to concurrent application of anisomycin and presynaptic stimulation. No statistical difference in LTP values was observed between anisomycin-treated slices and control (Figure 5A), nor with the LTP values measured at the end of the ten hours of recording for anisomycin treated slices obtained in the experiments shown in Figure 4A and Figure 4C (156 ± 7%, n = 8; 171 ± 9%, n = 8).

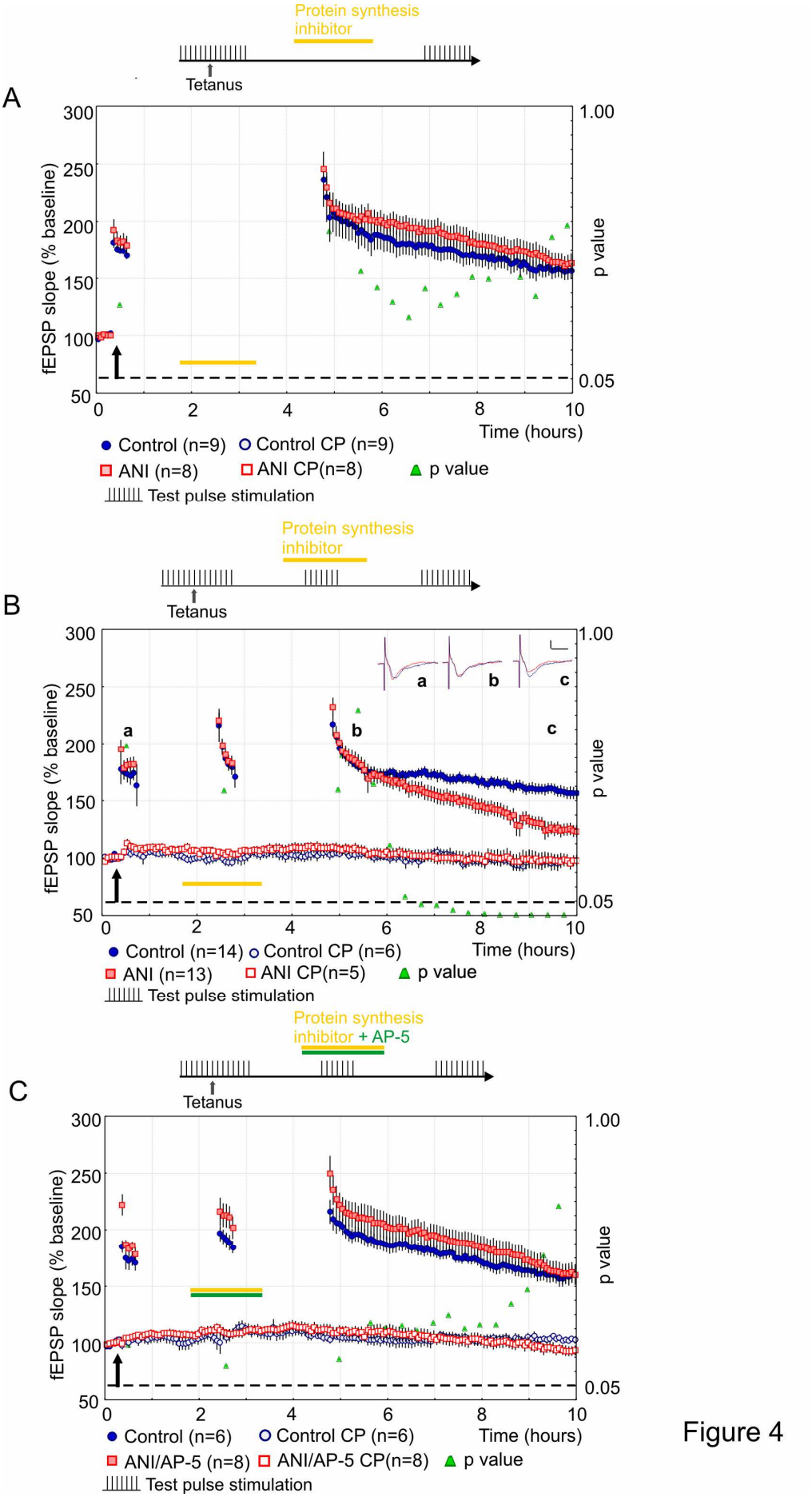
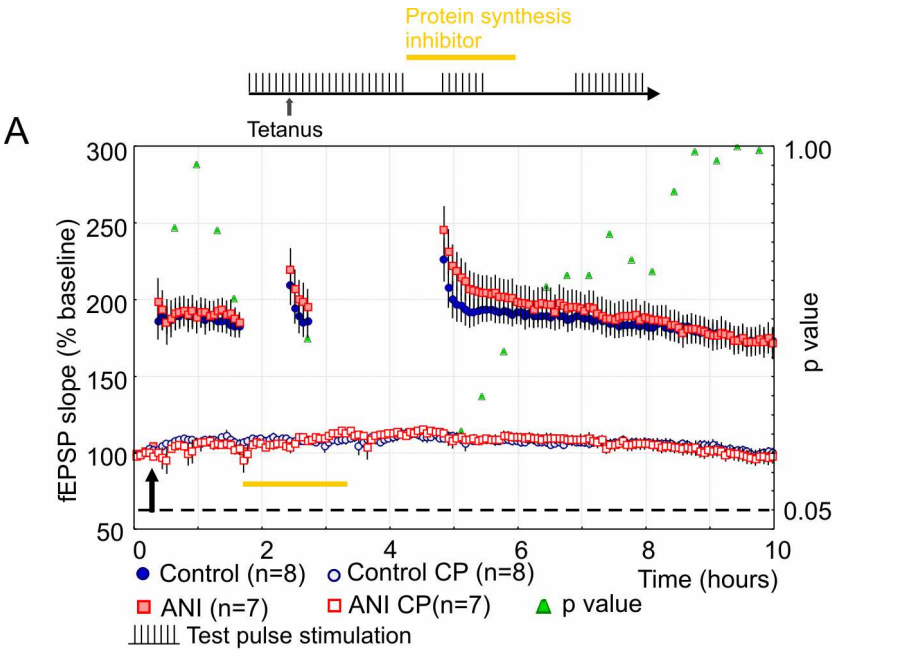

A
Protein synthesis inhibitor
Tetanus
fEPSP slope (% baseline)
p value
Time (hours)
Control (n=9)
Control CP (n=9)
ANI (n=8)
ANI CP(n=8)
p value
Test pulse stimulation
B
Protein synthesis inhibitor
Tetanus
a
b
c
Control (n=14)
Control CP (n=6)
ANI (n=13)
ANI CP(n=5)
p value
Test pulse stimulation
C
Protein synthesis inhibitor + AP-5
Tetanus
Control (n=6)
Control CP (n=6)
ANI/AP-5 (n=8)
ANI/AP-5 CP(n=8)
p value
Test pulse stimulation

Figure 4

**Figure 4. The disruption of L-LTP maintenance by protein synthesis inhibition is dependent on concurrent synaptic activation and requires NMDA receptor activation. A.** If LTP was recorded intermittently after its induction, protein synthesis inhibition during its maintenance phase did not lead to any change in LTP values. LTP was recorded, at a frequency of 0.1 Hz, for 20 minutes after induction and resumed continuously 4 hours later. Anisomycin was bath applied for 1hour and 40 minutes during the maintenance phase of LTP, starting 1 hour after the interruption of the test-pulse stimulation. Protein synthesis inhibition did not lead to any change in LTP values compared to controls (filled red and blue symbols; green triangles denote p values). **B.** If, however, synaptic activation was present during the time window of anisomycin application, LTP values were reduced in a pronounced way, as measured later on. (filled red symbols). Experiment similar to figure 3A but synaptic activation was now present concurrently to protein synthesis inhibition (yellow bar). In some of the experiments a second control pathway was also recorded (open symbols) showing no appreciable change in basal synaptic transmission over the recorded time. Inset, individual fEPSPs traces for ANI and control conditions at times indicated scale: 1mV, 10ms.**C.** Experiment similar to the one depicted in Figure 3B, but the NMDA receptor antagonist AP-5 was co-applied with anisomycin. No difference was observed for LTP values between ANI/AP-5 treated slices (red symbols) and only AP-5 treated slices (blue symbols), green triangles for p values. A second, naive control pathway was monitored during the ten hours of recording and no appreciable change was observed in basal synaptic transmission for anisomycin/AP-5 or AP-5 treated slices (open symbols). n: number of slices.


A
Protein synthesis inhibitor
Tetanus
fEPSP slope (% baseline)
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250
200
150
100
50
1.00
p value
0.05
0
2
4
6
8
10
Time (hours)
Control (n=8)
Control CP (n=8)
ANI (n=7)
ANI CP(n=7)
p value
Test pulse stimulation

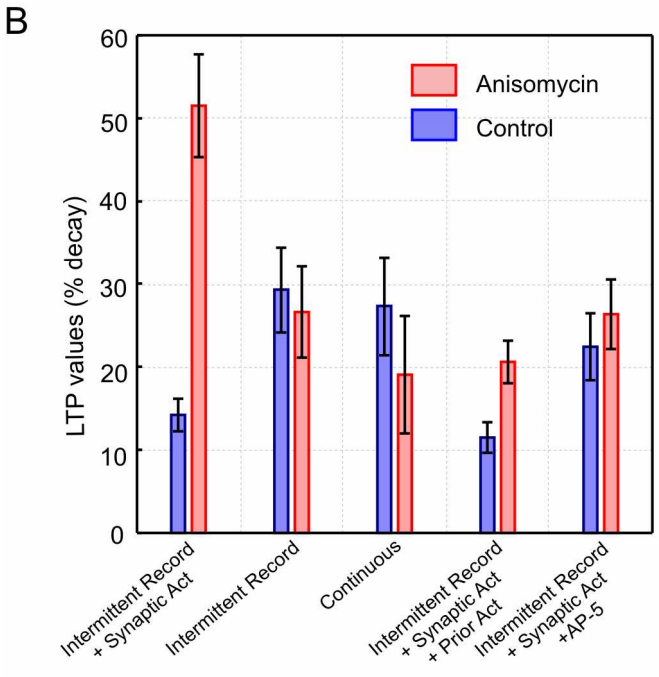
B
LTP values (% decay)
60
50
40
30
20
10
0
Anisomycin
Control
Intermittent Record + Synaptic Act
Intermittent Record
Continuous
Intermittent Record + Synaptic Act + Prior Act
Intermittent Record + Synaptic Act +AP-5

Figure 5

**Figure 5. The sensitivity of L-LTP to concurrent synaptic activation and protein synthesis inhibition is abolished by preceding synaptic activation. A.** If the test pulse is applied for one hour after LTP induction until the onset of anisomycin application, L-LTP disruption induced by concurrent synaptic activation and protein synthesis inhibition was rescued (filled red and blue symbols; green triangles denote p values). **B.** Summary plot showing the percentage decay in LTP values between two 20 minutes data bins at 300-320 minutes and 560-580 minutes for all performed experiments (Fig 3A, 3B and 3C; Fig 4A). The decay in LTP was largest for the experiment where synaptic activation was absent after LTP induction but synapses were concurrent activated with protein synthesis inhibition ($p<0.001$). n: number of slices.

## DISCUSSION

While L-LTP has always been postulated to depend on the ability to produce new proteins during the induction procedure it has been posited that E-LTP is largely insensitive to protein synthesis inhibition (Frey et al., 1988;Huang et al., 1996). Moreover, it has been suggested that protein synthesis inhibition after the initial induction procedure would not affect E-LTP nor L-LTP (Huang et al., 1996;Barco et al., 2002;Calixto et al., 2003;Cammalleri et al., 2003). Our results show that things are more complicated. Whereas low test-pulse stimulation frequencies of 1/minute makes it seem as if E-LTP is indeed not affected by application of anisomycin during and after the induction procedure, tests at higher stimulation frequencies show that also E-LTP can in fact depend on protein synthesis. We found that anisomycin and other protein synthesis blockers applied during and after the LTP inducing stimulus results in a clear dependency of the strength of LTP on the frequency of test stimulation. Higher stimulation frequencies led to a stronger decay in LTP values. Interestingly a similar effect of protein synthesis inhibition in the early-phase of LTP was reported for the mossy fiber LTP using a test-pulse frequency of 0.1 Hz. However, the link to different frequencies of the test-pulse stimulation that have been used in the literature was not established (Calixto et al., 2003).

Although we cannot come forward with a molecular explanation for this observation this finding is consistent with a scenario where the synaptic activation contributes for a increased susceptibility of LTP to protein synthesis inhibition by a process of synaptic destabilization. If the synaptic activation is weak or moderate (e.g. at stimulation frequencies of 0.017 Hz) the destabilization is lower and the requirement for *de novo* protein synthesis is accordingly lower. If however activation becomes more frequent substantial destabilization occurs and results in a clear decrement of LTP when protein synthesis is inhibited.

This interpretation is in line with the observations from our second series of experiments where the decay of LTP does not occur if the protein synthesis inhibitor was applied during a phase where no test-pulse stimulation is present, while LTP is diminished when test stimulation and protein synthesis are applied simultaneously. This scenario proposes that proteins are produced, either constitutively or due to synaptic activation, which protects the potentiated synapses against the destabilizing effect of synaptic activation. If the production of these proteins is blocked by a protein synthesis inhibitor, synaptic activation will cause a decay of the previously potentiated synapses. Our data do not allow us to pinpoint the proteins needed for the protection of LTP, but they underscore the possible confounding effects that the test–pulse stimulation classically used in LTP recordings may exert on the time course and protein synthesis dependence of LTP.

As mentioned above, our results show a striking similarity to the reconsolidation phenomenon observed *in vivo* (Nader et al., 2000). Studies initially performed in an amygdala-dependent learning model of auditory fear conditioning (Nader et al., 2000) and later extended to other systems (Debiec et al., 2002;Eisenberg et al., 2003), have shown that memory reactivation re-sensitizes the respective forms of memory to protein synthesis blockade. If a memory is reactivated during the application of protein synthesis blocker to the respective brain region this memory is subsequently abolished. In analogy, our results show that synaptic reactivation by the test pulse stimulation can re-sensitize LTP to protein synthesis inhibition. Moreover, the effect of activity on destabilization of LTP is dependent on NMDA receptor activation, which is also required for the disruption of memories as described for reconsolidation phenomena (Debiec et al., 2002). Furthermore, a recent study has shown that multiple learning sessions produce ‘stronger’ memories less susceptible to disruption by protein synthesis inhibition during reactivation (Suzuki et al., 2004). Our data again bear some similarities to this finding as synapses, when activated sufficiently after

LTP induction, become resistant to protein synthesis inhibition during the maintenance phase. Although we are intensely aware of the danger of such analogies we were struck by the similarities between our in vitro experiments and the behavioral data on (re-) consolidation. Whether our approach can be carried further to provide a cellular analogue to the *in vivo* reconsolidation phenomenon or not, we think that it will provide a useful procedure to probe for molecular mechanisms that might be involved in the different steps of LTP induction and maintenance.

## CHAPTER III

# The late phase of long-term potentiation (L-LTP) requires proteosome activity for its expression


## SUMMARY

The rates of protein synthesis and protein degradation are key determinants for the availability of proteins inside neurons. Although the role of protein synthesis for synaptic plasticity is well documented, the roles of protein degradation, and thus protein turnover in general, have only recently come into focus. Here, I examine the effects of blocking both protein degradation as well as protein synthesis during the induction phase of LTP on the stability of late-phase LTP. I show that pharmacological inhibition of proteosome-dependent protein degradation results in a blockade of late-phase LTP establishment. Surprisingly, late-phase LTP was partially rescued when protein degradation and protein synthesis were blocked at the same time. My results show that establishment of the late phase of LTP depends on a highly dynamic interplay between protein synthesis and degradation.

## INTRODUCTION

Synaptic activity can induce a variety of changes within synapses, relying on rapid posttranslational modifications as well as sustained synthesis of proteins and mRNAs. Although it is well established that long-term changes in synaptic efficacy require *de novo* protein synthesis (Frey et al., 1988;Huang et al., 1996), the role of the ubiquitin-proteosome dependent degradation in synaptic plasticity is only starting to be revealed. Providing a cellular mechanism that can fine-tune protein availability in the cell, ubiquitination is a general mechanism by which proteins can be targeted for degradation via de proteosome pathway or transiently sequestered from the available functional pool (Hegde and DiAntonio, 2002;Cline, 2003;Cremona et al., 2003). Although proteosome inhibition by pharmacological means can indeed result in an accumulation of proteins involved in LTP (Ageta et al., 2001a;Ageta et al., 2001b;Ehlers, 2003), the effect of proteosome blockade in LTP has not been directly investigated. If protein degradation is merely a mechanism to counterbalance protein synthesis, the net effect of inhibiting the proteosome-dependent degradation pathway would be expected to result in an accumulation of "plasticity" proteins and thus elevated or prolonged LTP expression levels.

To address this question I induced LTP in the presence of lactacystin, an irreversible inhibitor of the proteosome (Dick et al., 1996). I observe, however, that inhibition of proteosome-dependent protein degradation blocked the establishment of L-LTP. Moreover, the decay of LTP values for lactacystin-treated slices depended on the frequency of the test pulse stimulation, indicating an activity-dependent requirement of protein degradation for L-LTP. Interestingly, co-application of pharmacological blockers of protein synthesis and protein degradation partially rescued the disruptive effect on L-LTP when either drug is

applied alone, suggesting that the effects of both pharmacological agents offset each other when applied at the same time. This is surprising because the direction of their individual effects is the same, namely disruptive for the stability of L-LTP, but indicates that a interaction between the two processes might regulate LTP induction and maintenance.

## MATERIAL AND METHODS

*Slice preparation and electrophysiological recordings*

The detailed procedure for acute hippocampal slice preparation and recording of extracellular fEPSPs was described previously (see Material and Methods, Chapter II). In brief, 400 µm acute slices were prepared from male wistar rats (3-4 weeks old). Recordings started after a 20 min resting period in the recording chamber. Schaffer collaterals were stimulated with 0.2 ms pulses using monopolar tungsten electrodes. Stimulus intensities were set to evoke 50% of the maximal fEPSP slope and LTP was induced after recording a stable 20 min baseline of fEPSPs. The test pulse frequency was 0.1 Hz, unless stated otherwise. Two stimulating electrodes were positioned in the *stratum radiatum* layer, allowing us to stimulate two independent sets of Schaffer collaterals. After baseline stimulation, one of the pathways was arbitrarily chosen to receive a tetanus at a frequency of 100 Hz for 1 sec. The control pathway was monitored continuously. Offline data analysis was performed using a customized LabView-program (National Instruments). As a measure for synaptic strength the initial slope of the evoked fEPSPs was calculated and expressed as percent changes from the baseline mean. Error bars denote SEM values. To test for group differences between LTP values across conditions, a two-tailed Student's t-test was used (green triangles indicate p values). To this end LTP values were averaged over 10 min data bins.

*Proteosome inhibitor treatment*

Lactacystin (Sigma) was dissolved in DMSO, and diluted down to achieve a final concentration of 10 µM (in 0.01% DMSO). Lactacystin was bath applied for 40 min before and washed out one hour after LTP induction depending on the experiments. For the control experiments only DMSO (0.01%) was added to the ACSF.

## RESULTS

*Protein degradation is required for L-LTP establishment*

To test whether protein degradation is important for late-phase LTP (L-LTP) establishment, LTP was induced in the presence of lactacystin (10μM), an irreversible proteosome inhibitor. Lactacystin was bath-applied for 40 minutes and washed out one hour after LTP induction. fEPSPs were recorded extracellularly at a frequency of 0.1 Hz and LTP was induced by tetanic stimulation (100 Hz for 1 sec; 0.2 ms pulse duration). Proteosome inhibition by lactacystin blocked L-LTP establishment (Figure 6A). LTP values for lactacystin treated slices (filled red symbols) were significantly lower than control values at the end of the recording (139.57 ± 5.32; n=9 compared to 168.96 ± 4.89; n=8; green triangles denote p values). A separate control pathway was recorded simultaneously, showing no decrement, indicating that lactacystin application had no effect on basal synaptic transmission. Previously, we have shown that the requirement of LTP for protein synthesis is strongly dependent on the frequency of synaptic activation, i.e. the frequency of test pulse stimulation (Figure 3; Chapter II). To test whether a similar synaptic activity-dependent effect on the decay rate of LTP exists when the proteosome-dependent protein degradation is blocked, the test pulse stimulation frequency was increased to 0.2 Hz. Figure 6B shows that increasing the test pulse frequency to 0.2 Hz in the presence of lactacystin resulted in a profound reduction in LTP values. LTP values for lactacystin-treated slices (filled red symbols) are significantly lower than control values (112.71 ± 3.02; n=6 compared to 172.02 ± 13.44; n=5; green triangle denote p values) at the end of the recording. The percentage decay in LTP values over the first half an hour was significantly increased for higher test

pulse frequencies (0.2 Hz vs 0.1 Hz) for lactacystin-treated slices (Figure 6C; 16.5 ± 3 % compared to 27.7 ± 4 %; $p = 0.03$ ).

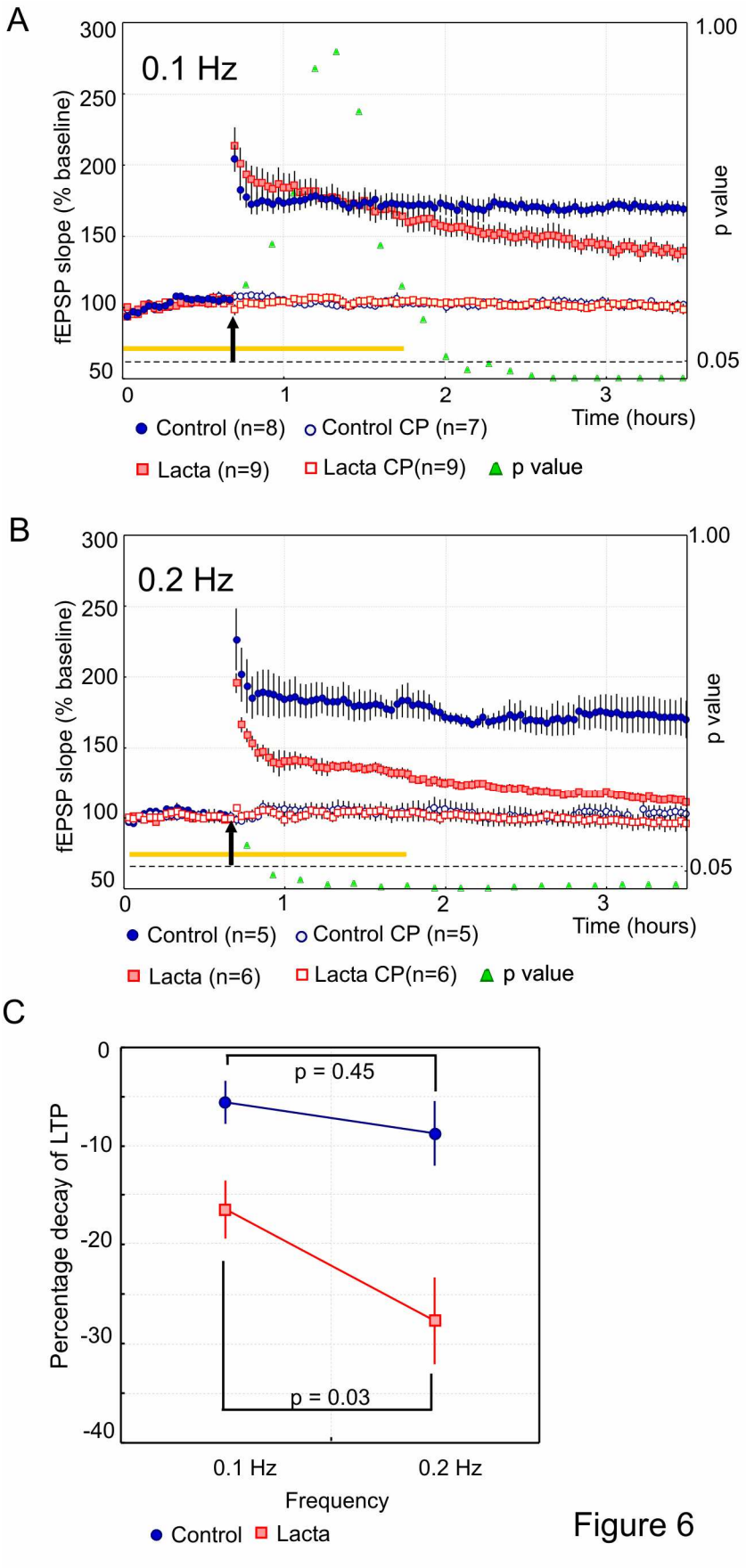
A
0.1 Hz
fEPSP slope (% baseline)
p value
Time (hours)
Control (n=8)
Control CP (n=7)
Lacta (n=9)
Lacta CP(n=9)
p value
B
0.2 Hz
fEPSP slope (% baseline)
p value
Time (hours)
Control (n=5)
Control CP (n=5)
Lacta (n=6)
Lacta CP(n=6)
p value
C
Percentage decay of LTP
p = 0.45
p = 0.03
0.1 Hz
0.2 Hz
Frequency
Control
Lacta

Figure 6

**Figure 6. Protein degradation is required for late-phase LTP establishment. A.** L-LTP establishment was blocked by inhibition of proteosome-dependent protein degradation. fEPSPs were recorded at a frequency of 0.1 Hz. LTP values for lactacystin treated slices (filled red symbols) were significantly lower than control values (blue filled symbols) at the end of the recording (139.57 ± 5.32; n=9 compared to 168.96 ± 4.89; n=8; green triangles denote p values). A second independent control pathway was recorded simultaneously, showing no decrement in basal synaptic transmission (open symbols). The green triangles denote p values (two-tailed Student's T-test) for 10 minutes data bins (see methods) **B.** Same experiment as in **A**, but fEPSP were recorded using a test pulse frequency of 0.2 Hz. LTP values for lactacystin-treated slices (red filled synbols) are significantly lower than control values (blue filled symbols) at the end of the recording (112.71 ± 3.02; n=6 compared to 172.02 ± 13.44; n=5; green triangles denote p values). **C.** Summary plot for the effect of the test pulse stimulation frequency on the decay of LTP. Plotted is the percentage decay of LTP values for lactacystin and control slices over the first half hour after LTP induction. n: number of slices.

*Protein synthesis inhibition can partially rescue the effect of proteosome inhibition on LTP*

Protein synthesis and degradation are antagonistic mechanisms that determine the availability of proteins in the cell. Even though inhibiting protein degradation is expected to lead to an increase in the availability in "plasticity" proteins and therefore to an increase in LTP maintenance, we observed the opposite effect. Inhibiting protein degradation caused a pronounced blockade in L-LTP establishment. This can be because the degradation of particular proteins that might act as negative regulators is required for L-LTP. Alternatively, inhibition of protein degradation can result in an accumulation of these proteins leading to synapse saturation. This last hypothesis makes the prediction that if protein degradation and protein synthesis are simultaneously inhibited than this accumulation of proteins would not occur and L-LTP establishment might be rescued. To address this question, LTP was induced in the presence of anisomycin (25μM), a reversible protein synthesis inhibitor, and lactacystin (10μM) (Figure 7A). Both drugs were bath-applied 40 minutes before and washed out 1 hour after LTP induction. LTP values for the anisomycin/lactacystin treated slices (filled red symbols) were significantly lower than controls (162.35 ± 4.64; n=9 compared to 182.56 ± 6.5; n=7; green triangles denote p values) at the end of the recording. Figure 7B and 7C shows that, however, LTP values for anisomycin/lactacystin treated slices were significantly higher than for lactacystin alone (162.35 ± 4.64; n=9 compared to 139.57 ± 5.32; n=9; p = 0.003) or for anisomycin alone treated slices (162.35 ± 4.64; n=9 compared to 126.57 ± 3.5; n=19; p = 0.00005).

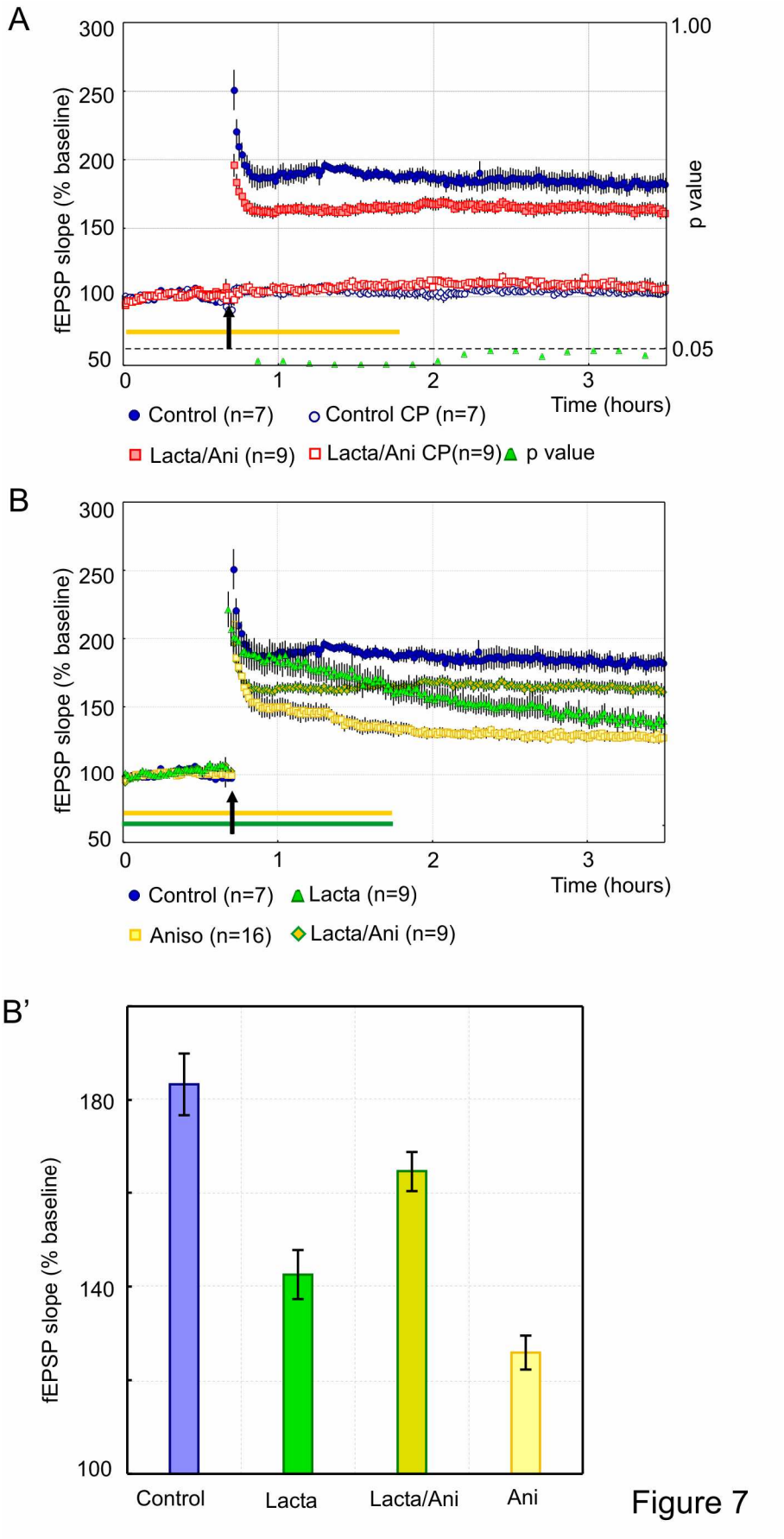
A
300
250
200
150
100
50
fEPSP slope (% baseline)
1.00
p value
0.05
0
1
2
3
Time (hours)
Control (n=7)
Control CP (n=7)
Lacta/Ani (n=9)
Lacta/Ani CP(n=9)
p value
B
300
250
200
150
100
50
fEPSP slope (% baseline)
0
1
2
3
Time (hours)
Control (n=7)
Lacta (n=9)
Aniso (n=16)
Lacta/Ani (n=9)
B'
180
140
100
fEPSP slope (% baseline)
Control
Lacta
Lacta/Ani
Ani

Figure 7

**Figure 7. A. Blocking protein synthesis partially rescues the disruptive effect of proteosome inhibition on L-LTP.** LTP was induced in the presence of anisomycin and lactacytin and recorded using a test pulse frequency of 0.1 Hz. LTP was induced and maintained for more than three hours. LTP levels in anisomycin/lactacystin treated slices were lower than obtained in control experiments (162.35 ± 4.64; n=9 compared to 182.56 ± 6.5; n=7; green triangles denote p values). **B.** Inhibition of protein synthesis can partially reverse the effect of proteosome inhibition on L-LTP establishment. LTP values for anisomycin/lactacystin treated slices were higher than for lactacystin treated slices (162.35 ± 4.64; n=9 compared to 139.57 ± 5.32; n=9; p = 0.003) or for anisomycin treated slices (162.35 ± 4.64; n=9 compared to 126.57 ± 3.5; n=19; p = 0.00005).

## DISCUSSION

Our results show that proteosome-dependent degradation is required for the expression of the long-lasting component of LTP. Moreover, the requirement of protein degradation for L-LTP establishment is dependent on the frequency of the test pulse stimulation, in a way akin to when protein synthesis is inhibited (see Chapter 2). Interestingly, concurrent application of a protein synthesis inhibitor can partially rescue the effect of proteosome inhibition on L-LTP establishment. When the proteosome inhibitor, lactacystin, and anisomycin, a protein synthesis inhibitor, were co-applied, L-LTP is maintained for at least 3.5 hours. This indicates that protein synthesis and degradation are both required for L-LTP expression and suggests that inhibiting protein degradation can partially reverse the effects brought on by protein synthesis inhibition.

Even though the observation that proteosome inhibition results in an impairment in L-LTP establishment was surprising, there are several reports in the literature that are in line with this finding. Proteosome inhibition was shown to block long-term memory formation in rodents (Lopez-Salon et al., 2001) and long-term facilitation in *Aplysia* (Chain et al., 1995;Chain et al., 1999). Also an impairment in LTP was also observed for mice with maternal deficiency in the E6-AP ubiquitin ligase (Jiang et al., 1998). However, these results are not readily compatible with some other reports (Ageta et al., 2001b;Zhao et al., 2003) that show, for example, that inhibition of the proteosome results in the accumulation of proteins like Vesl-1S/Homer-1a, which would be expected to lead to “improved” LTP (Ageta et al., 2001a;Ageta et al., 2001b). Taken together, our results delineate a novel role for protein degradation in L-LTP establishment and suggest that a highly dynamic interplay between protein synthesis and protein degradation regulates the stability of L-LTP.

## CHAPTER IV

# Competing for memory: hippocampal LTP under regimes of reduced protein synthesis


## SUMMARY

The persistence of synaptic potentiation in the hippocampus is known to depend on transcription and protein synthesis. We now report that under regimes of reduced protein synthesis, competition between synapses for the relevant intracellular proteins can be demonstrated. Under such circumstances, the induction of additional protein synthesis-dependent long-term potentiation for a given set of postsynaptic neurons occurs at the expense of the maintenance of prior potentiation on an independent pathway. This new phenomenon, that we term 'competitive maintenance', has important functional consequences and it may be explained in terms of dynamic interactions between synapses and 'plasticity factors' over extended periods of time.

## INTRODUCTION

A common experience is that some events of a day are remembered while others are not. We have discovered what might be a physiological correlate of this experience and refer to it as the 'competitive maintenance' of long-term potentiation (LTP). LTP in the hippocampus is a prominent cellular model of memory formation (Bliss et al., 2003) which, in its later phases, is thought to involve gene transcription and translation (Krug et al., 1984;Goelet et al., 1986;Frey et al., 1988;Huang et al., 1996). Exactly how the persistence of LTP depends on protein synthesis and, in particular, how the relevant synapse-to-nucleus and nucleus-to-synapse signaling are achieved is still a matter of debate (Deisseroth et al., 1996;Silva et al., 1998). Recent experiments point to a mechanism that sets 'synaptic tags' at potentiated synapses whose role is to sequester 'plasticity factors' in order to stabilize the expressed potentiation in an input-specific manner (Frey and Morris, 1997;Martin et al., 1997;Frey and Morris, 1998b). These experiments established the concept and function of synaptic tagging by revealing that plasticity factors induced through activation of one input might be shared with other synapses upon their subsequent activation. However, sharing is only one side of the coin and we reasoned that, when the availability of plasticity factors is limited, tagged synapses would compete for them and it should then be possible to observe this competition. We achieved this by limiting protein-synthesis during the reactivation of LTP and observed that stabilizing the potentiation of synapses on one input pathway afferent to a population of postsynaptic neurons was at the expense of maintained potentiation on another independent pathway.

## MATERIAL AND METHODS

*Slice preparation and electrophysiological recordings*

The detailed procedure for acute hippocampal slice preparation and recording of extracellular fEPSPs was described previously (see Material and Methods, Chapter II). In brief, 400 µm acute slices were prepared from male wistar rats (3-4 weeks old). Recordings started after a 20 min resting phase in the recording chamber. Schaffer collaterals were stimulated with 0.2 ms pulses using monopolar tungsten electrodes. Stimulus intensities were set to evoke 50% of the maximal fEPSP slope and LTP was induced after recording a stable 20 min baseline of fEPSPs. The test pulse frequency was 0.1 Hz, unless stated otherwise.

*Induction of Long Term Potentiation*

For the induction of associative LTP two sets of Schaffer collaterals were stimulated. We always checked for pathway independence by applying two pulses with 25 ms interpulse interval to the two pathways and checking for the absence of paired pulse facilitation. Arbitrarily chosen, one of the pathways received a weak tetanus (0.2 ms pulse duration, 100 Hz for 0.25 s). After 10 min the other pathway received a strong tetanus (0.2 ms pulse duration, 100 Hz for 1 s). Associative LTP was induced by concurrent tetani (weak and strong) of both pathways. LTP was monitored for a period of up to four hours, after which the pathway which had received the weak stimulus earlier received another tetanus, in the presence or absence of protein synthesis inhibitors such as anisomycin or emetine. It was verified that the experimental outcome was the same if we selected the initially strongly stimulated pathway to receive the last tetanus (data not shown). See Figures for a schematic timeline of the experimental paradigms.

*Drug treatment*

Anisomycin (Sigma) or emetine (Sigma) were dissolved in DMSO, and diluted down to achieve a final concentration of 25 μM and 50μM respectively (in 0.01% DMSO). Anisomycin and emetine at this concentration reliably block protein synthesis(Stanton and Sarvey, 1984;Frey et al., 1988). D-AP-5 was dissolved in ACSF to achieve a final concentration of 50μM. All drugs were bath applied for 40 min or 2 hours before and washed out one hour after the second tetanus. For the control experiments only DMSO (0.01%) was added to the ACSF.

*Data Analysis*

Offline data analysis was performed using a customized LabView-program (National Instruments). As a measure for synaptic strength the initial slope of the evoked fEPSPs was calculated and expressed as percent changes from the baseline mean. Error bars are always displayed as SEM. To test for group differences between LTP values across conditions, a two-tailed Student's t-test was used (green triangles indicate p values). To this end LTP values were averaged over 20 min data bins. The degree of correlation between potentiation in one pathway and decay in the other for individual experiments was calculated using time windows were the effect was largest: potentiation of the reactivated pathway was taken 30 min after the last tetanus and the decrease of the synaptic responses in the test pathway was measured 5 hours later. These values were taken to produce the correlation plots in the Figures. The precise time points did not matter. We tried several other time points and got qualitatively similar results (data not shown). For normalization of LTP values the 20 minute window before reactivation was taken as baseline.

## RESULTS

In adult hippocampal brain slices, competition was set up between two independent pathways hereafter called the 'reactivated pathway' (RP, red symbols in Figure 8A and thereafter; single traces shown in Figure 8B) and 'test pathway' (TP, blue symbols in Figure 8A and thereafter; single traces shown in Figure 8B) respectively. After recording baseline postsynaptic potentials in independent pathways (see Material and Methods), the RP received a weak tetanus that, by itself, induced little or no LTP (100 Hz for 0.25 s; open arrows in Figure 1a and thereafter), whereas the TP received strong tetanization that resulted in robust LTP (100 Hz for 1 s; filled arrow in Figure 8A, and thereafter). Both, weak and strong tetanizing stimuli were then applied simultaneously (concurrent open and filled arrow in Figure 8A; see also insert in Figure 8A), and the resulting associativity between the two inputs induced LTP on both pathways (Barrionuevo and Brown, 1983). This protocol serves as a control for possible heterosynaptic interactions between the two pathways during potentiation, while also enabling associative LTP on both inputs.

The key finding of 'competitive maintenance' emerged in the next phase of our protocol. LTP on both the 'red' and 'blue' pathways was maintained for the next 4 hours (Figure 8A) indicating that the long-lasting, protein synthesis-dependent form of LTP (L-LTP) had been induced. Four hours after the start of the experiment, anisomycin was applied to the slice to block any ongoing or further synthesis of new proteins (yellow bar in Figure 8a and thereafter). Forty minutes later, a further weak tetanus (open arrow in Figure 8A, blow-up in Figure 8C) was applied to the RP, thereby reactivating it (hence the name "reactivated pathway"). According to the tagging hypothesis, this reactivated pathway should display further early-LTP, at the same time setting further synaptic tags that would sequester the now limited supply of plasticity factors available due to the earlier tetanization and so enabling its

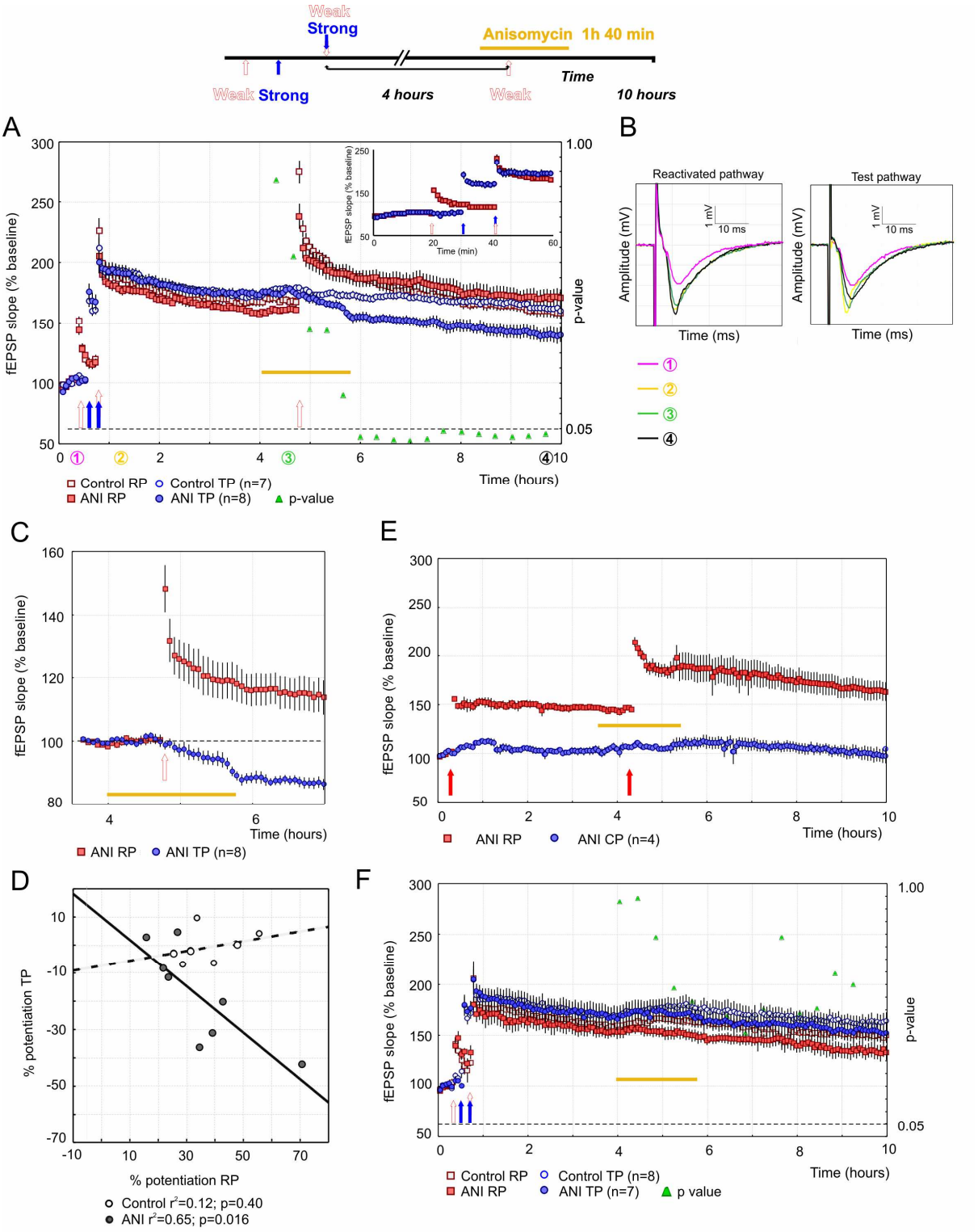
Weak
Strong
Anisomycin 1h 40 min
Time
Weak Strong
4 hours
Weak
10 hours
A
B
fEPSP slope (% baseline)
p-value
Time (hours)
Control RP
Control TP (n=7)
ANI RP
ANI TP (n=8)
p-value
fEPSP slope (% baseline)
Time (min)
Reactivated pathway
Test pathway
Amplitude (mV)
1 mV
10 ms
Time (ms)
C
fEPSP slope (% baseline)
Time (hours)
ANI RP
ANI TP (n=8)
E
fEPSP slope (% baseline)
Time (hours)
ANI RP
ANI CP (n=4)
D
% potentiation TP
% potentiation RP
Control r²=0.12; p=0.40
ANI r²=0.65; p=0.016
F
fEPSP slope (% baseline)
p-value
Time (hours)
Control RP
Control TP (n=8)
ANI RP
ANI TP (n=7)
p value

Figure 8

**Figure 8**. **Competitive interaction between two potentiated pathways. A:** Weak and strong tetani were used to induce associative LTP in two CA3 to CA1 synaptic pathways (see also inset in the upper right corner). Four hours after the start of the experiment the protein synthesis inhibitor anisomycin (ANI) was bath applied to the slice (yellow bar). Forty minutes later one of the pathways (reactivated pathway or RP; red symbols) received another (weak) tetanus, while the other pathway (test pathway or TP, blue symbols) continued to receive only test pulses. Potentiation of the reactivated pathway is at the expense of persistence of LTP in the test pathway, provided the experiment is performed under anisomycin (filled symbols). In the control case (open symbols) no such effect can be observed. In this and subsequent figures the statistical significance of the difference between test pathways in the presence or absence of anisomycin, expressed in p values, is plotted by the green triangles. The significance threshold of p=0.05 is always displayed as dashed line. **B:** Example traces of fEPSPs for the test and reactivated pathway at times indicated by the colors and the circled numbers (see also Figure 1A). **C:** The competitive maintenance effect at an expanded timescale (synaptic transmission before the second potentiation in the test and reactivated pathway was normalized to 100%). **D:** Correlation between the potentiation of the reactivated pathway and the decrease of the test pathway are plotted for each experiment, either in the presence (filled symbols) or absence (open symbols) of anisomycin. A linear regression shows for the anisomycin experiments a clear correlation of the amount of potentiation in the reactivated pathway with the amount of decay in the test pathway while there is no correlation for the control cases **E:** The effect only occurs on a potentiated pathway: an unpotentiated control pathway (CP, blue symbols) does not show decay in response to reactivation of the other pathway (RP, red symbols) which was subjected to an initial tetanus (filled arrow) and then a second tetanus (filled arrow) in the presence of

anisomycin.**F:** Time course of the synaptic responses in two pathways that both expressed associative LTP. Anisomycin alone does not cause decay in either pathway (RP and TP only in analogy to Figure 1a; no reactivation is applied). n: number of slices.

stabilization as persistent or late-LTP. This prediction was upheld (Figure 8A and C, enhancement of the RP; filled red squares after ~5 hours), in line with earlier reports (Frey and Morris, 1997). The application of anisomycin, however, had an important additional effect: the potentiation of the reactivated pathway was at the expense of the enhancement of the test pathway (filled blue circles) which started vanishing after the potentiation of the reactivated pathway had set in (Figure 8A, C).

Our proposed explanation of this unexpected 'competition' between the two input pathways is that sustained enhancement of the reactivated pathway 'uses up' the plasticity factors at the expense of stabilization of the test pathway that gradually weakens as a result. Such a competition hypothesis is bolstered by plotting the magnitude of the strengthening of the RP against the decay in the TP on an experiment-by-experiment basis (Figure 8D, filled data points and regression line). This reveals a clear correlation between enhancement and decay ($r^2$-values and p-values always in the Figures). Importantly, this correlation only held under anisomycin, i.e. when the pool of putative plasticity factors was limited (controls in Figure 8D as open data points and dashed regression line).

It was important to check that this phenomenon could not be explained by established principles, for the decay of maintained potentiation on the test pathway is reminiscent of a range of activity-dependent depression phenomena that are sometimes observed during the induction of LTP. However, none of these is likely to provide an explanation for the effect. First, as the test pathway did not itself receive tetanization when the reactivated pathway was re-stimulated, the decay cannot be due to homosynaptic long-term depression (Mulkey and Malenka, 1992;Dudek and Bear, 1992;Bear and Abraham, 1996) or depotentiation (Staubli and Lynch, 1990). Secondly, the competitive maintenance effect did not occur unless protein synthesis was blocked (control experiments in Figure 8A, 9A, 9C, 10A) or if synapses had not been potentiated previously (Figure 8E). This argues against heterosynaptic LTD (Lynch

et al., 1977;Abraham and Goddard, 1983;Abraham et al., 1985;Scanziani et al., 1996) as a potential underlying mechanism, as heterosynaptic LTD should occur regardless of the potentiation history of the synapses. Third, the effect is not some inevitable consequence of protein synthesis inhibition as a further control showed that the application of anisomycin without synaptic reactivation (Figure 8F) yielded no effect. Thus, the competition effect is in the domain of LTP maintenance, not its induction.

Before further characterizing the determinants of competitive maintenance, we wanted to ascertain that the effect is genuine and cannot be explained by side effects of the stimulation or the drugs used. Two types of control experiments were conducted. First, to rule out any unspecific effect of the stimulation to the reactivated pathway, we examined what would happen to the competitive maintenance effect if the attempted induction of LTP in the RP was blocked by AP-5 (Figure 9A,B). The experiment was – except for the presence of AP-5 – identical to the one described in Figure 1a with an initial phase in which the TP and RP were pre-potentiated. Here however, for reasons of clarity, we display only the relevant portion of the experiment (see colored and grayed-out portions of the experiment in the scheme above Figure 9A) and normalized the electrophysiological responses before the last (weak) tetanus to 100%, (see Material and Methods). We observed that, in the presence of AP-5, competitive maintenance does not occur indicating that successful enhancement in the RP is needed to observe the decrease in the TP (Figure 9A,B). An additional feature of these experiments was that a third control pathway was also recorded simultaneously (diamonds in Figure 9A). Its stability demonstrates that the decay observed in the TP in the absence of AP-5 (blue circles), must be genuine and cannot be attributed to any overall 'run-down' of the slice. Second, we also wanted to ensure that the observed effect was not caused by a pharmacological side effect of anisomycin. We therefore repeated the experiment with

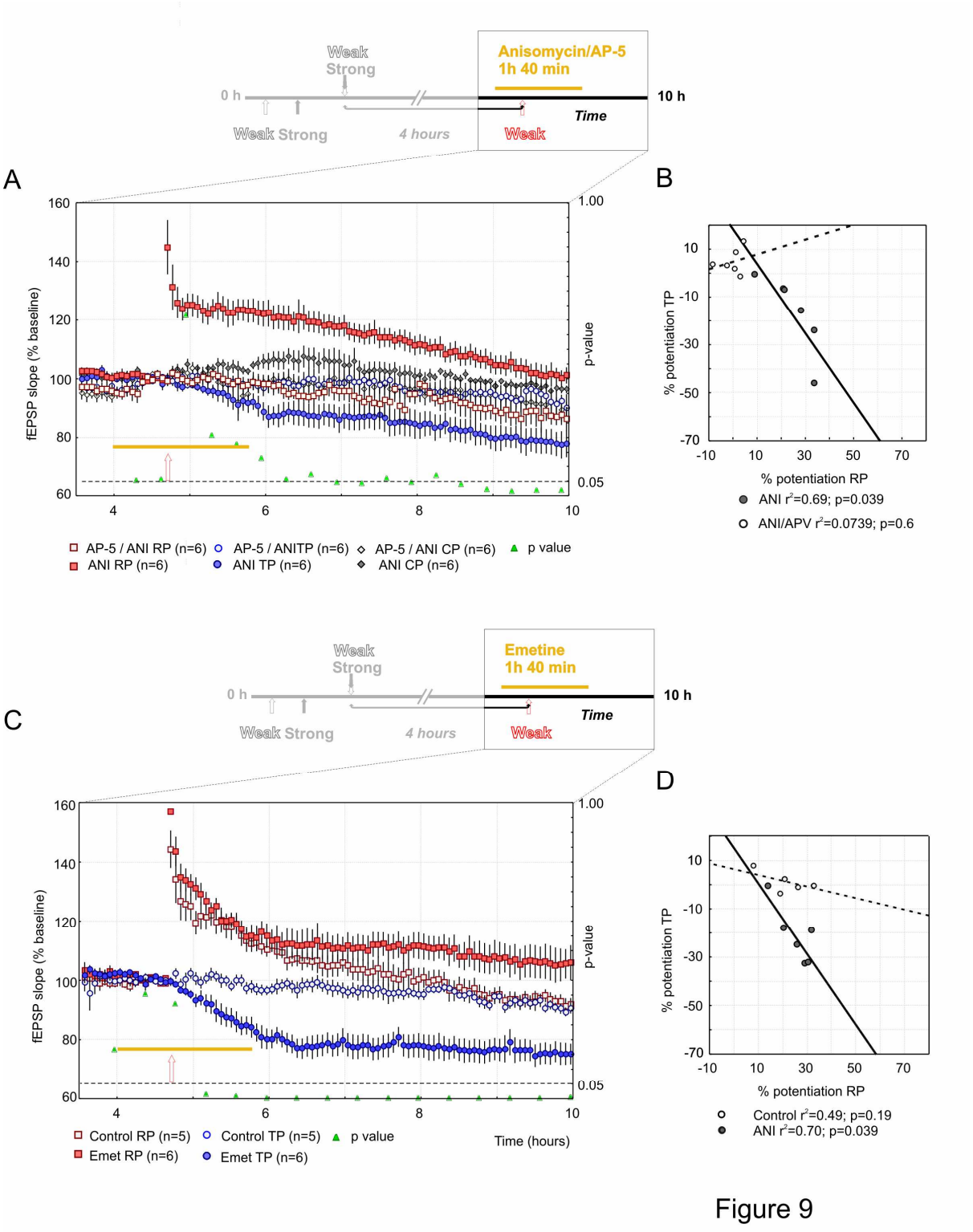
A
Weak
Strong
Anisomycin/AP-5
1h 40 min
0 h
10 h
Time
Weak Strong
4 hours
Weak
fEPSP slope (% baseline)
p-value
AP-5 / ANI RP (n=6)
AP-5 / ANITP (n=6)
AP-5 / ANI CP (n=6)
p value
ANI RP (n=6)
ANI TP (n=6)
ANI CP (n=6)
B
% potentiation TP
% potentiation RP
ANI r²=0.69; p=0.039
ANI/APV r²=0.0739; p=0.6
C
Weak
Strong
Emetine
1h 40 min
0 h
10 h
Time
Weak Strong
4 hours
Weak
fEPSP slope (% baseline)
p-value
Control RP (n=5)
Control TP (n=5)
p value
Emet RP (n=6)
Emet TP (n=6)
Time (hours)
D
% potentiation TP
% potentiation RP
Control r²=0.49; p=0.19
ANI r²=0.70; p=0.039

Figure 9

**Figure 9**. **A,B, Competitive interaction does not occur if AP-5 is present during the reactivation. A:** Experiment similar to the one shown in Figure 1a but in the presence of AP-5 and anisomycin or anisomycin alone. For clarity only the relevant 6.5 hours of the experiment are shown (responses 20 min before the last tetanus were normalized to 100%). Under AP-5 NMDA receptors are blocked and no further potentiation is obtained by the reactivation. No decay was observed for the test pathway in turn. Without APV the same competitive interaction displayed in Figure 1a occurs. **B:** The experiments performed with anisomycin alone (solid circles) show a clear correlation between potentiation in the reactivated pathway and decay in the test pathway whereas the experiments performed with anisomycin and APV yield no correlation (compare also Figure 1d). n: number of slices. **C,D, Competitive interactions are also induced when protein synthesis is blocked with emetine C:** Experiment similar to the one shown in Figure 1a only with emetine as protein synthesis blocker instead of anisomycin. Again, only the relevant 6.5 hours of the experiment are shown (responses 20 min before the last tetanus were normalized to 100%). **D:** Also the emetine experiments show a clear correlation between potentiation in the reactivated pathway and decay in the test pathway. n: number of slices.

another protein synthesis blocker, emetine. It yielded an identical competitive maintenance effect (Figure 9C,D).

If competitive maintenance is to do with protein availability, several further predictions can be made. First, decay of the test pathway should be enhanced by increasing the duration of the anisomycin application, thereby decreasing protein availability and intensifying competition. With the protein synthesis inhibitor applied for a longer time (3 h instead of 1 h 40 min; Figure 10A,B), we observed that the decay of the test pathway was increased. This effect can be seen in the population analysis of the data (Figure 10A) as well as in the correlation analysis (Figure 10B).

On the other hand, the competition effect should be decreased when the time between induction and reactivation is reduced because a greater supply of plasticity factors should still be available from the initial potentiation. In keeping with this prediction, when the interval between the LTP induction and its reactivation was reduced from 4 to 2 h, enhancement of the reactivated pathway resulted in almost no decay of LTP in the test pathway (Figure 10C). The correlation function also became flattened (Figure 10D).

Third, the distance between the potentiated synapses should influence the extent to which the two input pathways can compete for diffusing plasticity factors. To limit competition, one stimulating electrode was placed in the *stratum radiatum* (test pathway), stimulating synapses localized on the apical dendritic tree, and a second electrode in the *stratum oriens* (reactivated pathway), stimulating synapses localized on the basal dendrites (Figure 11B). Apart from the greater distance of the stimulating electrodes the experimental conditions were identical to those in Figure 1 a. Under these circumstances, we observed no interaction between the two pathways: the RP was enhanced but no concomitant decay was observed in the TP (population data: Figure 11A; correlation analysis: Figure 11C). This again supports our hypothesis that competition may explain the observed effect.

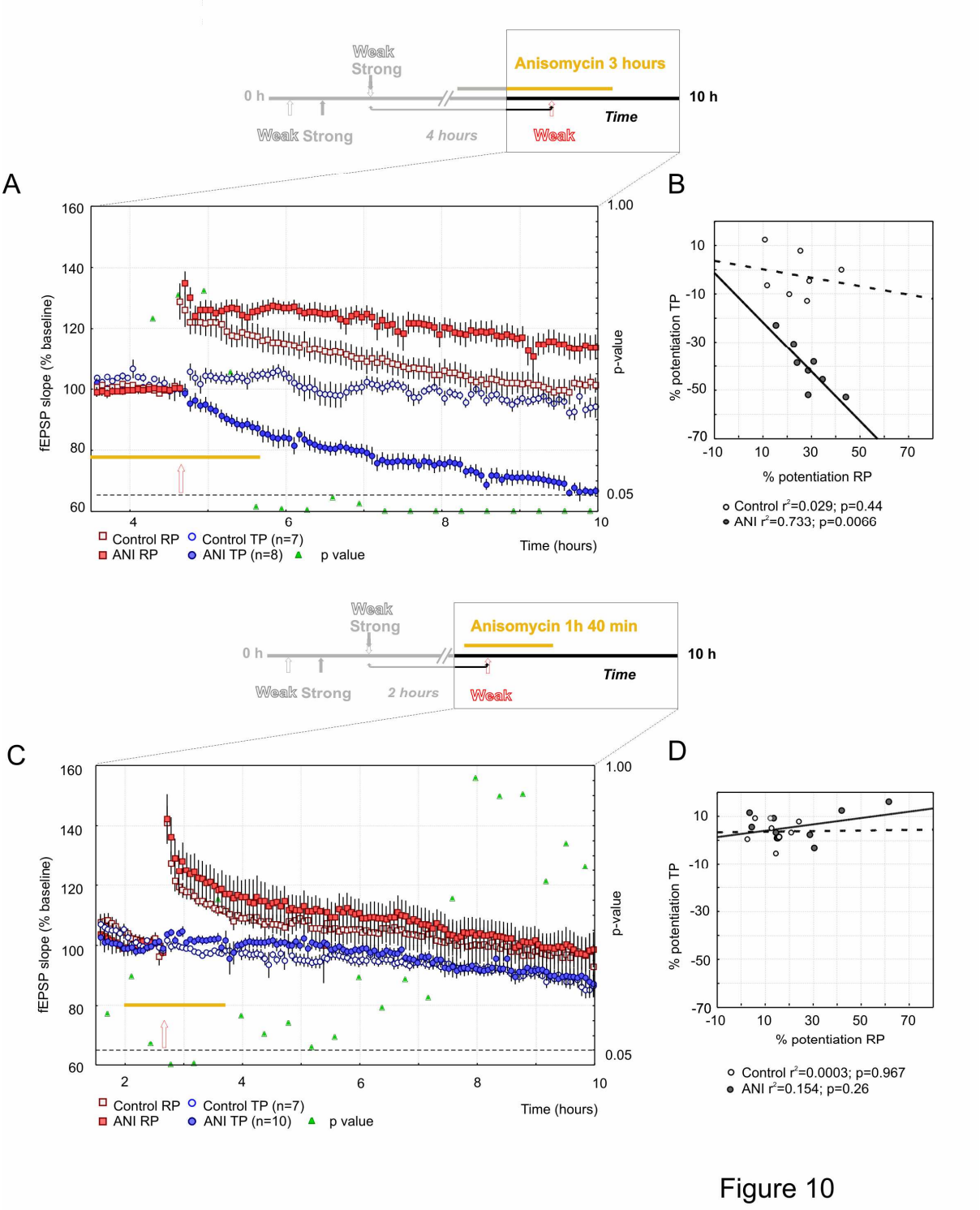
A
Weak
Strong
0 h
Weak Strong
4 hours
Anisomycin 3 hours
10 h
Time
Weak
fEPSP slope (% baseline)
p-value
Control RP
Control TP (n=7)
ANI RP
ANI TP (n=8)
p value
Time (hours)
B
% potentiation TP
% potentiation RP
Control r²=0.029; p=0.44
ANI r²=0.733; p=0.0066
C
Weak
Strong
0 h
Weak Strong
2 hours
Anisomycin 1h 40 min
10 h
Time
Weak
fEPSP slope (% baseline)
p-value
Control RP
Control TP (n=7)
ANI RP
ANI TP (n=10)
p value
Time (hours)
D
% potentiation TP
% potentiation RP
Control r²=0.0003; p=0.967
ANI r²=0.154; p=0.26

Figure 10

**Figure 10**. **A,B: Competitive interaction is increased after prolonged application of anisomycin. A:** Experiment similar to the one shown in Figure 1a, however anisomycin was bath applied for a period of 3 hours, presumably limiting the availability of plasticity factors even further. The decline observed in the test pathway due to the reactivation is increased. For clarity only the relevant 6.5 hours of the experiment are shown (responses before the last tetanus were normalized to 100%). **B:** Potentiation in the reactivated pathway plotted against the potentiation (resp. decay) in the test pathway, either in the presence (filled symbols) or absence (open symbols) of anisomycin. The correlation analysis reveals a stronger correlation between potentiation in the reactivated pathway and decay in the test pathway as well as a stronger effect (denoted by the steeper slope of the regression line). **C,D: Competitive interaction is decreased by reducing the time period between initial LTP induction and reactivation. C:** - In this case the reactivating tetanus was applied already after two hours, causing the competition to vanish (p-values as green triangles). For clarity only the relevant 8.5 hours of the experiment are shown (responses 20 min before the last tetanus were normalized to 100%). **D:** Linear regression returns no correlation. n: number of slices.

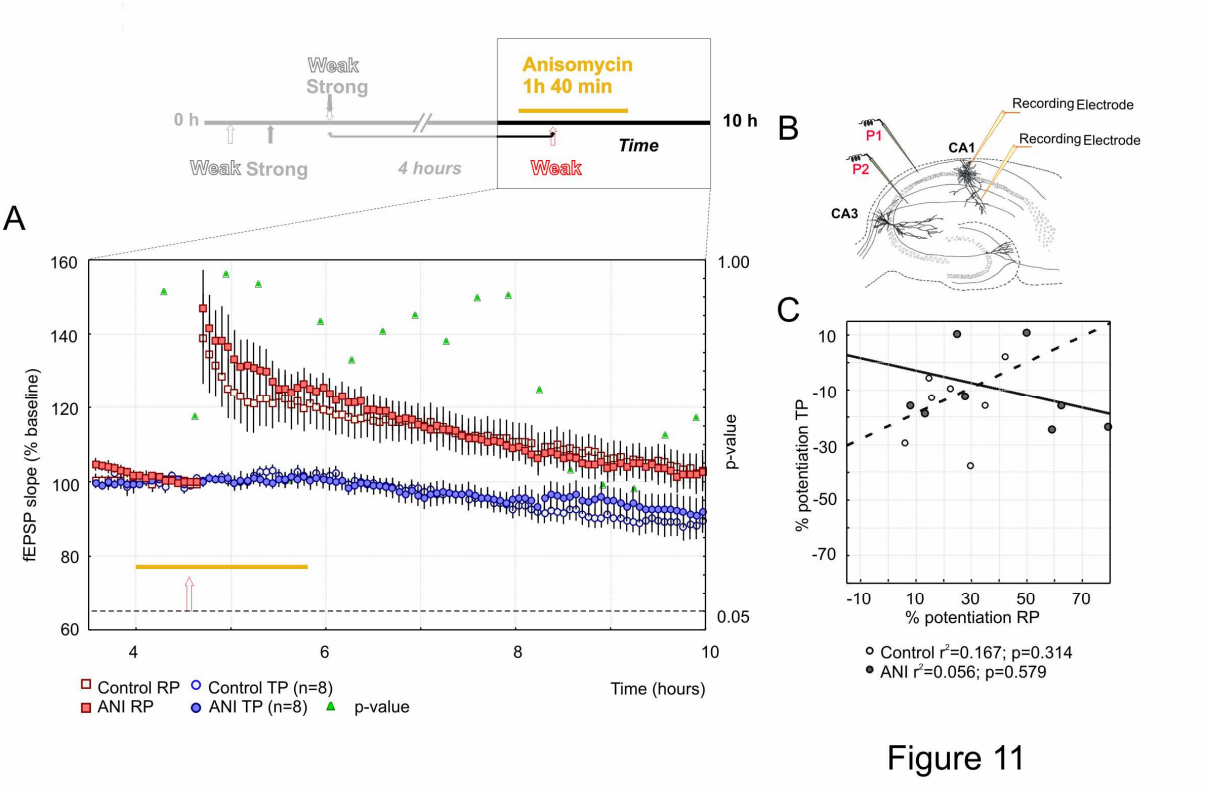
A
Weak
Strong
Anisomycin
1h 40 min
0 h
10 h
Time
Weak Strong
4 hours
Weak
fEPSP slope (% baseline)
160
140
120
100
80
60
1.00
p-value
0.05
4
6
8
10
Time (hours)
Control RP
ANI RP
Control TP (n=8)
ANI TP (n=8)
p-value
B
P1
P2
CA1
CA3
Recording Electrode
Recording Electrode
C
% potentiation TP
10
-10
-30
-50
-70
-10
10
30
50
70
% potentiation RP
Control r²=0.167; p=0.314
ANI r²=0.056; p=0.579

Figure 11

**Figure 11**. **Competitive interaction is absent when input pathways are in separate dendritic domains**. **A:** Experiment similar to the one shown in Figure 1a. This time, however, stimulation electrodes were spatially separated. Fibers in the *stratum oriens* served as the reactivated pathway, whereas the fibers of the *stratum radiatum* defined the test pathway. With this spatial separation the competitive interaction between the two potentiated pathways vanished. For clarity only the relevant 6.5 hours of the experiment are shown (responses 20 min before the last tetanus were normalized to 100%). **B:** Illustration of the positions of recording and stimulation electrodes. Two stimulation electrodes placed in the *stratum oriens* and the *stratum radiatum* were used to evoke fEPSPs. In this case we used two recording electrodes placed within the basal and apical dendritic region of CA1 neurons to faithfully record the synaptic potentials generated on different sides of the cell body. **C:** Linear regression shows no correlation between potentiation in the reactivated pathway and decay in the test pathway. n: number of slices.

Finally, fourth, we reasoned that if the number of additional tags set on the competing pathway was varied, for instance by tetanizing the reactivated pathway with successively stronger stimulation, decay of the test pathway should be enhanced proportionately. Indeed, when we increased the number of pulses in the high-frequency re-tetanizing train, we found that decay in the test pathway increased accordingly (Figure 12A,B). This is consistent with the view that stronger reactivation tilts the competition for plasticity factors in favor of the reactivated pathway at the expense of the test pathway. Note, however, that more tetanizing pulses do not trigger a stronger potentiation of the reactivated pathway. This is puzzling, but might be explained in terms of a dissociation between the potentiation at an individual synapse, which rapidly reaches an asymptote, and the number of tags that can be set at it.

To explore whether the effect of competitive maintenance occurs under more physiological conditions and not only under conditions of protein-synthesis blockade, we performed further experiments, in which the initial LTP was achieved by associatively combining stimulation with two weak tetani (see diagram and inset in figure in Figure 13A). We reasoned that if the degree of LTP persistence is related to the amount of plasticity factor synthesized, inducing associative LTP by pairing two weakly stimulated pathways would be expected to result in a lower concentration than that induced by strong tetanization and thus stronger competition at the time of reactivation. Figure 13A shows that indeed, also under such conditions, and in the absence of anisomycin, stimulation of the RP was associated with depression of the test pathway. There was again a correlation between the amount of potentiation in the RP and the amount of decrease in the TP (Figure 13B).

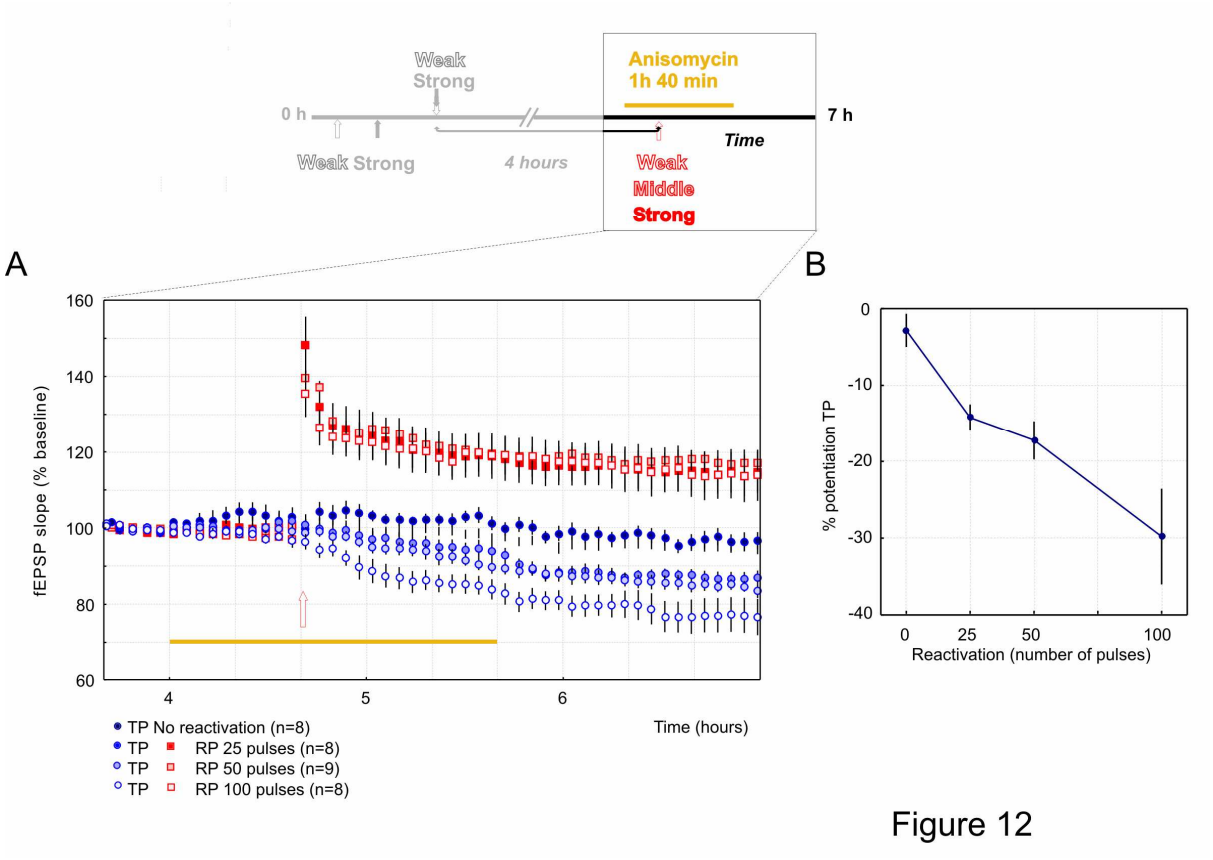
Weak
Strong
Anisomycin
1h 40 min
0 h
7 h
Time
Weak Strong
4 hours
Weak
Middle
Strong
A
B
160
140
120
100
80
60
fEPSP slope (% baseline)
4
5
6
Time (hours)
TP No reactivation (n=8)
TP RP 25 pulses (n=8)
TP RP 50 pulses (n=9)
TP RP 100 pulses (n=8)
0
-10
-20
-30
-40
% potentiation TP
0
25
50
100
Reactivation (number of pulses)

Figure 12

**Figure 12**. **Competitive interaction is a function of LTP reactivation strength. A:** Experiment similar to the one shown in Figure 1a. For clarity only the relevant 3.5 hours of the experiment are shown (responses 20 min before the last tetanus were normalized to 100%). In contrast to the experiment in Figure 1a, high-frequency trains differing in the number of pulses were used to reactivate the second pathway. The more pulses are applied to the reactivated pathway (the "stronger" the stimulation) the more pronounced is the decay in the test pathway. **B:** Relationship between the number of pulses used for the reactivating tetanus and the subsequent decay produced in the synaptic responses of the test pathway. n: number of slices.

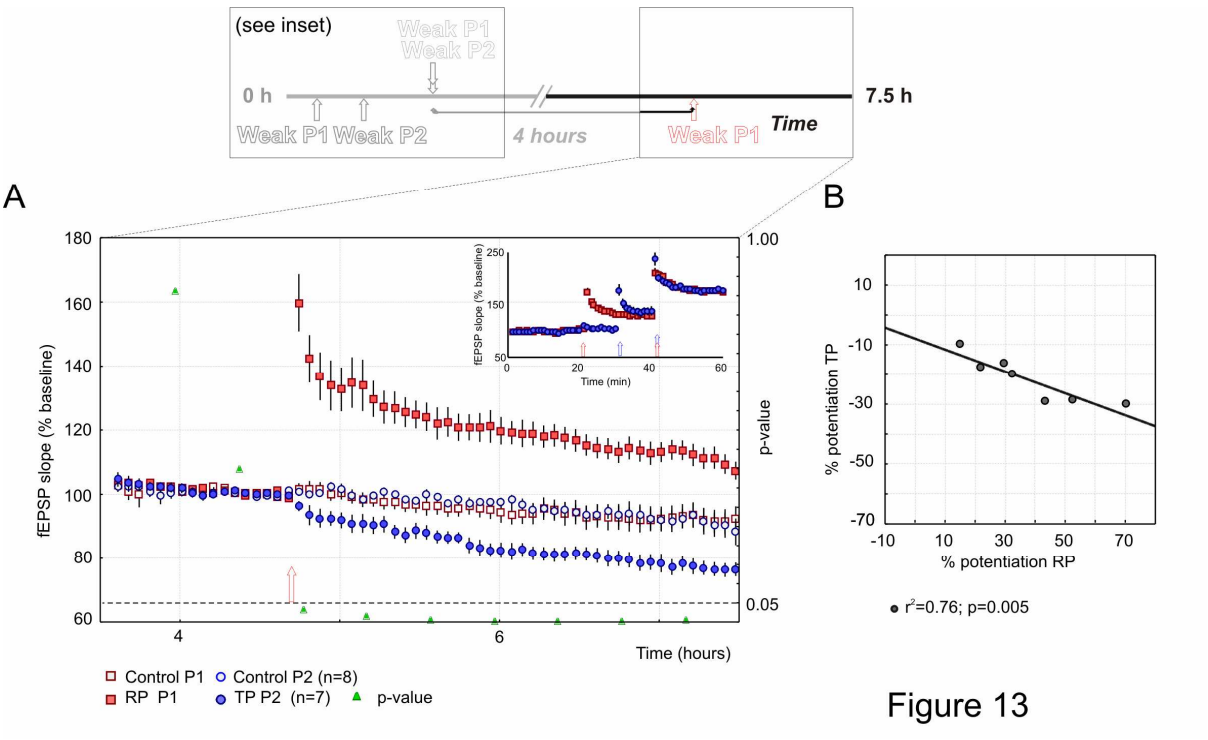
(see inset)
Weak P1
Weak P2
0 h
7.5 h
Weak P1 Weak P2
4 hours
Weak P1
Time
A
B
fEPSP slope (% baseline)
p-value
Time (hours)
Time (min)
Control P1
Control P2 (n=8)
RP P1
TP P2 (n=7)
p-value
% potentiation TP
% potentiation RP
r²=0.76; p=0.005

Figure 13

**Figure 13**. **Competitive interaction under conditions of prior weak associative tetanization. A:** Experiment similar to the one shown in Figure 1a but in the absence of anisomycin. Associative LTP was induced by concurrently applying weak tetanus to both pathways (see inset). Four hours later, one of the pathways (P1) received a second weak tetanus, this time in the absence of anisomycin. This results in potentiation of the reactivated pathway, again at the expense of a decay in the test pathway. In the control experiments (open symbols) no second tetanus was applied. For clarity only the relevant 4 hours of the experiment are shown (responses 20 min before the last tetanus were normalized to 100%). **B:** Linear regression shows a clear correlation between potentiation in the reactivated pathway and decay in the test pathway. n: number of slices.

## DISCUSSION

Our experiments describe a novel phenomenon - "competitive maintenance" - in which the successful persistence of synaptic potentiation in a reactivated input to a population of neurons is at the expense of sustained potentiation in an independent input. This phenomenon is consistent with there being intracellular competition for plasticity factors responsible for the persistence of synaptic potentiation.

The scenario of competitive interactions between these factors makes a number of strong predictions that we have tested. First, if protein synthesis normally occurs for a sustained period after tetanization, longer application of anisomycin should lead to lower amounts of plasticity factor and thus stronger competition. This is precisely what we observed. Second, a shorter interval between initial potentiation and reactivation should result in a higher abundance of plasticity factors and reduced competition. Third, increased spatial distance between the activation sites should lead to less competition and therefore a smaller competitive maintenance effect. Fourth, stronger reactivation should lead to more tags being set, thus a higher demand for plasticity factors, and hence a stronger competition. With respect to this fourth prediction, a distinction should be recognised between the *magnitude* of LTP and its *persistence*. At first sight, the findings of Fig 12 are puzzling, but an asymmetry exists between the reactivated and test pathways in that a graded alteration in the availability of new tags on the reactivated pathway will, paradoxically, more rapidly alter the rate of decline of the test pathway than the impact on the magnitude of LTP in the reactivated pathway (RP). Plasticity factor molecules need not augment the magnitude of LTP, only its persistence. Finally, competitive maintenance also occurred when competition was engineered in the absence of anisomycin using weak tetanization. All these predictions were upheld, and the data are therefore consistent with our interpretation that competition for

plasticity factors might account for the competitive maintenance effect. While this evidence is indirect inasmuch as we do not yet know the identity of the tags or plasticity factors (Martin and Kosik, 2002), the status of each of these predictions is logically independent of their identities.

Our results are consistent with the view that plasticity factors synthesized upon activation of relevant signal transduction cascades – presumably proteins although they could be mRNAs (Steward and Schuman, 2001) – are a limited resource to be distributed in the cell. They indicate that distribution and synthesis occurs over a sustained period of time – at the time of induction and also during the later maintenance phase of LTP expression. The synaptic tagging hypothesis implies that reactivation of one of the potentiated pathways sets new tags at the activated synapses, and these tags serve as a functional sink for plasticity factors. Experiments in which weak tetanization had been given before strong tetanization to independent pathways had earlier suggested (Frey and Morris, 1998a) that synaptic tags lasted no longer than 1-2 hr (at 32°C). However, these studies were conducted such that tags set on the weak pathway would not have had access to any plasticity factors throughout that time. The present findings suggest that synaptic tags can be maintained for longer times provided that plasticity factors are present, pointing to dynamic tag-plasticity factor interactions over time during the maintenance phase of late-LTP. Competitive interactions can be observed in potentiated but not yet stabilized pathways for as long as after 4 hr after initial LTP induction.

The effect described here is, at first glance, reminiscent of heterosynaptic depression (Lynch et al., 1977;Abraham and Goddard, 1983;Abraham et al., 1985;Scanziani et al., 1996). There are three reasons to doubt that this could account for our finding. First, the decay in the test pathway was only observed for pathways that had been potentiated previously (e.g. Figure 8E), whereas for ‘classical’ heterosynaptic depression, it makes no

difference whether the pathways were initially potentiated or not. Second, we could only observe a competitive effect under conditions where the levels of plasticity proteins were expected to be low - during protein synthesis inhibition or after prior weak associative LTP induction. If the same experiments were performed without applying protein synthesis inhibitors, competition was not observed (control cases in Figures 8A, 9A, 9C, 10A). Finally, LTD lasting several hours has been reported to be protein synthesis-dependent (Kauderer and Kandel, 2000;Sajikumar and Frey, 2003). If the mechanisms underlying competitive maintenance overlapped with those of this form of LTD, it should not occur and be maintained with protein synthesis blocked. The key difference between classical heterosynaptic depression and competitive maintenance is that the former relates to the induction and expression of decreased synaptic strength, whereas we have described an effect that reflects the maintenance of synaptic potentiation.

We favor and regard as most parsimonious the interpretation that competition for a limited pool of proteins is the basic mechanism underlying competitive maintenance, but there are other possibilities. In particular, as mentioned above and suggested earlier (Martin and Kosik, 2002), mRNAs could well serve as "tags" and proteins controlling translation could be the "plasticity factors" for which the mRNAs compete. Also, it is conceivable that, instead of competing for strengthening proteins, enhanced synapses release an inhibiting factor that acts on nearby (tagged) synapses to cause a decrement in their strength. In this scenario, however, it is less easy to understand why the effect should only occur under regimes of reduced protein availability (but see (Woo and Nguyen, 2003)). Importantly, while describing different molecular underpinnings of the effect, all these alternative mechanisms, are functionally equivalent in that they cause enhancement of one pathway at the expense of another.

One of the potential functional consequences of competitive maintenance is that it could provide a means for selective information storage when multiple inputs converge. That we could enhance competitive interactions by increasing the number of pulses delivered during reactivation suggests that the number or potency of tags is determined by the strength of input stimulation. Competition could, therefore, be used to scale cellular responses and thereby maintain a balance of overall synaptic strength during the maintenance phase of L-LTP (Turrigiano et al., 1998). Even though competition was initially revealed under artificial circumstances, namely protein synthesis inhibition, later experiments revealed that the more physiological conditions of prior weak associative LTP enables a similar effect to occur during the reactivation of one pathway. We hypothesize that competition for plasticity factors can provide the means for the selective memory storage of events, even if they have occurred at different times, provided they have taken place within a finite time window. Such circumstances may be common in remembering the events of daily life. Although clearly speculative, our findings may provide a physiological handle on the long-established interference theory of forgetting (Baddeley, 1990).

The phenomenon of competitive maintenance offers further support for the synaptic tagging hypothesis and extends it in an important way. Plasticity factors are shared according to need amongst synapses whose activity may have played no part in their provenance. However, under circumstances of reduced availability, competition defines which synapses will win. Equitable sharing and ruthless competition are two sides of the tagging coin.

# CHAPTER V

# CONCLUSIONS AND PERSPECTIVES

Memory is fundamental for everyday life and memory loss has devastating consequences for the individual and the society. Understanding the neuro-physiological and cellular basis of memory formation promises to open the way to potential therapeutic targets for neurological diseases that lead to memory loss. Long-Term Potentiation (LTP) has served as the dominant experimental model to study the cellular and synaptic mechanisms that lead to memory formation, in large part because of its long-lasting nature (Malinow et al., 2000;Malenka, 2003;Abraham and Williams, 2003). Therefore, understanding the mechanisms underling LTP induction, and in particular its maintenance, has become an intensively studied field. The present work was aimed to further extend our understanding of the role of *de novo* protein synthesis for the induction and maintenance of synaptic enhancement. The requirement of *de novo* protein synthesis for late-phase LTP (L-LTP) expression is well established, since protein synthesis inhibitors can block the establishment of L-LTP when applied during LTP induction (Frey et al., 1988). Generally, it is assumed that the induction of LTP triggers the transcription of several gene products and the translation of proteins required for the expression of L-LTP (Abraham and Williams, 2003). After L-LTP is established (typically 1-2 hours after induction), *de novo* synthesis of proteins is thought not to be required anymore. This was supported by reports showing that application of protein synthesis inhibitors after the induction of LTP had no effect on its expression and maintenance (Huang et al., 1996;Barco et al., 2002;Calixto et al., 2003;Cammalleri et al., 2003). This suggested that protein synthesis is triggered by the LTP

inducing stimuli and is required for L-LTP establishment in a transient as opposed to an on-going manner.

There is compelling evidence that synaptic activation can regulate protein synthesis and degradation (Wells et al., 2000;Wells et al., 2001;West et al., 2002;Ehlers, 2003), thus regulating the turn-over of possible key proteins involved in LTP. However, the possible roles of synaptic activation for the protein synthesis-dependent late-phase of LTP had not been characterized. This led us to ask whether synaptic activation might influence the extent of which *de novo* protein synthesis is critical for L-LTP. To address this question, I experimentally varied the level of synaptic activation that the potentiated synaptic pathways experienced, and looked at the effect on the dependence of LTP on *de novo* protein synthesis. LTP was recorded using different frequencies of test-pulse stimulation, while protein synthesis was inhibited during LTP induction. L-LTP was blocked by protein synthesis inhibition and the decay of LTP was strongly dependent on the frequency of synaptic activation. For the higher frequencies tested (0.2 and 0.1 Hz), protein synthesis inhibition led to a pronounced decay in LTP from very early on (less than 10 minutes), during the so-called early phase of LTP. This came as a surprise, since the early-phase of LTP is thought to be independent of protein synthesis. In the control experiments, where no protein synthesis inhibitor was applied to the bath, no difference in LTP values was observed for the different frequencies tested. Moreover, I did not observe any decrement in basal synaptic transmission (control pathways) for slices treated with protein synthesis inhibitor, demonstrating that the decrement in LTP values associated with the different frequencies is restricted to potentiated synapses (see Figure 2A and 3A and 3B, chapter II). This indicates that synaptic activity has an important role in determining the requirement of *de novo* protein synthesis for the induction and expression of the late phase of LTP. These results also suggest that the distinction of an early versus a late phase of LTP based on their requirement for *de novo*

protein synthesis might not be as clear-cut as previously thought. A key parameter is the frequency of test-pulse stimulation at which the recordings are performed. In previous studies in the literature the frequency of synaptic activation was much lower (Frey et al., 1988;Huang et al., 1996;Barco et al., 2002) than any of those used here and this may explain the difference in duration of what is considered to be the protein synthesis-independent early-phase LTP. The results shown here suggest that protein synthesis is required for early-phase LTP and that this requirement is intimately linked to the level of synaptic activation. The requirement of *de novo* protein synthesis for the early phase has been described previously for hippocampal mossy fibers LTP (Barea-Rodriguez et al., 2000;Calixto et al., 2003) demonstrating that also in this form of LTP the distinction of early versus late-phase LTP based on their requirement on protein synthesis is not sharp.

Interestingly, the level of synaptic activation after the induction of LTP also influences the maintenance of LTP and its sensitivity to protein synthesis inhibition. In this experimental condition synaptic activation was switched off for most of the time after LTP induction and resumed continuously during its maintenance phase. If the potentiated synapses are stimulated in this intermittent fashion, concurrent NMDA receptor activation and protein synthesis inhibition applied during the maintenance phase of LTP (more than 1.5 hours after induction), causes LTP to decay. Taken together, these results suggest that synaptic activation increases the turn-over of proteins required for the expression and maintenance of LTP. When protein synthesis is blocked the increase in turn-over may reduce the availability of these proteins required for the stabilisation of the potentiation. However, can activity have a stabilizing role if present before the time window of protein synthesis inhibition? To test this, synapses were stimulated for an additional hour, after LTP induction and before protein synthesis inhibition. Under such conditions, concurrent synaptic activation and protein synthesis inhibition no longer results in a disruption of LTP. Although we can only speculate

about the molecular mechanism that might underlie this observation, it is clear that synaptic activation plays an important role in the "consolidation" of LTP. Interestingly, the observation that synaptic activation plays an important role for long-lasting LTP *in vivo* was reported (Abraham et al., 2002), but the link to the role regulation of protein synthesis was not established. Taken together, this suggests that synaptic activation might have a role in regulating protein synthesis and thus the maintenance of synaptic enhancement. This may represent a mechanism by which a certain trace is maintained based on an activity-dependent resource allocation. In other words, if a synapses are potentiated, but then not further stimulated, the allocation of newly synthesized proteins to the enhanced synapses might lower, leading to destabilization of the LTP.

If activity increases protein turn-over, any increase in protein synthesis or availability must be matched by an increase of protein removal and ultimately protein degradation. In a simple model, one could predict that blocking protein degradation might increase the availability of those proteins important for LTP, resulting in an increase in the expression or in the stability of LTP. To test this, I inhibited the proteosome-dependent protein degradation during the induction of LTP. This treatment resulted in an impairment in L-LTP. Interestingly, by recording LTP at different frequencies of synaptic stimulation, while blocking the proteosome-dependent protein degradation, I obtained evidence for an activity-dependent requirement of protein degradation for LTP, in much the same way as the requirement for protein synthesis. This result, although not entirely surprising due to previous reports (Chain et al., 1995;Jiang et al., 1998;Chain et al., 1999;Lopez-Salon et al., 2001), indicates that the role of protein degradation cannot be just a counter-balance mechanism of protein synthesis. However, since no direct measurements of protein levels in the slice were performed, the impairment in LTP by inhibition of protein degradation can actually result from an accumulation of proteins that leads to synapse saturation. No change in the basal

transmission was observed during the inhibition of the proteosome-dependent protein degradation, arguing against the saturation hypothesis, but it is conceivable that we are looking at maintenance and not expression processes. In other words, protein degradation might not affect directly the expression of potentiation (for example insertion of new AMPA receptors), but result in an accumulation of scaffolding proteins (Colledge et al., 2003;Ehrlich and Malinow, 2004), making the synapses "superstable" and therefore resistant to modifications. To test this hypothesis, I induced and recorded LTP in the presence of protein synthesis and protein degradation inhibitors. Interestingly, L-LTP establishment was not blocked by the co-application of the inhibitors. Although the expression levels of LTP are lower than controls, the observation that LTP could be induced and maintained in the presence of protein synthesis and protein degradation inhibitors, suggests that the requirement of *de novo* protein synthesis for the expression of LTP is to counterbalance the activity-dependent increase in turn-over of proteins. Moreover, this is consistent with the hypothesis that the observed impairment in inducing LTP when protein degradation was blocked is due to an accumulation of proteins, whose synthesis is up-regulated upon LTP induction.

Consistent with this hypothesis is the report that for LTD induction the degradation of PSD-95, a scaffolding protein of the post-synaptic density (PSD), is required (Colledge et al., 2003) and that over-expression of PSD-95 leads to an occlusion of LTP induction, suggesting that accumulation of PSD-95 leads to synapse saturation (Ehrlich and Malinow, 2004). Moreover, the induction of LTP requires a dynamic actin cytoskeleton (Kim and Lisman, 1999;Krucker et al., 2000;Fukazawa et al., 2003), probably linked to changes in protein composition on the PSD complex. Whereas after the induction of LTP the PSD complex may undergo a transient disassembly state allowing for additional proteins to be added, the maintenance phase might involve a re-assembly of the PSD complex with new scaffolding proteins being synthesised to perform this task (Lisman and Zhabotinsky, 2001). What might

be the role of activity in this scenario? Synaptic activation might render synapses unstable and therefore susceptible to insertion of new protein complexes, which are subsequently stabilized. If, however, protein synthesis is inhibited, the required stabilization of these protein complexes is compromised and LTP maintenance is impaired. It is conceivable that calcium entry and subsequent activation of signalling cascades may lead to this destabilization. My observation that the impairment in LTP maintenance could be rescued, induced by concurrent synaptic activation and protein synthesis inhibition, by blocking NMDA receptor activation, suggests that the test-pulse stimulation can, at least to some extent, activate NMDA-receptors. If synaptic activation leads to the destabilization of synapses, the NMDA-receptor activation by the test-pulse stimulation may provide an explanation for the requirement of *de novo* protein synthesis for LTP maintenance.

In the experiments described in Chapter IV I took advantage of the associative property of LTP to demonstrate that proteins, synthesised upon LTP induction are available in limited amounts and that they can be redistributed in the cell among distinct inputs. Two independent input pathways to the CA1 pyramidal cells of the hippocampus were used to induce associative LTP. Four hours after induction one of these pathways is reactivated by a tetanic stimulation. Under protein synthesis inhibition, the enhancement of the non-reactivated pathway rapidly deteriorates in a fashion that is correlated to the amount of potentiation expressed by the reactivated pathway. This novel phenomenon, called "competitive maintenance" of LTP, shows that synaptic potentiation in a pathway occurs at the expense of the maintenance of the potentiation of a second pathway. According to the "tagging" hypothesis, an LTP-inducing stimuli leads to the formation of a "tag" at the stimulated synapses so that newly synthesised proteins can have a specific local action (Frey and Morris, 1997;Frey and Morris, 1998a;Frey and Morris, 1998b;Martin and Kosik, 2002). In the same way, the second reactivating LTP inducing stimuli generates a new "tag", which

serves as a sink for the limited "plasticity" proteins. Resources will be therefore allocated to the input strength resulting in competitive interactions between the two input pathways. Although the idea that proteins can be re-distributed among two sets of inputs is not new, I have shown that this redistribution occurs not only during induction but also during the maintenance phase of LTP and that under a regime of limited availability of these proteins, they will be distributed according to the strength of the input resulting in competitive interactions. Although the molecular nature of the tag and the plasticity proteins is not known, the "synaptic competition" results shown here suggest that the interaction "tag-plasticity protein" has to be a highly dynamic one, since the reactivation of one of the inputs results in a re-allocation of resources at the expense of the non-reactivated input.

The competition hypothesis is based on an activity-dependent redistribution of protein inside the presumably post-synaptic cell. But could the competition be played out on the presynaptic side? As the protein synthesis inhibitor is applied extracellularly, protein synthesis is blocked both pre- and postsynaptically, and a presynaptic effect can not be excluded *a priori*. However, a presynaptic mechanism seems unlikely as it should result in an interaction between neighbouring output neurons rather than in competition between different inputs. As we have not examined such interactions, we cannot exclude that a presynaptic mechanism is also at work, resulting in an additional crosstalk on the output side. Furthermore, one might also speculate that competition could occur extracellularly for secreted proteins. While our approach does not allow ruling out this possibility, we find this a less likely scenario in view of the existing literature on candidate intracellular tags and plasticity proteins (Martin and Kosik, 2002). Moreover, competition and selection of inputs is a general mechanism during the development of circuits and here deem it likely that the post-synaptic component the predominant in terms of selection of the input pathways that are maintained (Lichtman and Colman, 2000). For example, intracellular recordings from muscle

fibers provide support for the idea that during the development of the neuromuscular junction, changes in the post-synaptic cell, namely expression and localization of the acetylcholine receptors, occur before the nerve has withdrawn and seems to determine the selection between neighbouring competitive innervating fibers (Lichtman and Colman, 2000).

Another open question is whether the morphological organization of the input fibers determines the degree of competitive interactions between neighbouring synapses? Several reports have pointed out the importance of the morphological organization of the input fibers in defining functional dendritic domains, which are key determinants for the induction of synaptic plasticity (White et al., 1988;Lichtman and Colman, 2000). To address this question whether this morphological organization fibers plays and important role in the induction of the competitive interaction, I spatially separated the stimulation electrodes as to segregate the termination zones of the input fibers on two different post-synaptic dendritic domains (apical dendrites versus basal dendrites; see figure 11 in chapter IV). With this configuration I failed to observe competitive interactions when protein synthesis was blocked. However, this approach does not fully address the above question, since different mechanisms may be involved in LTP induction in basal versus apical dendrites (Haley et al., 1996). To make a stronger case it would have been necessary to demonstrate that competitive interactions can also occur at all in the basal dendritic domain. Another open question relates to the time window in which synapses are sensitive to the competition. Does the potentiation become insensitive to disruption by protein synthesis inhibition after some time? This question could be addressed by extending the time of the recordings, but at present it is difficult to do so given the limitations of the acute slice preparation. Alternative approaches relying on organotypic slice cultures or *in vivo* preparations may help, since they provide the means to maintain the preparation viable for longer periods.

Taken together, the present work has demonstrated a key role of *de novo* protein synthesis for the maintenance of synaptic enhancement. I have shown that this requirement depends on the patterns of activity experienced by the enhanced synapses after LTP induction. The results shown here outline the importance of synaptic activation is regulating the requirement of LTP maintenance on protein synthesis and degradation and are consistent with recent reports that describe the role of turnover of specific synaptic proteins for synaptic plasticity. Consistent with the idea that *de novo* protein synthesis is required during the maintenance phase, I showed that if protein synthesis is inhibited, induction of additional potentiation is achieved at the expense of the maintenance of a second pathway. This indicates that proteins synthesised upon LTP induction are limited and can be redistributed among two sets of inputs according to their synaptic strength.

Overall, the analogy between the results shown here and the "reconsolidation" hypothesis is striking. I have shown that during the maintenance of LTP synaptic reactivation renders LTP unstable and therefore susceptible to protein synthesis inhibition, as much as happens during memory reactivation (Nader et al., 2000;Nader, 2003). Although several questions remain unsolved, the results shown here are consistent with the view that analogous to memory formation, long lasting forms of synaptic enhancement are highly dynamic and may require an on-going synthesis of proteins to stabilize the enhancement state. Taken together, the experimental approaches followed here may provide a powerful model that can be further used to study in more detail the molecular mechanisms that underlie memory reconsolidation.

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**Curriculum Vitae**

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**Education:**

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|---|---|
| 2000-04 | Start of PhD in Munich at the Max-Planck-Institute of Neurobiology with Tobias Bonhoeffer, PhD, in the project "Remembering makes memories fragile: the cellular basis of reconsolidation". This project is part of a Human Frontier Science Research Grant with Prof. Joseph LeDoux, Dr. Karim Nader, Dr. Richard Morris and Dr. Yadin Dudai. |
| 1999–00 | First year of biology courses from the PGDBM. |
| 1999 | Enrolment in the Gulbenkian PhD Program in Biology and Medicine (PGDBM). |
| 1998–99 | Carried out scientific research in the project: "Purines and the neuron-astrocytic communication using slices and cell culture", under the supervision of Alexandra Rodrigues, PhD, in the laboratory of Neuroscience, Medicine School, University of Lisbon. |
| 1998–99 | Biology teacher in a high school in Lisbon. |
| 1994–98 | Degree in Biology, from the Science School, University of Lisbon. Final classification 17 (out of 20). |

**List of Publications:**

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**Fonseca R.**, Nägerl U.V., Morris R.G.M. and Bonhoeffer T: "Competitive interactions between potentiated synapses", 2004, Channels, Receptors and Synapses, CSHL meeting, Cold spring Harbor Laboratory, USA

**Fonseca R.**, Nägerl U.V., Morris R.G.M. and Bonhoeffer T: "Competitive interactions between potentiated synapses", 2004, Symposium, "Synaptic Tagging, Synaptic Capture: Key to Memory encoding?", International Graduate School of Neuroscience (IGSN), Ruhr University, Bochum, Germany

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