Re-evaluation of the hypothesis that LTP has two temporal phases and that the late phase is protein synthesis-dependent

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To memory of my father

List of Publications

This thesis is based on the following papers, which will be referred by their Roman numerals:

I. Abdul-Karim Abbas, Mikhail Dozmorov, Rui Li, Fen-Sheng Huang, Fredrik Hellberg, Jonas Danielson, Ye Tian, Jörgen Ekström, Mats Sandberg, Holger Wigström (2009). Persistent LTP without triggered protein synthesis. Neuroscience Research 63: 59-65.

II. Abdul-Karim Abbas, Fen-Sheng Huang, Rui Li, Jörgen Ekström, Holger Wigström (2011). Emetine treatment masks initial LTP without affecting long-term stability. Brain Research 1 4 2 6: 1 8-2 9.

III. Abdul-Karim Abbas (2013). Evidence for constitutive protein synthesis in hippocampal LTP stabilization. Neuroscience 246: 301-311.

IV. Abdul-Karim Abbas, Agnés Villers, Laurence Ris (2015). Long-term potentiation (LTP) temporal phases: myth or fact? Rev. Neurosci. (In Press).

Abstract

Long-term potentiation (LTP) is an activity-dependent increase in synaptic efficacy that is most studied in the hippocampus and that is considered a cellular substrate for learning and memory. Accepting the belief that the durability (persistence in time) of LTP is analogical to long-standing store of hippocampus-dependent memories warrants the necessity for understanding the mechanisms underlying LTP stabilization. Although the great majority of neuroscientists assume that LTP induction, akin to the formation of memories triggers the synthesis of proteins that are instrumental for subsequent consolidation neither the identity of such presumed proteins nor the mechanisms by which they act to consolidate LTP are clear. Based on this notion LTP is distinguished temporally into an early phase (E-LTP), which is protein synthesis-independent and a late phase (L-LTP), which is protein synthesis-dependent. However, several behavioral and electrophysiological findings cast doubts on this notion. In the present thesis I have examined the effect of protein synthesis inhibitors (PSIs) on the stabilization of LTP in hippocampal slices obtained from young rats. Treating hippocampal slices with PSIs using a temporal window relative to the induction of LTP that has previously been used in the literature failed to block L-LTP, a result in contrast with published data. However, long-lasting pretreatment with the PSI emetine blocked LTP by LTP-unrelated mechanism as the drug showed deteriorating effect on the baseline response. In contrast, depleting the protein repertoire in the slice by long-lasting pretreatment with the PSI cycloheximide deteriorated the stabilization of LTP. Additionally, acceleration of protein degradation using hydrogen peroxide after the induction of LTP resulted in decay of LTP. Addition of cycloheximide induced additive decay of LTP stabilization. These contradictory findings have recently been replicated by other laboratories. In this thesis I present a working model that aims to explain the discrepant findings regarding PSI and LTP. The model concedes that knowing the kinetics of protein turnover during the induction of LTP may provide a prediction for the subsequent stabilization of LTP. This can explain the wide variability in the time course of the presumed protein-synthesis independent E-LTP. The model gains support from experiments in which a low concentration of the proteasome inhibitor MG-115 improved the stability of LTP induced by a weak induction protocol. In summary, my results suggest that 1) the temporal distinction of LTP into E- and L-LTP is a false dichotomy and 2) the rate of protein degradation may explain whether PSIs would, or would not, have an effect on LTP stabilization.

Abbreviations

4E-BP1	eukaryotic translation initiation factor 4E (eIF-4E)-binding protein 1
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
Arc/Arg3.1	activity-regulated cytoskeleton-associated protein
BDNF	brain-derived neurotrophic factor
CaMKII	calcium-calmodulin dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CREB	cAMP response element-binding protein
DG	dentate gyrus
E-LTP	early LTP
eIF4E	eukaryotic initiation factor 4E
eIF4F	eukaryotic initiation factor 4F
fEPSP	field excitatory postsynaptic potential
HFS	high-frequency stimulation
IEG	immediate early gene
L-LTP	late LTP
LTD	long-term depression
LTM	long-term memory
LTP	long-term potentiation
LFS	low-frequency stimulation
MAPK	mitogen-activated protein kinase
mGlu receptor	metabotropic glutamate receptor
mTOR	mammalian target of rapamycin (mechanistic target of rapamycin)
NMDA	*N*-methyl-D-aspartate
PI3K	phosphoinositide 3’ kinase
PSD	postsynaptic density
PKA	protein kinase A
PKC	protein kinase C
PKMζ	protein kinas Mzeta
PSI	protein synthesis inhibitor
S6K	40S ribosomal protein S6 kinase
STP	short-term potentiation
TBS	theta burst stimulation
TrkB	tropomyosin-like kinase B
UPS	ubiquitin-proteasome system
VGCC	voltage-gated calcium channel

Contents

1. Introduction

Research in the field of memory constitutes a central core in neuroscience, not merely aiming to understand the underlying physiological mechanism of memory but also to find suitable ways to cure several neuropathological and neuropsychiatric problems accompanied by memory disorders. Early attention placed on neurons and synapses as plausible sites for all psychological attributes was the materialist understanding that psychological attributes simply reflect anatomical function located at synaptic units (e.g. Martin et al., 2000; Abraham and Williams, 2008). This proposal was supported by subsequent advancements in neurophysiological and neurochemical approaches, indicating that synaptic modifications are an important aspect of neural function (Bliss and Lømo, 1973; Martin et al., 2000; Lynch, 2004; Diamond et al., 2005; Abraham and Williams, 2008; Baudry et al., 2011; Yin et al., 2011; Park et al., 2014). Despite these advancements, there is still a gap bridging the relevance of synaptic modifications to memory. The available data are sufficient to presume that they constitute, at least partly, an important mechanism for many forms of memory.

An important type of synaptic modification is long-term potentiation (LTP). The plentifulness of the molecules implicated, in some way or another, in LTP generation (Sanes and Lichtman, 1999) endows it a heterogeneous character in sense different underlying mechanisms are involved in its induction, expression, and maintenance (for reviews see Lynch, 2004; Malenka and Bear, 2004). One aspect representing the plurality of LTP, akin to memory, is the temporal division of the LTP into several phases: early-, intermediate-, and late-phase (E-LTP, I-LTP and L-LTP, respectively). However, despite the presumption that LTP persistence in time represents a strong criterion for its relevance to memory (Paper IV, for references), the underlying mechanisms for its persistence are poorly understood. Several hypotheses have been developed (Lisman, 1994; Frey and Morris, 1997; Frey and Morris, 1998a; Routtenberg and Rekart, 2005; Routtenberg, 2008; Lisman et al., 2012). Protein synthesis has been considered as, in some way or another, one of the main mechanisms that renders plasticity durable and long-standing, via a presumed "gating" or "switching" mechanism that converts "E-LTP" into "L-LTP" (Frey et al., 2003; Atkins et al., 2005) in a similar way to that which memories are supposed to be consolidated (e.g. Kandel, 2012). This idea, first established in several learning tasks (McGaugh, 1966; Geller et al., 1969; Squire and Barondes, 1972; Davis and Squire, 1984; Goelet et al., 1986; Dudai, 2004; Meeter and Murre, 2004; Inda et al., 2005; Klann and Sweatt, 2008), was not without serious challenges (Paper IV, for review), and the question whether protein synthesis plays a memory/synaptic plasticity-related role or is required for general brain operations has remained debatable (Paper IV, for references).

The obvious inconsistent findings about the dependence of LTP stabilization on newly synthesized proteins raised serious challenges to the hypothesis (Paper IV, for review). For example, it has been shown that, under certain conditions, LTP can be stabilized under a state of protein synthesis inhibition (*cf*. Stanton and Sarvey, 1984) even for longer periods. In addition, several other studies interpreted under the umbrella of the dominant notion that LTP is composed of a phase that is protein synthesis-dependent, can be re-interpreted in ways that disprove the notion (Paper IV, for references and discussion). It could also be suggested that LTP in young animals exhibits different mechanisms that could explain part of the discrepancies in the literature (*cf*. Kleppisch et al., 2003). Alternatively, age might have no significance (Villers et al., 2012 vs. Papers I, III) or it has significance only in conjunction with other variables (Paper IV, for discussing this issue). Against this background, this thesis tries to contribute to this enigmatic issue, which might be crucial for understanding

memory/learning mechanisms. Choosing the hippocampus is related to its essential role, as a part of the medial temporal lobe, in acquisition, consolidation, retrieval, and/or storage of some forms of memory.

2. Background knowledge

2.1. Hippocampus

There are three major reasons underlying the interest in the hippocampus: its role in physiological, psychiatric and pathological cognitive and emotional function (O'Keefe and Nadel, 1978; Cotman and Lynch, 1989; Zola-Morgan and Squire, 1990; Sakimura et al., 1995; Martin et al., 2000; Buffalo et al., 2006; Howard and Crandall, 2007; Kemp and Manahan-Vaughan, 2007; Citri and Malenka, 2008; Neves et al., 2008); it is a "simple" distinctly laminated anatomical structure (Hjorth-Simonsen, 1973; Altman and Bayer, 1975; Lynch and Cotman, 1975); and most importantly it has the ability to express a robust LTP as compared to other brain regions (Bliss and Lømo, 1973; Douglas and Goddard, 1975; Racine et al., 1983).

2.1.1. Anatomical and physiological considerations

From slice-like view, the hippocampus is characterized by trisynaptic unidirectional connectivity (Fig. 1A). However, given the hippocampal formation is essentially a class of association cortex, all types of sensory information gain access to this structure (Swanson et al., 1978; Braitenberg and Schuz, 1983; Swanson, 1983) and the afferent pathways to the hippocampus usually synapse with all its fields (Fig. 1B), renders the slice-like view an oversimplification of the anatomical connections in the hippocampus. An important functional significance of the alternative "parallel and multiple connections" conception (for an example, see Hölscher, 1997) explains why interruption of an afferent pathway (e.g. blocking the LTP at that pathway) was not always associated with a learning deficit (Robinson, 1992; Bliss and Richter-Levin, 1993; Sutherland et al., 1993; Lynch, 2004).

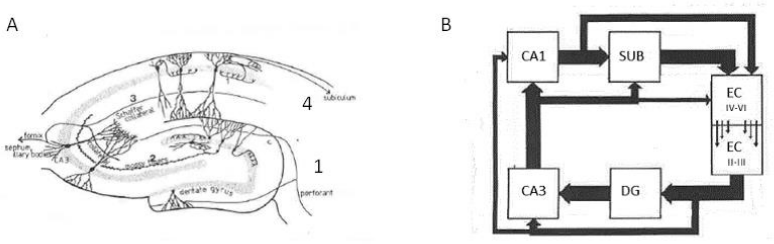

Fig. 1. *Hippocampal circuit and connections*. A) Partial schematic representation of intrinsic hippocampal circuit: inputs from the entorhinal cortex reach the hippocampus through the perforant path (1), which makes synapses with the dendrites of the dendate granule cells and also with the apical dendrites of the CA3 and CA1 (to CA1 is not shown) pyramidal cells. The dendate granule cells project via the mossy fibers (2) to the CA3 pyramidal cells. The well-developed recurrent collateral system of the CA3 cells is indicated. The CA3 pyramidal cells project via the Schaffer collateral (3) to the basal (not shown) and apical CA1 dendrites. CA1 have connections (4) to the subiculum (Rolls, 1989). B) Diagram of the hippocampal connections. Dark arrow thickness represents degree of functional significance of the connections as in a. Completion of loop is through the entorhinal cortex (EC) connections between layers IV-VI and II-III (Modified from Deadwyler et al., 1988).

The hippocampal formation is composed of the subiculum, the hippocampal proper (cornu ammonins (CA) fields), and dentate gyrus (DG). The hippocampal complex includes the hippocampal formation, and the entorhinal cortex, the perirhinal cortex, and the parahippocampal region. The major fiber systems connected to the hippocampus are composed of fibers from the entorhinal cortex and other fields of the hippocampal formation; the associational fibers of the fimbria-fornix system through which the hippocampal formation interconnects with the subcortical brain structures; and, the commissural pathways which interconnect the hippocampal formation from either side of the cerebral hemispheres. The entorhinal cortex is considered to be the first step in the intrinsic hippocampal circuit as it provides inputs (perforant paths), to the DG, CA3 and CA1. It is the fact that the DG does not project back to the entorhinal cortex underlies the concept of unidirectionality. The fiber system, originating from several subcortical areas (e.g. the medial septum and the diagonal band of Broca, the anterior thalamic area, the mammillary complex, the ventral tegmental area) via fimbria-fornix (Mosko et al., 1973; Azmitia and Segal, 1978; Wyss et al., 1979; Loy et al., 1980), constitutes an important contributor, i.e. modulator, to hippocampal function and synaptic plasticity via modulating effects of its neurotransmitters (see next section for references).

All CA subfields are divided into several layers. The stratum lucidum of CA3 receives DG axons (mossy fibers). The stratum oriens contains the basal dendrites of the pyramidal cells and several classes of interneurons. It contains also CA3-CA3 collateral connections, and CA3-CA1 Schaffer connections. The stratum radiatum is the location of major parts of the apical dendritic trees of the pyramidal neurons and in this layer most projections from CA3 to CA1 terminate. The stratum lacunosum-moleculare of CA1 area is the site where fibers from entorhinal cortex (e.g. Blackstad, 1958; for a review, see Vinogradova, 1975) and dopaminergic system (for references, see Spruston and McBain, 2007) terminate. The CA1 neurons projects directly to the subiculum but also to the medial and orbital prefrontal cortices (Barbas and Blatt, 1995).

2.1.2. Synapse/spine as basic unit for information retention

The currently dominant view is that the unit of long-term memory (LTM) storage is the synapse (Bourne and Harris, 2008; Mayford et al., 2012; Murakoshi and Yasuda, 2012). Accordingly, a Hebbian mechanism, i.e. the pre- and postsynaptic association, in single spines of hippocampal CA1 neurons has been confirmed (Matsuzaki et al., 2004) and long-term potentiation could be induced in one-to-one connections (Tsien and Malinow, 1990; but see Debanne et al., 1996). However, this does not rule out a continuous presynaptic-to-postsynaptic dialogue (Routtenberg and Rekart, 2005), postsynaptic-to-cell body cross-talk process (Dudai and Morris, 2000), neuron-to-neuron interactions within the network (Royer and Paré, 2003; Turrigiano and Nelson, 2004; Abraham and Robins, 2005; Sutton et al., 2006), or neuronal firing (Destexhe and Marder, 2004) as contributors in plasticity.

The excitatory synapse (Fig. 2) is usually located at one spine in both young and adult animals (Westrum and Blackstad, 1962; Harris and Stevens, 1988; Andersen et al., 1990). The synapse has, in general, four main components: the pre-synaptic terminal, post-synaptic end, synaptic cleft and astrocytic surround. The electron-dense thickening of the postsynaptic membrane, known as the postsynaptic density (PSD), is separated, by about a 12-20 nm synaptic cleft, from another synaptic specialization located in the presynaptic end, known as the active zone (AZ) (Hu et al., 2001). The importance of the PSD and the key proteins it contains for synaptic plasticity has led to the conclusion that the major unit for memory storage is the PSD (Lisman and Goldring, 1988).

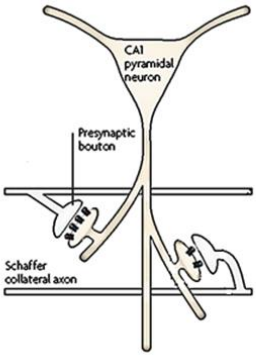

Fig. 2. *A simplified schematic representation of the Schaffer collateral synapse to the CA1 apical dendrites* (modified from Kerchner and Nicoll, 2008).

The most important receptors present in the PSD of the excitatory synapse of CA1 are of the glutamate-type (Coba et al., 2009). They are primarily divided into ionotropic (ligand-gated channels: AMPA and NMDA) and metabotropic (G-protein coupled, metabotropic glutamate (mGlur)) receptors. The α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors are made of the subunits GluA1-A4 (Seeburg, 1993). They mediate a fast synaptic current (Spruston and McBain, 2007). The GluA2 subunit is critical for the determination if the AMPA is impermeable or permeable to calcium ions (Hollmann and Heinemann, 1994). The *N*-methyl-D-aspartate (NMDA) receptors are also made of different subunits GluN1 and GluN2A-2D (Seeburg, 1993; Scannevin and Huganir, 2000; Ogden and Traynelis, 2011). Each subunit is comprised of a core channel associated with scaffolding and regulatory proteins (Scannevin and Huganir, 2000; Sheng and Pak, 2000). They are highly permeable to Ca^{2+} and mediate a slow synaptic current. They are endogenously blocked in a voltage dependent manner by Mg^{2+} (Mayer et al., 1984; Nowak et al., 1984) and the blockade is intensified by GABA-mediated synaptic inhibition (Collingridge et al., 1992).

Other receptors are also implicated in certain types of plasticity in the CA1 area. Most important among them are the mGlu (Bashir et al., 1993b; Gerber et al., 2007; Kroker et al., 2011a), dopamine (Abraham, 2003; but see Shires et al., 2012), acetylcholine (Ge and Dani, 2005; Gipson and Yeckel, 2007; Kroker et al., 2011b), adrenergic (Gelinas and Nguyen, 2005; Dommett et al., 2008), adenosine (de Mendonca and Ribeiro, 1994; Rex et al., 2005) and tyrosine kinase (Bekinschtein et al., 2007; Bekinschtein et al., 2008) receptors.

2.1.3. Synaptic potential/transmission

The electrical signals within neurons are based on movement of inorganic ions (Na^+, K^+, Cl^-) across the cell membrane. This is triggered by membrane voltage change from a "resting" potential state into a depolarization state which when it reaches a threshold level, generates an action potential in the hillock region of the soma. Axonal firing leads to release of neurotransmitter from the axonal terminals, which in turn, stimulates the post- and presynaptic receptors specified to that transmitter (e.g. McCormick, 2008). In the excitatory synapses, glutamate (and aspartate) release activates the

postsynaptic AMPA receptors leading to influx of positive charged ions into the postsynaptic sites; this inward ion flow constitutes the excitatory postsynaptic current (EPSC). This current will depolarize the postsynaptic end and gives rise to what is called excitatory postsynaptic potential (EPSP). Furthermore, depolarization caused by Na^+ inflow will act to remove Mg^{2+} from the NMDA-coupled channels, relieving the latter from the blocking effect of Mg^{2+} (see above) and allows inflow of Ca^{2+} ions. This slow process, in comparison to the fast AMPA receptors stimulation, composes the far-late component of the excitatory postsynaptic potential.

2.2. Synaptic plasticity

The proposal that memory is encoded by changes in connections between the brain's "nervous elements" and becomes stabilized during the first several minutes following its acquisition belongs to the nineteenth century (described by Shor and Matzel, 1997; Lynch et al., 2008). In the middle of last century, Jerzy Konorski coined the term synaptic plasticity to denote persistent and activity-driven changes in synaptic strength (Konorski, 1948). However, this concept turned out to be problematic as to whether the change in synaptic weight is stable or should only be transient. A stable synaptic weight change was proposed to render possible a stable storage of information although it was argued that this stability would decrease the storage efficiency of the whole memory system by early saturation (e.g. Abraham and Robins, 2005; Abraham and Williams, 2008; Citri and Malenka, 2008; Turrigiano and Nelson, 2004; Routtenberg and Rekart, 2005).

2.2.1. Forms of synaptic plasticity

2.2.1.1. Long-term depression (LTD)

A long-lasting decrease in the efficacy of synaptic transmission was first observed as a heterosynaptic phenomenon, being a reversible reduction of synaptic response in the non-stimulated pathway following induction of LTP in a separate pathway (Lynch et al., 1977). Subsequently, homosynaptic depression of basal responses that is restricted to the pathway that has been stimulated by low-frequency stimulation (LFS), or other protocols, has been observed (Dudek and Bear, 1992; Mulkey and Malenka, 1992). The most prominent investigated form of homosynaptic activity-dependent LTD is the NMDA receptor-LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Bear and Abraham, 1996; Kemp and Manahan-Vaughan, 2004).

The second common form of LTD is mGlu receptor-dependent (Oliet et al., 1997; Gladding et al., 2009; Ireland and Abraham, 2009). The expression of this form of LTD was suggested to be dependent on triggered protein synthesis (Kemp and Bashir, 1999; Huber et al., 2000; Huber et al., 2001; Snyder et al., 2001; Zakharenko et al., 2002; Neyman and Manahan-Vaughan, 2008). However, more recent studies suggested that mGlu receptor-LTD in adult (Moult et al., 2008; Mohammad, 2010) and juvenile (Mohammad, 2010) CA1 is independent of protein synthesis.

2.2.1.2. Depotentiation

Depotentiation was the first case of homosynaptic depression observed in the hippocampus (Barrionuevo et al., 1980). It is proposed that depotentiation is activity-dependent erasure of LTP (Huang and Hsu, 2001). Alternatively, it could merely represent an LTD of the current level of synaptic transmission (Wagner and Alger, 1996). The form of depotentiation induced by LFS has been

found to be NMDA receptor- (Fujii et al., 1991; O'Dell and Kandel, 1994; Wagner and Alger, 1996) and/or mGlu receptor-dependent (Bashir et al., 1993a*;* Bashir and Collingridge, 1994; Chen et al., 2001; but see Selig et al., 1995; Chinestra et al., 1993).

2.2.1.3. Long-term potentiation (LTP)

In 1966, Lømo (1966) reported that a single, short test shock, following an initial period of conditioning test shocks to the perforant path, elicited a potentiated response in the DG. This work was followed by full quantitative description of LTP *in vivo* (Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973). These findings were immediately replicated both *in vivo* and *in vitro* (Douglas and Goddard, 1975; Schwartzkroin and Wester, 1975; Alger and Teyler, 1976).

2.2.2. Relevance of hippocampal LTP to learning/memory

Memory impairment of anterograde amnesic form is typically associated with bilateral damage to the medial temporal lobe (Cotman and Lynch, 1989; Zola-Morgan and Squire, 1990). Human amnesic studies confirmed (patient H.M) that the hippocampus is essential for the formation of new episodic memories and might also have a role in their long-term storage (Maguire, 1997; Nadel et al., 2000; Squire et al., 2004). The case of R.B., a patient who as the result of an ischemic episode sustained a lesion involving the entire CA1 field of the hippocampus, has provided further evidence for its significance in enduring amnesia (Squire et al., 1990). Additionally, animal studies revealed that controlled lesions, pharmacological inactivation, or molecular knockouts limited to the hippocampus result in either a failure to learn or a loss of spatial or recognition memory (O'Keefe and Nadel, 1978; Sakimura et al., 1995; Martin et al., 2005; see also Kemp and Manahan-Vaughan, 2007; Neves et al., 2008 for reviews).

Since the discovery of LTP, a number of correlations and interactions between behavior and LTP have been described (Shor and Matzel, 1997; Martin et al., 2000; Lynch, 2004; Bliss et al., 2007; Fedulov et al., 2007; Hernandez and Abel, 2008). The Hebbian nature of this form of synaptic plasticity was confirmed by several well-described characteristics, which include cooperativity (Bliss and Lomo, 1973; McNaughton et al., 1978), associativity (Levy and Steward, 1979; Barrionuevo and Brown, 1983; Kelso et al., 1986; Steward et al., 1988; Debanne et al., 1996), input specificity (Levy and Steward, 1979; Andersen et al., 1980; Barrionuevo and Brown, 1983; Kelso et al., 1986), and durability (but see Hölscher, 1997; Abraham and Robins, 2005; Paper IV)[1]. Additionally, the rapid inducibility of LTP (Gustafsson and Wigström, 1990) is seen to be compatible with reports revealing rapid memory acquisition and encoding (O'Keefe and Nadel, 1978); drugs studies, such as NMDA receptor antagonists (Morris et al., 1986; Davis et al., 1992; but see Bannerman et al., 1995), and mGlu receptor antagonists (Bashir et al., 1993b; Manahan-Vaughan and Braunewell, 2005) causing both impairment of LTP and disruption of memory; genetic studies showing that transgenic animals carrying mutant forms of molecules necessary for normal hippocampal plasticity also possess deficit

[1] Martin et al. (2000) have proposed four additional criteria that should be met for synaptic plasticity to serve as a mechanism for learning and memory: detectability ("memory" should be observed as a change in synaptic efficacy; Kemp and Manahan-Vaughan, 2004), mimicry, anterograde alteration and retrograde alteration (see Dudai, 1995; Andersen et al., 2007).

in hippocampal-dependent behaviors (e.g. Sakimura et al., 1995; Wood et al., 2005; Yin et al., 2011); and the recent optogentic approaches which reveal activation/inactivation of some forms of memory by LTP/LTD (e.g. Nabavi et al., 2014), are considered to be evidence supporting the hypothesis that LTP may be a biological substrate for at least some forms of memory.

2.2.3. Mechanisms of LTP generation

The mechanisms responsible for enhancement in synaptic weight can be divided into induction, expression and maintenance.

Induction. It refers to the sequence of events that starts with initial triggers followed by events (signal transduction) that set into motion the process of synaptic modification (Brown et al., 1988). The induction phase is in the range of seconds, 20-30 sec (Gustafsson et al., 1989; Gustafsson and Wigström, 1990; Ben-Ari et al., 1992) or even of millisecond (Stäubli and Chun, 1996; Stäubli et al., 1998), and requires 2-5 min for stabilization (Arai et al., 1990b; Arai et al., 1990a).

The most important class of receptors that function as a trigger for LTP is the NMDA receptor (Collingridge et al., 1983; Wigström and Gustafsson, 1984). The postsynaptic depolarization is necessary to relieve the Mg^{2+} block in the calcium channel that is associated with the NMDA receptor (see above for reference) and likely to be enhanced by upstream tyrosine phosphorylation (O'Dell et al., 1991; Smart, 1997). The constriction in dendritic spine necks may participate in an amplification of the depolarization attained in the vicinity of the synapse (Harris and Kater, 1994; but see Guthrie et al., 1991; Miller, 1992). There is also evidence that mGlu receptor (Bashir et al., 1993b; O'Connor et al., 1995; Breakwell et al., 1996; Lu et al., 1997; Bortolotto et al., 1999; but see Chinestra et al., 1993) and voltage-gated calcium channel (VGCC) (e.g. Little et al., 1995) activation may have a role in the induction of LTP.

Following the transient receptors triggering events, second-messenger systems are activated. A major second-messenger is calcium. The source of the calcium that is involved in induction of LTP was considered to be mainly the ligand-gated channels (Lynch et al., 1983; Harvey and Collingridge, 1992; Malenka et al., 1992). Other second messengers such as cyclic adenosine monophosphate (cAMP), IP3 and diacylglycerol (DAG) might also be involved in induction and/or maintenance of LTP (Brostrom et al., 1975; Musgrave et al., 1993). The transducers (effectors) implicated in induction of LTP involve second-messenger-dependent kinases such as calcium/calmodulin-dependent kinase II (CaMKII), protein kinase C (PKC), and protein kinase A (PKA), and second-messenger-independent kinases such as mitogen-activated protein kinases (MAPK) and tyrosine kinase (Wang and Feng, 1992; Huang et al., 2000; Hudmon and Schulman, 2002; Huang and Reichardt, 2003; Sweatt, 2004).

Expression. It refers to those neurophysiological and biophysical changes that represent an ultimate consequence of the induced modification process and constitutes the proximal cause of the observed synaptic enhancement (Brown et al., 1988). Expression of the most common forms of synaptic plasticity is likely to involve pre- (e.g. Bekkers and Stevens, 1990; Bolshakov and Siegelbaum, 1995; Choi et al., 2000), post- (e.g. Kauer et al., 1988) or pre- and postsynaptic mechanisms (e.g. Larkman et al., 1992; Bliss and Collingridge, 1993; Lisman, 2003; Antonova et al., 2001). A key postsynaptic change downstream activated kinases involves glutamate receptors modifications that might result in an increased synaptic efficacy (e.g. Yao et al., 2008).

***Maintenance*.** Simply, it can be considered as persistence of expression. However, emergent changes might be crucial in distinguishing early maintenance events from late ones. There are several lines of evidence supporting the idea of persistent presynaptic (e.g. Bliss et al., 1986; Malinow and Tsien, 1990; Larkman et al., 1992; Voronin et al., 1992; Lynch et al., 1994), postsynaptic (e.g. Malinow, 1994; Liao et al., 1995) or some combination of the two components (Davies et al., 1989; Bliss and Collingridge, 1993) in maintenance of LTP.

2.3. Protein synthesis and its inhibition

2.3.1. Introduction

Since Flexner et al. (1963) initiated their studies for a possible role of protein synthesis in memory formation[2], it became a central tenet in the contemporary neurobiological models of memory that its formation passes through two major phases, an early protein synthesis-independent phase and a later, *de novo* protein synthesis-dependent phase. Those observations and their interpretations were followed by, and were concurrent to, findings with synaptic plasticity in a trial to essentially parallel them with behavioral studies (Paper IV, for review).

2.3.2. Protein synthesis and control mechanisms

The genetic information of the cell is stored and transmitted in the nucleotide sequences of DNA and expression of this information requires its selective transcription into molecules of mRNA that carry specific and precise messages from the nuclear "data bank" to the cytoplasmic sites of protein synthesis.

Cap-dependent protein translation, the most common pathway, is controlled by various translation factors (Sachs et al., 1997). Many of them are phosphoproteins, and the state of their phosphorylation determines their effect on protein synthesis (Morley and Traugh, 1993). Translation rates are primarily regulated at the initiation phase (reviewed in Dever, 2002); a multiple step process involving, in eukaryotes, the recruitment of the 40S small ribosomal subunit to the 5´ end of an mRNA and the positioning of the ribosome at an initiation codon (Merrick and Hershey, 1996; Trachsel, 1996; Dever, 1999; Gingras et al., 1999; Sheikh and Fornace, 1999). One feature that all eukaryotic mRNAs have in common is the presence of a 7-methylguanosine 5-triphosphate cap structure (m7GpppN) at the 5´end. With few exceptions, the 3´-end contains a poly(A) tail (Jacobson, 1996; Sachs, 2000). This poly(A) tail has been confirmed to play a role in enhancement of cap-dependent translation (Richter, 1999), especially *in vivo* (Gallie, 1991).

Before translation initiation starts, the cap structure should be recognized by the eukaryotic initiation factor 4F complex (eIF4F; Fig. 3), which contains three initiation factors: (1) eukaryotic initiation factor 4E (eIF4E), the cap-binding factor, which is responsible for recognition of the m^7GpppN cap structure (Sonenberg et al., 1978; Carberry et al., 1992); (2) eIF4A, an RNA-dependent ATPase (Grifo et al., 1984; Ray et al., 1985) that participates in RNA helicase activity (Rozen et al., 1990); and (3) eIF4G, a large protein that acts as a scaffold binding eIF4E to eIF4A. The recognition step becomes

[2] However, it was also the Flexners who incited the ongoing debate regarding the actual role of *de novo* protein synthesis in memory formation (Hernandez and Abel, 2008; Paper IV).

possible when eIF4E is phosphorylated in response to a variety of extracellular stimuli (reviewed in Raught and Gingras, 1999), which activate two converging MAPK pathways, extracellular regulated kinase (ERK) and p38 MAPKs (Raught and Gingras, 1999; Sweatt, 2001). However, other levels of initiation regulation are also required for efficient translation. Those involve phosphorylation of one of three related inhibitory binding proteins (eIF4E-binding protein; 4E-BPs) (Altmann et al., 1997; Raught et al., 2000) and of a poly(A)-binding protein (PABP) (Gingras et al., 1999; Sachs, 2000; see also Klann and Sweatt, 2008, for a review). Moreover, studies of the signal transduction cascade that lead to the phosphorylation of 4E-BP1, show the importance of three cascadic pathways: a phosphoinositide 3´-OH kinase (PI3K)/serine/threonine kinases Akt/protein kinase B or PKB, and mammalian target of rapamycin (mTOR) (Gingras et al., 1998; Dufner et al., 1999).

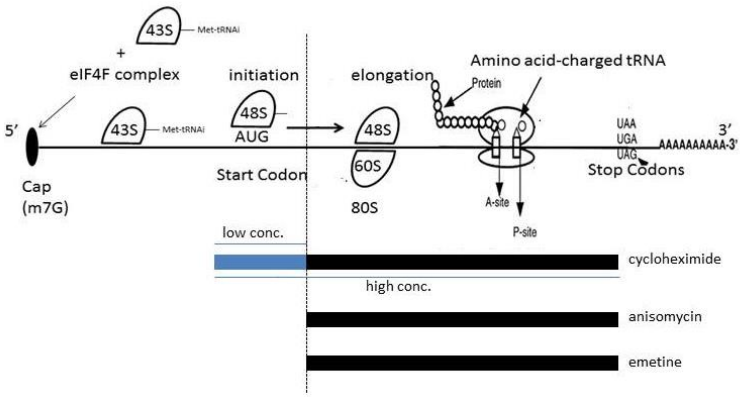

Fig. 3. *An overview showing protein translation on progress*. The first step in translation initiation is the binding of the initiator Met-tRNAi to the small 40S ribosomal subunit to form the 43S pre-initiation complex. This is enhanced by the eukaryotic initiation factor 4F complex (eIF4F). The second step is the recruitment of the 43S complex to the initiation codon (AUG) of an mRNA to form the 48S complex. Next, a release of the initiation factors from the ribosome enables the large 60S ribosomal subunit to be added to generate a translation-competent 80S ribosome that is now able to proceed with translation elongation. Low concentrations of cycloheximide act on initiation steps. Elongation involves binding of the aminoacyl-tRNA in the ribosomal A site, peptide bond formation, and translocation of the mRNA and peptidyl-tRNA on the ribosomal surface. At each step of protein synthesis now, the ribosomal peptidyl transferase transfers the growing peptide from its carrier tRNA to the α-amino group of the amino acid residue of the aminoacyl-tRNA specified by the next codon of the messenger. Anisomycin and high concentrations of cycloheximide (the concentrations that used in this work) block this step of protein synthesis reversibly while emetine blocks it in irreversible way. Anisomycin block this step by binding to 60S ribosomal subunits and blocking peptide bond formation while cycloheximide acts by inhibiting the translocation of aminoacyl-tRNA from the acceptor to the donor site. On the other hand, emetine inhibits the movement of ribosomes along the mRNA. The process of synthesis continues until one of the three stop codons are reached at which point the translation is terminated. (Adapted from Merrick and Hershey, 1996; Trachsel, 1996; Dever, 1999; Gingras et al., 1999; Sheikh and Fornace, 1999).

2.3.3. Protein synthesis inhibitors (PSIs)

The initial interesting findings that revealed the effect of PSIs on memory and aimed to support the hypothesis of initial lability of new memories (Dudai and Morris, 2000; for a review see Paper IV) have encouraged researchers to continue their work with this pharmacological tool despite the contradictory findings, and the consequent controversial interpretations that immediately followed. The debate about the role of protein synthesis inhibitors in memory and synaptic plasticity lies in

between two major perspectives: the first claims that PSIs block LTP/learning-specific processes, i.e. a triggered *de novo* protein synthesis induced by LTP-inducing or learning paradigms, while the second perspective claims that the positive effect of PSIs on memory, and expectedly, on synaptic plasticity, is due to other, non-protein synthesis effects, i.e. either due to side effect of the drugs or due to their global effect on the house-keeping processes of neurons. The antibiotics are classified into several major families. Those which are used in this work are:

Anisomycin. An antibiotic isolated from cultures of various *Streptomyces*. It is a reversible (Flood et al., 1973) translational inhibitor that blocks the peptidyl transferase reaction (peptide bond formation) on ribosome of eukaryotes (Grollman and Huang, 1976; Jiménez and Vásquez, 1979).

Emetine ($C_{29}H_{40}N_2O_4$). An alkaloid derived from ipecac ("Brazil root"), the ground roots of *Uragoga ipecacuanha*, or prepared synthetically. It is an irreversible inhibitor in HeLa cells (Grollman, 1968), if in high concentration. However, it is reversible in Chinese hamster ovary cells (Gupta and Siminovitch, 1976) even at a higher concentration. It prevents protein synthesis by inhibiting the translocation of peptidyl-tRNA on the ribosome and/or the ribosome translocation along mRNA (Vazquez, 1974). Thus, it is an inhibitor of the elongation steps in protein synthesis (Grollman and Huang, 1976). It has also the ability to inhibit the mitochondrial protein synthesis (Lietman, 1970; O'Brien, 1976). The latter effect might have a marked influence on its amnesic and synaptic plasticity decay effects (Paper II).

Cycloheximide. A reversible inhibitor (Grollman, 1968; Grollman and Huang, 1976), is produced by *Streptomyces griseus*, and inhibits the translation on the initiation step when used at low concentrations (Lin et al., 1966; Baliga et al., 1969). However, at higher concentrations, it also inhibits elongation step via acting against aminoacyltransferase II (Baliga et al., 1969) preventing, similar to emetine, the translocation of aminoacyl-tRNA (Lin et al., 1966; Baliga et al., 1969).

2.3.4. The complex outcome of PSIs

As mentioned above, one perspective proposes that the amnesic effect of PSIs relies on global and non-selective effects as well as on a suppressive effect exerted by the inhibitors on extra-brain tissues (e.g. Randt et al., 1973; Canal et al., 2007). This has caused a series of interpretive difficulties (Paper IV, for addressing this issue in details). Unfortunately, such a global inhibitory effect, i.e. at least 90-95% inhibitory effect that is obtained by high toxic concentrations, is required to get positive findings with regard to learning and synaptic plasticity (for a review see Dudai and Morris, 2000). PSIs also cause superinduction of immediate early genes (IEGs) such as c-*fos*, activity-regulated cytoskeleton-associated protein (*Arc/Arg3.1*), and c-*jun* (Paper IV). However, at higher concentration, anisomycin most likely disables the translation of these gene transcripts (Karpova et al., 2006). On other hand rebound effect, i.e. a general inhibition of protein synthesis can improve the translational fidelity at the synapses by selecting mRNAs that can be translated, has also been reported (Walden et al., 1981; Sorrentino et al., 1985; Jacobson, 1996; Scheetz et al., 2000). Furthermore, emetine, but not cycloheximide exhibits mitochondrial toxicity which decrease the axonal ATP level (Hillefors et al., 2007). This might have an effect on axonal firing mediated via the ATP-sensitive K^+ channels (Jiang and Haddad, 1991). These kinds of side effects probably were implicated in emetine's effect on synaptic plasticity (Paper II).

2.4. Protein synthesis, memory and LTP

2.4.1. Early studies

According to one idea (e.g. Matthies, 1973), lability of memory has to have two characteristics, the first is a temporary state à la Hebb (1949), that almost always follows the initial phase of memory acquisition, and the second is an interruption-resistant and permanent phase. Two consequent ideas have emerged: the ideas of a time-window and of consolidation (Paper IV, for review). The discovery of the PSIs in the late 1950s (Yarmolinsky and de la Haba, 1959) provided a direct test for the role of protein synthesis in memory and the outcome provided further evidence for both ideas (Paper IV). However, the dependence of LTM formation on macromolecular synthesis was demonstrated not only by the inhibitors, but also by numerous correlative experimental data revealing an increase of RNA-, protein, and glycoprotein synthesis during acquisition (e.g. Glassman, 1969; Dunn et al., 1974; Jork et al., 1978; see also Dunn, 1980, for a comprehensive review).

2.4.2. LTP time-courses: from observation to explanation

The early characterization of LTP into decremental and non-decremental was based on stimulation paradigms such as kindling (Racine et al., 1983), or weak vs. strong tetanization protocols. The observations led to dividing LTP into phases dependent on their time courses: LTP1, LTP2, LTP3 (Paper IV, for review). The introduction of pharmacological tools such as PSIs, or transcription inhibitors has led to a proposal that the maintenance process of the NMDA-dependent LTP in CA1 and DG is divided into two or three phases; the first one is an early phase (E-LTP) is protein synthesis-independent, and a late phase (L-LTP), which is transcription- and/or protein synthesis-dependent, both *in vivo* and *in vitro* (Paper IV, for review).

To further corroborate the hypothesis that *de novo* protein synthesis is required for induction of the late LTP phase several indirect strategies have been used. For example, as PKA is presumed to play an important role in protein synthesis, the effect of PKA inhibitors on LTP is considered as further evidence demonstrating the mechanistic LTP distinction based on triggered protein synthesis (Frey et al., 1993; Matthies and Reymann, 1993; Huang and Kandel, 1994; Nguyen et al., 1994; Impey et al., 1996; Nguyen and Kandel, 1996; Nguyen and Kandel, 1997; Nayak et al., 1998; Young et al., 2006; Habib and Dringenberg, 2010). Similarly, cAMP response element-binding protein (CREB) inhibition, IEGs antisense, brain-derived neurotrophic factor (BDNF) and tropomyosin-like kinase B (TrkB) receptor inhibition, or genetic manipulation targeting molecules implicated in some way or another in protein synthesis have led to similar arguments regarding the protein synthesis dependence of LTP (Paper IV, for review).

Whatever the temporal distinction is based on, E-LTP has been phenomenally characterized to start at around 30-45 min or less following an initial component which is called short-term potentiation (STP). However, the lifetime of E-LTP seemed to be widely variable lasting from less than one hour to up more than 5 h (Fig. 1A, 1B of Paper IV). Regardless the underlying reason, this wide life time of LTP denoted as E-LTP illustrates the difficulties in extrapolating *a priori* a definite time-course into different experimental conditions (Paper IV, for more elaboration).

2.4.3. Neurobiology of LTP maintenance

2.4.3.1. Protein synthesis-dependent mechanisms (Paper IV)

Initial studies using PSIs have shown that cycloheximide, emetine or puromycin reduced the frequency of occurrence of LTP (Stanton and Sarvey, 1984; Deadwyler et al., 1987). These

observations were concurrent to those findings shown in the hippocampus that strong synaptic stimulation evoked the synthesis of new proteins and increased the release of certain classes of proteins into extracellular space (Duffy et al., 1981; Bliss et al., 1987; Charriaut-Marlangue et al., 1988; Fazeli et al., 1988; Otani et al., 1992; Balkowiec and Katz, 2000; Hartmann et al., 2001; Gärtner and Staiger, 2002). The time course of protein synthesis in the postsynaptic cell following LTP induction appeared, in most cases, to be rapid because new proteins were seen within few minutes. Likewise, other reports have demonstrated a blockade of LTP induction by PSIs in areas CA1 or CA3 of a hippocampal slice if they were applied within a short time frame, but not when LTP was already established. Two correlated concepts were derived from those observations: the critical time-window(s) and the triggered protein synthesis. Another widely accepted idea is synaptic tagging and capture. This idea was introduced to explain how the wide-spread non-specific somatic transcription and translation processes are able to maintain input-specificity of synaptic plasticity (Frey et al., 1988; Frey and Morris, 1998a; Casadio et al., 1999).

However, several problems arise from such ideas. For example, ongoing, delayed, or several waves of, protein synthesis have been reported to be associated with synaptic plasticity induction as accompanied with learning and memory. Interestingly, a relatively recent review argues against the standard model that *de novo* synthesis of synaptic memory traces is necessarily triggered by neural activity associated with the actual events to be remembered. Rather, the synthesis of new plasticity-related proteins (PRPs) may be regulated in other ways than neuronal activity (e.g. emerged mechanisms during the course of memory formation) and over a longer time window (Wang and Morris, 2010).

2.4.3.2. Morphological and Structural changes

The second most widely accepted hypothesis for synaptic plasticity stability is the structural and morphological changes. The hypothesis is supported by LTP-induced rapid changes in the anatomy of spines and synapses (e.g. Matsuzaki et al., 2004; see also Baudry et al., 2011, for a review), an increase in the number of spines and/or change in spine shape (e.g. Bozdagi et al., 2000; Ackermann and Matus, 2003; see also Yuste and Bonhoeffer, 2001, for a review). Furthermore, some correlation between synaptic function and morphological changes has been confirmed (e.g. Brown et al., 1988; Matsuzaki et al., 2001; see Yuste and Bonhoeffer, 2001; Kasai et al., 2010, for reviews).

The time-course and role of morphological changes in LTP maintenance are not yet very clear. The increase in dendritic spine number returns back to pre-high-frequency stimulation (HFS) size and conformation within a few hours (Chang and Greenough, 1984). However, the increase in dendritic spines has been shown to persist for at least 8 h, as does LTP in the hippocampal slice (Chang and Greenough, 1984). Moreover, rapid changes are required for initial maintenance (Lang et al., 2004; Lynch et al., 2007) as they appear within few minutes following HFS (Chang and Greenough, 1984), which may represent precursors for mature synapses (Maletic-Savatic et al., 1999). The molecular pool supplementing these events is constitutive in nature (but see Fifkova et al., 1982). Later phase morphological changes are required for long-standing stabilization (e.g. Bozdagi et al., 2000; Murase et al., 2002; see also Miyashita et al., 2008 for a review). These changes are confirmed to require protein synthesis (Ostroff et al., 2002; but see Steward et al., 1988). However, whether this protein synthesis is triggered (instructive) or on demand (permissive) is unclear. Beside the requirement for protein synthesis, the molecular background for morphological changes (e.g. actin feedback polymerization, adhesion molecules, CREB phosphorylation and synthesis of fragile X mental

retardation protein (FMRP), Arc/Arg3.1, and AMPA receptors) is presumed to require "persistent activation of the appropriate protein kinase(s), resulting in continuous phosphorylation and rephosphorylation" (Dudai and Morris, 2000) to turn on them into functional substrate serving synaptic plasticity stabilization.

2.4.3.3. Protein synthesis-independent stabilizing factors

There is considerable body of evidence that attributes maintaining mechanisms of LTP to kinase(s)-mediated substrate modifications (Abraham and Williams, 2003; Lisman et al., 2012; Nicoll and Roche, 2013). Presuming that LTP stabilization (in such case it is L-LTP) requires newly synthesized proteins and/or morphological changes, either process may be insufficient *per se* to ascertain LTP stabilization before it is functional via phosphorylating/dephosphorylating-mediated modifications, otherwise, the accumulated new protein molecules would not be able serve for restructuring the emergent synaptic complexity.

The targets of signaling pathways downstream receptor activation (e.g. neuromodulatory, mGlu receptor, VGCC, TrkB) are not restricted to the translational processes but are also implicated in non-translational stabilization events, i.e. posttranslational modifications. The case is applicable to almost all the known downstream cascade of activated kinases including the PI3K, mTOR, tyrosine kinases, 40S ribosomal protein S6 kinase 1 (S6K1), BDNF, or various MAPKs (e.g. Passafaro et al. 2001; Lhuillier and Dryer, 2002; Pereira et al., 2006; Simsek-Duran and Lonart, 2008; Chan et al., 2011; Malik et al., 2013). This may entail that maintenance of LTP could be achieved even when one of their targets (e.g. protein translation) was curtailed (section 6.7).

2.4.3.4. Protein degradation and synaptic plasticity sustainability

An early indication for the importance of protein degradation in LTP mechanisms was the discovery that partial proteolysis of kinases renders them persistently active. The irreversibility of partial proteolysis of kinases underlies their persistent activity (Kishimoto et al., 1983; Melloni et al., 1985; Bayer et al., 2001; see also Schwartz and Greenberg, 1987; Micheau and Riedel, 1999 for reviews), which might result in relatively long-lasting synaptic changes (e.g. Hegde et al., 1997; Micheau and Riedel, 1999; Ahmed and Frey, 2005). Important kinases that undergo proteolysis-mediated persistent activation include PKA and PKC. One isoform of PKC is the atypical zeta form, and the proteolysis of this form leads to release of a catalytic subunit known as protein kinas Mzeta (PKMζ). It is probably that the effect of PKC on LTP is related, at least partially, to this persistent form of PKC (Kishimoto et al., 1983; Suzuki et al., 1992; Sacktor et al., 1993). However, there is also an evidence that PKMζ has no role for maintenance of LTP (e.g. Denny et al., 1990; Sajikumar and Korte, 2011; Wu-Zhang et al., 2012; Volk et al., 2013).

Another degradation system is the proteasome system, which is composed of an ATP-independent (20S) and an ATP-dependent (26S) component that involves ubiquitination, and has a role in controlling the half-lives of important regulatory proteins. Interestingly, LTP induction in the hippocampus has been found to lead not only to an increase in the rate of protein synthesis but also to an increase in the active degradation of proteins (Colledge et al., 2003; Ehlers, 2003). Furthermore, inhibition of protein ubiquitination, or proteasome activity leads to impaired "L-LTP" (Paper IV, for references).

The mechanism(s) by which the degradation mediated via the ubiquitin-proteasome system (UPS) is complex. For example, in contrast to *Aplysia* studies (e.g. Speese et al., 2003; Zhao et al., 2003), evidence in mammals provided a positive role for enhanced degradation in boosting synaptic plasticity and regulating the molecular architecture of synapses (Paper IV, for references).

Moreover, reactive oxygen species can modify macromolecules in several ways. Beside the direct free radical mediated modification, direct non-radical-mediated (e.g. hydrogen peroxide) protein modifications can also occur via reactions of carbonyl groups. All these modifications, without repair processes or degradation, lead to dysfunctional/hazardous proteins, among other cellular components, which might have deleterious effects such as inactivation of enzymes and at longer timescale may lead to formation of aggregates that are associated with several neuropathological conditions (e.g. Halliwell, 1992). Despite the repair systems that are available, for the vast number of amino acid oxidation products no repair mechanisms are known. Therefore, the removal of oxidized proteins and the re-synthesis of them seem to be the major pathway for repair. It has been demonstrated that the ATP-independent component of proteasome system (20S) is the major pathway for degradation of moderately oxidized proteins (Jentsch, 1992; Grune et al., 1997; Orlowski and Wilk, 2003).

3. Aims

Understanding the mechanisms for synaptic plasticity stabilization may have profound implications for many areas extending from memory, forgetting and learning to amnesic disorders, obsessive recollection, posttraumatic stress disorder, acquired phobia, drug addiction and schizophrenia. Unfortunately, there are still lots of "black boxes" for understanding stabilization, and the exact nature of the role of protein synthesis is far from clear. The NMDA receptor-dependent LTP in CA1 area was studied with respect to stabilization when the global protein synthesis was inhibited. The specific goals of this study were:

1. To examine whether LTP stabilization can be obtained under PSIs regimes when applied before, after, and during LTP induction
2. To evaluate to what extent the putative effects of PSI on LTP are specific for LTP.
3. To introduce a tentative model that contributes to explain the controversial findings regarding the role of PSI in LTP stabilization.
4. To test the hypothesis that protein turnover has a role in stabilization of LTP.

4. Methods and Materials

4.1. Methods

4.1.1. Animals

Sprague-Dawely rats, unless specified, of either sex thrived in the Experimental Biomedicine (EBM) animal facility accredited the Swedish Central Council for Laboratory Animals were used. Animal procedures were performed in ways approved by the Local Ethics Committee at University of Gothenburg. As almost, with few exceptions (Aakalu et al., 2001; Fonseca et al., 2004; Fonseca et al., 2006), relatively young adult or adult animals were used to investigate the role of PSIs in synaptic

plasticity, juvenile animals aged between 14 to 22 days were used in part of this work. The rational for choosing this age is the early reports that revealed significant age-related decrease in protein synthesis both *in vivo* (Gaitonde and Richter, 1956; Dunlop et al., 1977; Waterlow et al., 1978, p.455; Goldspink, 1988) and *in vitro* (Orrego and Lipmann, 1967). However, the fact that young animal is of greater ease of manipulation well as the finding that slices of younger rats "usually give more robust, clearer LTP" (McEachern and Shaw, 2001) also contributed to our choice. There was no worry whether the hippocampus at this age is of significance to the animal's behavior. The development of the hippocampus is complete following the first week of rat age (Vinogradova, 1975) and behavioral correlates tell us that the behavioral repertoire of the developing animals is rapidly increasing from 15 days old rat (e.g. Barnett, 1975; Campbell et al., 1969; Leblanc and Bland, 1979). These observations are consistent with the reported "maximal" hippocampal LTP expression, as induced by tetanus protocols, to be reached at 15 days (for a review, see Bennett, 2000) or even 11 days (Cao and Harris, 2012) of rat age.

4.1.2. *In vitro* slice preparation

Technical procedures. Animals were deeply anesthetized by isoflurane and decapitated between 13.00 and 14.00 p.m. to prevent variations caused by circadian rhythms or nonspecific stressors (Teyler and DiScenna, 1987). This type of anesthesia is less likely to interfere with the electrical (e.g. decreasing the EPSP size) or biochemical (e.g. decreasing the brain protein synthesis) responses as has been reported in other types of anesthetized animals (Yamamoto and McIlwain, 1966; Gaitonde and Richter, 1956).

The rat brain was removed and placed in an ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 119, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 26, NaH_2PO_4 1 and glucose 10. All solutions during experiments were oxygenated by O_2 95% and CO_2 5% and their pH was 7.4. The hippocampus was dissected out and transferred to the chopper. Only in one group of experiments (Fig. 1, Paper I), slices of 400 µm were obtained using a vibratome.

Slice pre-incubation interval. It is well-known that the improvement of slice preparation after the trauma of slicing depends on the pre-incubation period (deliberated in Sajikumar et al., 2005; Redondo et al., 2010, for examples). Biochemical studies conclude that a recovery period of at least 1 h is allowed prior to data acquisition in a hippocampal slice. However, despite the fact that it has been concluded that 1 to 2 h pre-incubation interval is sufficient for metabolic slice stabilization (e.g. Whittingham et al., 1984), several authors insist that at least 4 h pre-incubation period is required for reaching a metabolic stability in slices (Sajikumar et al., 2005; Redondo et al., 2010). In our hands, at least 90 min was sufficient for pre-incubation at room temperature before they were transferred to recording chambers.

4.1.3. Extracellular recordings

Chambers setup. For extracellular field potential recordings, a single slice was incubated in a submersion recording chamber. The slice was submerged between nylon net and a set of parallel nylon threads attached to a U-shaped platinum wire to stabilize the slice. The chambers consisted of circular well of a low volume (1-2 ml) and was perfused continuously with warm (31 °C), oxygenated ACSF (Ca^{2+}:Mg^{2+} was 2.5:1.3 mM) at a flow rate of 1.5-2 ml/min. Each chamber is provided with two stimulating electrodes and one recording electrode. Electrode positioning is performed under visual

guidance using an upright stereomicroscope**,** which was used to identify the CA1 region of the hippocampus.

Two monopolar tungsten stimulating electrodes were used to stimulate two distinct bundles of Schaffer collaterals. Recording of field excitatory postsynaptic potentials (fEPSPs) was made by a glass micropipette filled with 1 M NaCl (*R* = 2.5-5 MΩ) lowered into the CA1 stratum radiatum at equal distances (100-200 μm) between the stimulating electrodes to achieve as much as possible symmetrical responses. Negative, constant current pulses, 100 μs, were alternately delivered to the two stimulating electrodes, providing access to a pair of separate sets of afferents. The interval between the successive stimuli was either 20 or 30 s (40 or 60 s for each, respectively), depending on experimental design.

The validity of two inputs procedure. The two-pathway design is possible due to the fact that most fibers travel at the plane of the slice. Thus, stimulating electrodes positioned on either side of a population of neurons activate a non-overlapping set of axons projecting to the same target cells. Also, this configuration allows the investigation of the effect of drugs on synaptic plasticity or transmission both prior to and after perfusion (Bortolotto et al., 2001) on one hand, and the health of slice, on the other hand (e.g. Abraham et al., 1995). However, the method is not foolproof because one input can change independently of the other. For example, a stimulating electrode can move, one set of fibers can deteriorate selectively (Bortolotto et al., 2001). Although not straightforward, the control pathway, in our experiments, was monitored continuously, and experiments with control pathway decayed more than 30% below baseline were rejected (Paper I, III; *cf*. Fonseca et al., 2006).

Data analysis and Readouts from experiments. Signals were amplified, filtered, digitized and transferred to a PC computer for on- and off-line analysis. The AMPA-receptor mediated EPSPs component was measured using an early time window positioned just after the presynaptic volley. Measurements were calculated by integrating the EPSP curve along the specified time window after subtraction of the pre-stimulus baseline. Alternatively, the EPSP quantified by slope measurement was used which earlier have been shown to give similar results (Dozmorov et al., 2003). The amplitude measurement was generally conducted unless otherwise indicated.

Responses of the test pathway were expressed relative to the pre-LTP induction baseline and/or in some cases relative to responses of the control pathway. This procedure was used when there was no difference in the control input decay rate between the groups (but see Paper II, especially Fig. 2). The amount of LTP was estimated by measuring the response size during 5-10 min intervals positioned at certain times. Values are expressed as mean ± SEM.

Pharmacological compounds and drug treatments. Anisomycin (2-[p-methoxybenzyl]-3,4, pyrrolidinediol-3-acetate), emetine dihydrochloride hydrate (referred to as emetine), cycloheximide (4-{(2R)-2-[(1S,3S,5S)-3,5-dimethyl-2-ococyclohexyl]-2-hydroxyethyl}piperidine-2,6-dione), dimethysulfoxide (DMSO), ferrous sulfate ($FeSO_4$), Rp-adenosine 3´, 5´-cyclic monophosphorothioate triethylammonium salt hydrate (Rp-cAMPS), R(+)-SCH-23390 hydrochloride (SCH23390), and hydrogen peroxide 3% (H_2O_2) were obtained from Sigma-Aldrich (St Louis, MO, USA). The NMDA receptor antagonist D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5, referred to as AP5), AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), Z-Leu-Leu-Nva-al (MG-115), and the adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were purchased from Tocris Bioscience (UK) or Ascent Scientific Ltd (UK). [^{3}H]leucine was obtained from Amersham,

Buckinghamshire, UK. Milli-Q deionized water (Millipore, Bedford, MA, U.S.A.) was used in all preparations of buffers and solutions. Drugs were made up as stock solutions (10-1000 x final concentration) in H_2O for AP5, emetine, SCH23390, Rp-cAMPS, $FeSO_4$, and anisomycin, and in DMSO 99% v/v. for cycloheximide, DPCPX, MG-115, and CNQX. DMSO at final concentration of 0.1-0.5% was added to the solution of the control group as vehicle. The stocks were diluted in ACSF to achieve their desired final concentrations. For extracellular experiments, anisomycin was added to ACSF to a final concentration of 25 µM or 40 µM (in some experiments) from 5 mM stocks prepared at the time of each experiment. Cycloheximide was first dissolved in DMSO to a concentration of 100 mM and stored at -18 °C until then diluted in physiologic buffer to final concentrations between 35 to100 µM. Emetine was dissolved in distilled water to a 10 mM stock concentration before it was diluted by ACSF to final concentration (20 or 40 µM). AP5 and CNQX were prepared as stock solutions in distilled water (50 mM) or DMSO (5 mM), respectively, and stored at -18 °C until used at final concentration of 50 µM or 10 µM in the bath ACSF, respectively. SCH23390 and Rp-cAMPS were prepared as stock solutions (10 mM) in distilled water and stored at -18 °C until used at final concentration of 100 µM in the bath ACSF.

LTP-induction paradigms. Two LTP-inducing paradigms have been used in this work. Usually three trains of high-frequency stimulation, HFS (100 impulses at 100 Hz), separated by either 20 s (Paper I), or 5 s (Paper II, III) were used to induce LTP. Alternatively, three trains of theta burst stimulation (TBS), each composed of 10 bursts of four pulses at 100 Hz, repeated with a burst frequency 5 Hz, intertrain intervals of 15 min (Paper I) or 5 s (Paper I, III) were applied to induce robust LTP. This induction protocol is considered more “naturalistic”, given pyramidal cells in CA1 commonly fire short (30- to 40-msec) bursts of three to four spikes during learning (Kandel and Spencer, 1961; Vanderwolf, 1969), with the burst being repeated at 5-12 Hz, or theta frequency (Vanderwolf, 1969; Bland, 1986). For the MG-115 experiments, 50 pulses, at 100 Hz frequency were delivered to induce a “weak” LTP.

4.1.4. Protein synthesis effect tests

4.1.4.1. Effect of PSIs on baseline

Baseline monitoring can be used to indirectly judge the potency of the drug (e.g. emetine) when the tritiated leucine assay was not available. In baseline experiments, the same protocol for LTP experiments was followed, except that HFS was not given. Moreover, observing baseline pattern may give a clue for a “non-specific” effect of a PSI.

4.1.4.2. Yeast assay

Yeast growth observation was also used to evaluate the inhibitor effectiveness when the tritiated leucine incorporation method was not employed or when there was no effect on baseline pattern (e.g. anisomycin). One percent of yeast extract, i.e. yeast lacking cell wall (strain W303-1A of *Saccharomyces cerevisiae*) was grown in medium containing 2% peptone supplemented with 2% D-glucose as a carbon source (YEPD). Plate growth assays were performed by pregrowing the cells in YEPD liquid medium. Cells were resuspended in the same medium to an optical density measured at wavelength of 600 nm (OD600) of 1.0. A 10-fold serial dilution of this culture was made and 5 ml of each dilution was spotted onto agar YEPD plates. While one YEPD agar plate was used as control the

other was supplemented with 25 μM anisomycin. Yeast growth was monitored after 2–3 days at 30 °C (Paper I).

4.1.4.3. Leucine incorporation method

Potency of PSIs in whole slices was measured by [^{3}H]leucine incorporation into trichloracetic acid (TCA) precipitable macromolecules. Hippocampal slices from 13-24 days old rats were maintained under similar conditions as in the electrophysiological experiments but without electrical stimulation. Slices were put on a multiwell plastic dish (Corning Incorporated, Corning, N.Y.) and assigned to a PSI group or a control group in an interleaved manner to minimize interslice variability with respect to weight and metabolism. Both groups of slices were treated by tritiated leucine, final concentration 0.5–1 μCi/ml, incorporation of which was terminated by washing out the isotope in ACSF and placing slices in NaOH (5 mM). After protein precipitation, leucine incorporation was measured in a scintillation counter (LKB Wallace, 1219 Rackbeta, Finland). The degree of incorporation of labeled leucine into slice protein, which is regarded as a measure of the rate of protein synthesis, was calculated for each set of slices as the ratio of counts per minute in the precipitate to the total activity of tritiated leucine in the sample. Percentage inhibition of leucine incorporation produced by drug treatment was calculated by comparing counts in treated slices with those of control slices.

To test the effect of PSIs on rate of protein synthesis in dose-dependent manner, two groups of slices were pre-incubated for 10 min with different concentrations of the drug (or vehicle, if necessary) before addition of [^{3}H]leucine. Uptake and incorporation of leucine into proteins were allowed to proceed for 50 min either with or without the PSI.

For PSIs time-course reversibility test, a corresponding total duration of pre-incubation with the drug of 60 min was followed by different pre-incubation intervals (30, 60 or 90 min) in drug-free solution before allowing slices to be incubated in [^{3}H]leucine-contained solution for further 50 min. Radioactive amino acid incorporation was then terminated by the same way as mentioned above.

For the effect of hydrogen peroxide on the stability of the newly synthesized proteins, two groups of slices were incubated with tritiated leucine for 50 min at 31 °C following a pre-incubation period equivalent to that for electrophysiological experiments. Subsequently, slices were washed out from the leucine-containing ACSF and continuously perfused by new warm, oxygenated ACSF for a baseline period of 30 min before H_2O_2 was applied to one group of slices for 20 min under recirculation conditions identical to the LTP experiment while the other group was maintained under control condition. Switching solution to normal ACSF was then conducted for the first group and slices were terminated, rinsed and frozen (-80 °C) after different time intervals with their corresponding control slices.

4.1.5. PSIs application regimes

Slices are treated with different time intervals (Fig. 4). Initially, the traditional application time window is used, i.e. around 30 min before LTP induction to 30 min after (-30/+30). However, extended regimes are applied according to the lack of effect of PSI on LTP stabilization (Paper I). Emetine is applied either from 90-120 min before tetanization and washed out immediately or from 3 h before tetanization and kept throughout the experiment (5-6 h after tetanization) (Paper II). Another extreme protocol has also been performed whereby PSI is applied from 30 min before LTP induction and kept throughout the experiment (Paper III). The rationales include the lack of effect of

short application time interval (Paper I), the confounding effect associated with long emetine application times (Paper II), and the correlation between the PSI application time and the degree of LTP decay (Ris et al., 2009). Moreover, cycloheximide is applied for 4 h before LTP induction, either washed out immediately before induction or 1 h before (Paper III).

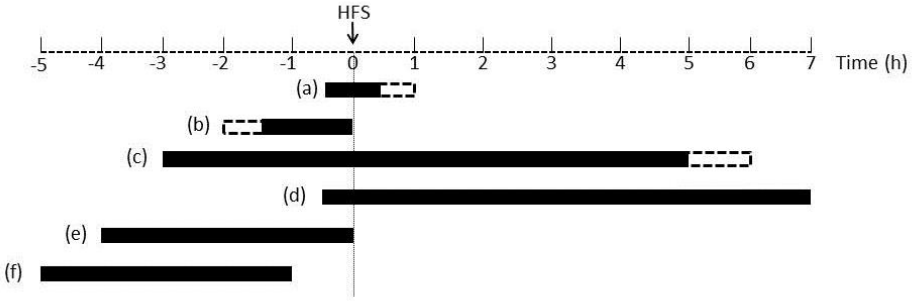

Fig. 4. *Schematic illustration of PSI treatment regimes*. Different PSIs are used at different time intervals with regard to LTP induction by HFS (0 h). Horizontal bars indicate PSI. a) Emetine or anisomycin was used at time interval extends 30 min before HFS and washed out 30 min after HFS. However, anisomycin experiments are pooled from two groups, one induced by "standard" HFS (see Methods) and the second with longer inter-train intervals. In the latter case, anisomycin treatment was extended for further 30 (dashed bar) after the last train of HFS (total 60 min) (Paper I). b) Emetine is applied for a time periods 90-120 min and washed out at 0 h (HFS), or c) for a time intervals starting 3 h before HFS and kept throughout the experiment (5-6 h following HFS) (Paper II). d) Either a mixture of cycloheximide and anisomycin, or cycloheximide alone is used from 30 min before HFS and kept throughout the experiment (Paper III). e) Cycloheximide is applied for 4 h before HFS and washed out immediately before HFS or f) 1 h before HFS (Paper III).

4.2. Statistical analysis

Field EPSP (mV/ms or mV) was calculated and data for each experiment were normalized relative to baseline recording. To test for group differences between LTP values across conditions a two-tailed Student's *t*-test was applied (Paper I, II, III, IV). When normality tests (Shapiro-Wilk test and Q-Q plot) revealed non-Gaussian distribution (Paper III), nonparametric tests were conducted (SPSS for windows, Version 18.0): Mann-Whitney *U* test to compare data between groups (drug vs. control). Numbers of experiments are indicated by *n*. Probability values of $p < 0.05$ were considered to represent significant differences.

5. Results

5.1. Short-time application of PSIs (Paper I)

Anisomycin or emetine, at concentrations reported to produce protein synthesis inhibition over 90% have been applied with a short time-window interval (-30/+30-60 min; Fig. 4(a)). The LTP-inducing paradigm for the anisomycin-treated slices was either tetanus or TBS. LTP recorded for 4-5 h revealed similar magnitude and time-course between the groups (Fig. 1, Paper I). Mechanisms of TBS induction of LTP may differ from those of continuous HFS (for references, see Cao and Harris, 2012). Therefore, the data was re-categorized revealing no difference between the paradigms on the stability of LTP in drug-treated vs. control (data not shown). Furthermore, as it was reported that

spaced protocol, i.e. induction protocol with long inter-train intervals, usually reveal long-standing LTP compared to the massed ones, i.e. with short inter-train intervals (Scharf et al., 2002; Woo et al., 2003) and as both protocols have been used in the above mentioned results, re-analysis of data has also not shown differential LTP resistance for anisomycin (data not shown). In analogy with anisomycin findings, substantial LTP was induced in either normal or emetine-treated slices (applied from 30 min before to 30 min after LTP induction), even when longer recording time reaching for up to 9 h was obtained. Similarly, no effect of emetine on LTP stabilization has been shown (Fig. 2, Paper I).

5.2. Verification the effect of drugs and chemicals

5.2.1. Effect of PSIs on baseline (Paper I, II, III)

In a first attempt to investigate whether the used concentrations of drug were actually potent, we carried out experiments where anisomycin or emetine was applied for 6 h periods and the effect on baseline synaptic responses was examined. Responses in anisomycin-treated slices (25 μM) started to decline steadily after about 3 h of treatment yielding no significant difference in the final synaptic response reduction at 6 h in comparison to control slice group (Fig. 4A, Paper I). Our findings with anisomycin were further replicated, in another set of experiments, where 4-5 h anisomycin (25 μM) together with AP5 were applied (Fig. 5E, Paper II). This pattern is also observed with cycloheximide (Fig. 5D, Paper II). The effect of cycloheximide on the control input was further verified by a separate set of experiments (Paper III) where cycloheximide at 100 μM was applied for 4 h and then washed out and the ongoing synaptic responses were monitored for up to 8 h. Again, there was no effect on baseline responses (data not shown).

However, following emetine application, responses started to decline after 1–2 h, and at 6 h there was above 65% reduction of responses. This was significant when compared to the decay of responses in untreated slices or slices treated with anisomycin (Fig. 4A, Paper I). Continuous emetine application revealed a serious effect on slice viability (Figs. 2A, 5A, Paper II), inconsistent with *in vivo* (Hagena and Manahan-Vaughan, 2013) and other *in vitro* (Manahan-Vaughan et al., 2000; Stanton and Sarvey, 1984) reports[3]. Although the findings did not tell us whether the emetine-induced depression in the basal responses was due to a block of protein synthesis, it is unlikely due to an apoptotic effect because similar concentration of cycloheximide has been shown to have nearly the same apoptotic effect to that of emetine (*cf*. Meijerman et al., 1999).

5.2.2. Yeast growth (Paper I)

Since even long time application of anisomycin had no clear effect on baseline transmission, the effect of the drug on yeast growth was investigated. Monitoring the growth of yeast after 2-3 days revealed a complete block in comparison with untreated cultures (Fig. 4B, Paper I), in accord with previous results (for a review, see Jiménez and Vásquez, 1979).

[3] One tentative explanation, though not meta-analyzed yet, is that the frequency of test stimulation might be a major player in the inconsistent data. This is based on our observations (unpublished data) that increasing the frequency of test stimulation was accompanied by more decaying effect of emetine on baseline responses.

5.2.3. Drug potency tests (Paper I, II, III)

Neither the drug effect on baseline response, nor the effect on yeast growth is conclusive whether the potency of the PSIs is or is not related specifically to protein synthesis. Thus, the level of protein synthesis inhibition was measured directly by leucine incorporation into the proteins of whole slices. Initial findings with anisomycin pre-incubated in the slices before the addition of tritiated leucine to the medium revealed about 93% of inhibition (Fig. 4C, Paper I). These values were further corroborated, by testing different concentrations of the drug on protein synthesis inhibition. Similarly, different concentrations of cycloheximide were tested revealing potent effect (Table 1, Paper II). For a more accurate assay, dose-dependent inhibition tests were conducted with anisomycin, as with cycloheximide (Figs. 2A, 2B, Paper III). The values confirm that the doses employed in the electrophysiological tests were efficient in term of a saturated level of protein synthesis inhibition.

In some experiments, a mixture of anisomycin (25 µM) and cycloheximide (70 µM) has been used to investigate whether such a drug cocktail would lead to a stronger inhibitory effect. However, the leucine incorporation assay revealed inhibitory levels not significantly higher than those obtained from either the drug alone at the same concentration (Fig. 2C, Paper III).

Emetine, again exhibited a distinct effect. An inhibitory value above 90% was obtained with 40 µM concentration whereas at 20 µM only 70% of the synthesis was blocked (Table 1, Paper II). However, the drug exhibited a time-dependent inhibitory effect. In a separate set of experiments designed to investigate the time-course pattern of the inhibitory effect of emetine, we observed an inhibitory rate of over 80% after 90 min of incubation with the same concentration of the drug, i.e. 20 µM, and this rate steadily increased over time, reaching 90% after 180 min (Fig. 5). As the values represent an average of inhibition following a total time of incubation with the drug, the "actual" time-course represents the sum of pre-incubation period + incubation period (50 min). This indicates that only following a total incubation interval of 90 min, emetine inhibitory effect reaches above 80% (Fig. 5). These data replicate the findings shown by Villers et al. (2012) as at around 80 min of emetine incubation there is about 69% of inhibitory effect. These values are also consistent with the observation that the baseline response begins to decay after a 90 min period of incubation with emetine. In contrast to emetine, anisomycin does not exhibit such property of time-dependency as maximum inhibitory effect is obtained with short incubation interval and does not increase when the application time is extended (data not shown).

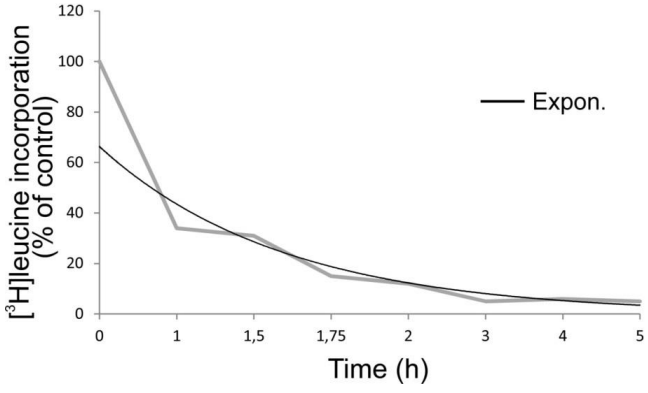

Fig. 5. *Time-depend inhibitory effect of 20 µM emetine on the protein synthesis*. Several groups of hippocampal slices were pre-incubated with emetine for different durations and their corresponding control groups were pre-incubated with ACSF for equivalent durations. Following pre-incubation period, slices incubated with ACSF containing tritiated leucine together with emetine for test groups and without emetine for the control groups, for further 50 min and treated ordinarily as described above. Each time point is based on pooled values (± S.E.M.) from 4 experiments compared to their own control (normalized at 100%). Each experiment is average of two slices. Exponential plot (Expon.) is superimposed on the actual curve.

5.2.4. Time-course of reversibility (Paper III)

In the experiment illustrated in Fig. 3A (Paper III), the inhibitor was first applied for 60 min, followed by a variable washout interval (0-90 min) before [^{3}H]leucine (without PSI) was applied for a 50 min period. The histogram data in Fig. 3B (Paper III) depicts that with no drug washout, there was a high blocking effect for both anisomycin and cycloheximide with values amounting to 96% and 90%, respectively. When using washout periods of increasing duration, 30-60-90 min, the inhibition by cycloheximide decreased substantially but always left a residual effect amounting to 20% with 90 min washout periods. The alternative drug, anisomycin, showed even slower reversibility with very little recovery of protein synthesis. The inhibition values were around 90% for all the three washout durations.

5.2.5. Proteins lifetime following hydrogen peroxide treatment (Paper III)

In a set of experiments in which slices were submitted to oxidative stress condition shortly following LTP induction (Paper III), a complementary study was performed to test whether oxidative stress had any contributing effect on protein degradation. Thus, a series of slices were assayed for leucine incorporation rate. The findings (Fig. 8, Paper III) revealed that the percentage of radiolabeled proteins 3 h following H_2O_2 (plus 100 µM $FeSO_4$) washout decreased significantly amounting to 85±4% of control value (< 0.05).

5.3. Emetine and LTP (Paper II)

Because of the time-dependent effect of emetine on baseline response stability (Paper I), emetine is not the drug of choice for testing LTP stabilization. However, this disadvantage has been exploited to investigate the mechanism(s) by which the long time application regime induces decay in LTP parallels to that induced on the control input.

5.3.1. Effect of long pretreatment of emetine on LTP

Fig. 1A (Paper II) depicts data from a set of experiments where emetine at a concentration of 20-40 µM was pre-incubated for 90-120 min before induction of LTP (Fig. 4(b)). Despite the long treatment with emetine, responses of the test pathway showed a clear potentiation during the first hour after induction. Comparison between test and control pathways revealed that the substantial decline of potentiated fEPSPs during the 8 h post-tetanus recording period was most likely due to a general decrease of slice viability. A comparison of responses in test vs control pathways at 8 h after induction, showed no significant difference for experiments with and without emetine (Fig. 1C, Paper II), which indicates further that the effect of the drug on LTP reflects its effect on basal synaptic transmission. However, there was a difference in that LTP, under emetine treatment, appeared to develop much slower. Whereas the “normal LTP” in Fig. 1 C (Paper II) peaked quickly after induction, the “emetine LTP” did not peak until after nearly 30 min.

5.3.2. LTP under extended emetine treatment

Additional experiments with emetine treatment starting earlier and ending later were carried out to further test the effects on LTP. Fig. 2A (Paper II) shows that such long application time had a serious effect on slice viability, as evidenced by complete disappearance of responses at 5-6 h after induction (corresponding to 8-9 h of total emetine treatment; Fig. 4(c)). Again, when calculating the degree of potentiation as test/control ratio (Fig. 2B, Paper II), "LTP" was presumed to be "similar" to normal LTP. Thus, it was concluded that LTP was stable at 4 h after induction and equivalent to that obtained under normal conditions (Fig. 1B, Paper II).

However, the data can be interpreted in a way that emetine, when applied for long time before tetanization, dramatically blocked LTP at its early stage rather than presuming that LTP was stable under emetine treatment (Paper II). In any case, the data confirm that this effect was not related to changes in LTP, but rather due to the effect of the drug on the basal synaptic transmission.

5.3.3. Time-dependent and emetine-specific transient depression

The nature of the slow onset of emetine-LTP was examined by performing additional experiments in presence of the NMDA receptor antagonist AP5 (50 μM). Fig. 4 (Paper II) depicts the first 90 min after tetanization. Due to the expanded time scale relative to Fig. 1 (Paper II), it is clear that the time to reach peak LTP in the emetine case was around 30 min. AP5 effectively blocked LTP, and in the slices lacking emetine pre-treatment (Fig. 4D, Paper II), there was no remaining "plasticity" (depression). When AP5 was present in combination with emetine treatment (Fig. 4B, Paper II), there was a remaining transient drug-dependent depression of fEPSPs, lasting for about 30 min.

Fig. 5 (Paper II) depicts an experiment with long-term emetine and AP5 treatment, illustrating the successive development of the transient depression under a "polytetanization protocol". It can be seen that after about 2 h of emetine treatment, the transient depression resulting from tetanization becomes more prominent with each successive test (Fig. 5B vs. 5C, Paper II). Application of AP5 together with either anisomycin or cycloheximide for more than 5-6 h failed to show quantitatively similar transient depression found with emetine (Fig. 5D, 5E, Paper II).

5.3.4. Fibre volley-dependent emetine-related depression

Further examination of recorded potentials revealed that during the emetine-dependent depression not only the fEPSP was depressed but also the fibre volley. As the trace in Fig. 5C (vs. Fig. 5B, Paper II) shows, a clear volley effect was seen under the long-term emetine treatment. We reasoned that if a change of fibre volley were the underlying cause of the fEPSP depression observed under emetine treatment, there would be a parallel change in these components of the response. Thus, we plotted average fEPSP vs. fibre volley during the transient depression after tetanization (Fig. 6A, Paper II) as well as during the gradual decline of baseline responses after long emetine treatment (Fig. 6B, Paper II). The curves reveal a linear relation in both cases. Fig. 6D (Paper II) shows that dividing the original fEPSP data with volley amplitude data yielded a normal-looking LTP curve.

5.5. Effect of continuous PSIs application on LTP (Paper III)

The above mentioned negative findings on LTP (section 5.1) and the LTP-non-specific changes (section 5.3) motivated us to optimize the experimental conditions. In line with a report indicating that longer post-induction drug application might be more effective (Ris et al., 2009), we omitted the

washout event, extending the period of drug application until the end of the experiment. According to the protocol illustrated in Fig. 1 (Paper III), a mixture of anisomycin and cycloheximide was applied from 30 min before HFS and kept for the rest of the recording period (Fig. 4(d)). HFS induced a substantial and stable LTP in both non-treated and treated groups of slices. The drift of the control pathway was small and similar in the two groups, i.e. a decline of about 20%, suggesting that slices were maintained under viable conditions and not harmed by the prolonged treatment. It is notable that LTP values of normal and treated slices, measured as percent of pre-tetanization baseline, were similar at both early and late time points (Figs. 1A, B, Paper III).

5.6. Extended pre-treatment of CHX with or without pre-treatment washout (Paper III)

We used another extended pre-treatment protocol of cycloheximide (100 μM) aiming to cause a significant depletion of the constitutive proteins and to observe whether there would be an effect on LTP. However, taking in consideration that total recording time was generally limited to 12-13 h the baseline recording interval was restricted to maximal 5 h. In the view of slow reversibility of cycloheximide (see 5.2.4), it might be that protein synthesis will also be blocked during roughly an hour of the post-tetanization period if the drug washout was immediate before LTP induction. We therefore carried out this series of experiments with a switch to drug-free solution occurring 1 h before tetanization and compared with a separate set of drug-free slices (Fig. 4(f)). Fig. 4 (Paper III) depicts a significant effect of the drug on LTP stabilization 6 h following induction compared to control LTP, i.e. LTP in slices not treated with cycloheximide.

In another series of experiments, LTP was induced following 4 h of drug treatment with washout been started immediately before LTP induction (Fig. 4(e)). Statistical testing revealed similar blocking effect of the drug on LTP stabilization to that shown in the above mentioned group of experiments (Fig. 5, Paper III). Taken together, the data indicate that the effect of the drug is not directly related to its blockade of triggered protein synthesis.

5.7. LTP under oxidative stress conditions and the sensitivity for PSIs (Paper III)

Five minutes following LTP induction, a 5 mM dose of hydrogen peroxide (H_2O_2) mixed with $FeSO_4$, (100 μM) was applied for 20 min interval to two separate groups of slices, one of them was already pre-incubated by cycloheximide (100 μM). DPCPX was applied to partially inhibit a plausible adenosine-mediated depotentiation (Huang and Hsu, 2001). AP5 was applied immediately following LTP induction to ameliorate the NMDA receptor-mediated elevation in cAMP, which in turn, decreases the likelihood of adenosine release, on one hand, and to alleviate the effect of peroxide-mediated glutamate effect on NMDA receptor, on the other hand. Hence, both agents might block any plausible activation of protein phosphatase 1 and consequently, avoiding the depotentiation phenomenon. H_2O_2 caused a depression of control input response followed by partial recovery which kept stable throughout the experiment (Figs. 6A, 7A, Paper III). There was also a proportional decay in the potentiated pathways, likely due to the same mechanisms underlying the control inputs decay (Figs. 6B, 7B, Paper III). However, the LTP from slices treated with cycloheximide exhibited a disproportional decay in order of 121 ± 12% and 90 ± 8% at 2 and 7 h, respectively) compared to that obtained from slices treated only with peroxide (Fig. 7C, Paper IIII).

5.8. Testing the LTP "component"

Presuming that the temporal distinction of LTP into an early and late phases is true, we wondered whether the stable LTP under protein synthesis inhibitory conditions can be considered as *bona fide* "L-LTP", i.e. L-LTP defined by its time-course to be > 3-4 h, or if it is a long-standing mechanistically "E-LTP". Relying simply on time-course for E-LTP to be in range of 1-3 h (Figs. 1A, 1B, Paper IV), the stable LTP observed in our slices up to 7-8 h is definitely an "L-LTP". It would also be illogical to conclude that the stable protein synthesis-independent LTP observed in our case is L-LTP, as "L-LTP" is defined as protein synthesis-dependent. Alternatively, it could be that the LTP, under our conditions, regarded as a long-lasting mechanistically "E-LTP" (but see section 6.4; Paper IV).

There are several ways, other than PSIs, for testing the temporal range of E-LTP. One widely used way is either application of cAMP analogue/activator to enhance "switching" of E-LTP, i.e. decremental LTP induced by weak induction protocol, into an L-LTP, i.e. non-decremental LTP, or using antagonists to block the induction of L-LTP induced by strong induction protocol. For this purpose, we reasoned that similar procedures might give a clue for the time course of LTP phase(s) in our preparation. Therefore, dopamine receptors D1/D5 antagonist, SCH23390 was used at concentration and time window already reported to block LTP in a similar way as that of PSIs. Following an initial reversible, non-significant depression observed immediately after the drug application in both the potentiated and non-potentiated inputs, LTP induced by three trains of TBS was not different in slices treated with SCH23990 from LTP in control slices (Fig. 6). However, as the rise in cAMP triggered by dopamine receptors activation might only partially contribute in the total rise of cAMP (Musgrave et al., 1993; Wong et al., 1999), a competitive inhibitor of PKA, Rp-cAMPS, was used in the same pattern of application. Again, no effect on LTP was observed, similar to that obtained with SCH23390 (Fig. 6).

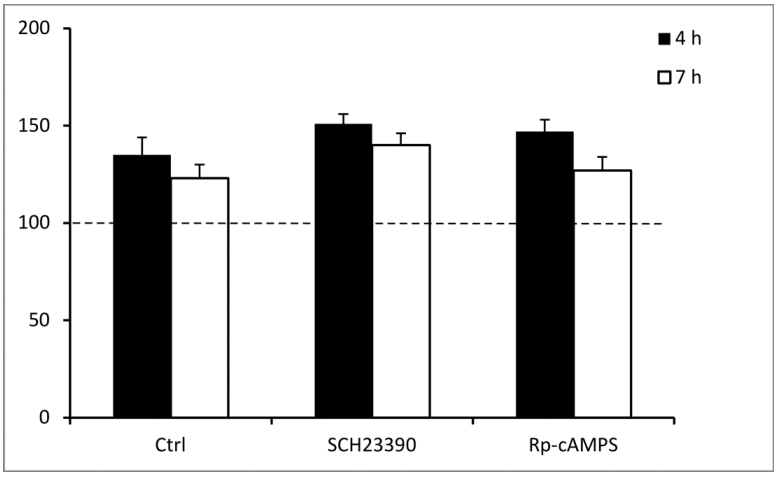

Fig. 6. *"L-LTP" is independent of PKA*. Bar diagram depicts that the PKA inhibitors SCH23390 (100 µM; *n* = 8) or Rp-cAMPS (100 µM; *n* = 8) applied for 40 min before and washed out 60 min after the induction of LTP in CA1 in slices obtained from 12-21 days old rats had no effect on the LTP measured for up to 7 h in comparison to control (Ctrl, *n* = 6). LTP was induced by 3 trains of TBS, each train composed of 10 bursts of four pulses at 100 Hz, repeated with a burst frequency 5 Hz, intertrain intervals of 5 sec. fEPSPs were measured as mean of values for 5 min bins ± SEM.

6. Discussion

The hypothesis that stabilization of synaptic plasticity depends on *de novo* protein synthesis is prevalent in the literature (e.g. Sajikumar and Frey, 2004; Frey and Morris, 1997; Frey and Morris, 1998b; Cammalleri et al., 2003). Beside the controversial findings in behavioral studies (e.g. PSI does not block memory retention, PSI-induced amnesia can be recovered spontaneously or by using some agents, etc.; for detailed discussion see Paper IV), several reports revealed stable LTP in slice preparation under a state of global protein synthesis inhibition (e.g. Stanton and Sarvey, 1984; Villers et al., 2012). In this work, we have shown for the first time that LTP in hippocampal slice from young rats could be maintained, for a time-scale equivalent to that attributed to "L-LTP", under conditions of protein synthesis inhibition. Similar results have been found in other independent laboratories (Gary Lynch, unpublished manuscript; Colin O'Carroll, personal communication; Maksym Kopanitsa, personal communication; Viller et al., 2012). The work thus went further to investigate the plausible underlying mechanism(s) for the protein synthesis-independent LTP stabilization. Subsequent findings (Paper III, IV) paved the way for introducing a tentative model, which is partly verified experimentally (section 6.7). The study supposes that the dichotomy of early vs. late LTP is false and discusses why LTP maintenance can be influenced by PSI.

6.1. Stable LTP under condition of brief PSI application

We were able to maintain LTP induced by either tetanus or TBS in slices from juvenile rats for several hours even when anisomycin or emetine was applied before, during and after induction. The drug concentration, at least of anisomycin, was demonstrated to have optimal inhibitory effect. However, the case with emetine was less clear as subsequent radiolabeled experiments revealed time-dependence effect that parallels its effect on baseline stability (Paper I, II). The temporal window for the drug application was chosen according to the idea that the events which generate the protein synthesis-dependent LTP are triggered immediately following induction. Given that the inhibitory effect of the PSIs could be achieved within a few minutes, a pre-HFS time interval would be sufficient for the drug to work. Even if the critical time window might be, under specific conditions, longer than 30 min, our experiments using extended post-tetanization PSI also revealed stable LTP (section 6.2).

Although it is generally stated that "E-LTP" has a time constant in a range of 1-3 h, there is in fact, extreme variability that extended beyond that range (Figs. 1A, 1B, Paper IV). Hence, while it could be justified to refer to such values for educational purposes, it is erroneous to extrapolate such values into other experimental contexts for either proving or disproving a hypothesis. This misconception appeared in our conclusion, i.e. "L-LTP" is protein synthesis-independent. If one accepts the notion that L-LTP is in essence protein synthesis-dependent, then it would be of logical contradiction to consider that it might be protein synthesis-independent. Rather, a stabilized LTP under conditions of protein synthesis inhibition may either be an "E-LTP" but with time-course at range of "L-LTP" or that the dichotomy is false (for a detailed discussion of these issues, see Paper IV)

6.2. Could LTP be maintained under conditions of prolonged post-tetanization protein synthesis inhibition?

The findings that protein synthesis can be expressed in several waves following a learning task, and that separate inhibition of each wave resulted in a significant loss of LTM, the findings shown in Ris et al.'s (2009) study (Paper IV) and our negative findings with regard to short time interval of PSI application (Paper I) have motivated us to test whether continuous PSI application lead to LTP blockage. However, our results seem not in accord to Villers et al.'s (2012) results, which revealed

that neither continuous anisomycin nor cycloheximide was able to block LTP maintenance for up to 9 h (Paper IV, for discussing this discrepancy).

6.3. Prolonged pre-tetanization protein synthesis inhibition

We reasoned that treating the slice with PSI for long pre-HFS intervals may have an effect on the stabilization of LTP. The earlier rationale for this reasoning was grounded on behavioral studies as well as on studies of LTP and PSIs suggesting that depleting constitutive proteins have deleterious effect on LTP (Paper IV, for a review), in case the basal electrophysiological events are intact. As achieving such objective is not easy given that the lifespan of the slice is limited, the protein half-lives are variable and the toxic effect of drugs is likely time-dependent. Despite these difficulties we developed a strategy where PSIs were applied in succeeding application periods before tetanization to ascertain a relatively long recorded LTP.

6.3.1. Emetine non-specifically blocks LTP

Despite its obvious effect on the baseline response (Paper I), we were curious to investigate the mechanisms and extent by which emetine might block LTP if it was pre-applied for long intervals. To our surprise, the pattern of LTP induction under emetine conditions was slowly developing compared to normal LTP or to LTP in slices treated with cycloheximide or anisomycin (Figs. 1, 4, Paper II). Further analysis of the phenomenon revealed that the slowly developed LTP was due to superimposing drug-mediated short-term depression during conducting the tetanus trains and that that depression is caused by an action potential fatigue (Figs. 5, 6, Paper II).

Whatever the mechanism(s) of action of the drug on the action potential generation (e.g. energy failure resulting from an effect on mitochondrial functions, an inhibitory effect on synthesis of axonal protein, disturbing the Na^+-K^+-ATPase pump system and/or dysfunction of $K_{IR}6.x$ subunit of the K^+-ATP channel), the time-dependent manner of the emetine-induced depression in the responses indicates at least 90 min of drug application is needed to observe the slowly developing LTP and the baseline decay. Longer application time intervals lead even to block the induction of LTP in a group of slices (data not shown). These observations were consistent with the results of tritiated leucine which show that only following 90 min incubation there was inhibition of protein synthesis reaching over 80% that steadily increased with time (section 5.2.3; Fig. 5). However, in the paper (Paper II) it was miss-judged that LTP was stable.

6.3.2. Cycloheximide blocks LTP stabilization without blocking protein synthesis during induction events

The findings of emetine experiments led us to pay attention to the fact that depleting constitutive proteins is infeasible without affecting slice viability. Our supplementary findings have shown that neither anisomycin nor cycloheximide has shown any significant effect on baseline responses when applied for several hours extending up to 5 h (Paper II). This gave us a rationale for using them for long pre-application intervals. However, another issue should be addressed before the usage of the drug for the purpose of depleting the protein repertoire of a slice before conducting LTP paradigm. Whether the pharmacological effect of the drug will be curtailed immediately after washout or it outlasts it, and how long the time lag between washing out and disappearance of its effect, i.e. its reversibility, may have significance on the hypothesis of triggered protein synthesis. While our reversibility assay revealed that anisomycin exhibits no reversibility for up to 90 min after washout

(*cf*. Villers et al., 2012), cycloheximide showed faster reversibility but still with time lag around 60 min for protein synthesis to be recovered at "reasonable" value (section 5.2.4). Another issue we put attention on is the mechanism of action of PSIs. Because anisomycin blocks only the elongation step, an enhancement of protein synthesis following cessation of its effect is likely to occur (e.g. Montarolo et al., 1986). In contrast, cycloheximide, at concentration used in this work reversibly blocks initiation and elongation simultaneously, ruling out the plausibility of rebound effect which may have confounding effect on LTP (Fig. 3, for illustration). Thus, cycloheximide was the drug of choice for long pre-application time window if it was washed out for a sufficient time before LTP induction. We considered that 4 h might be sufficient to cover the half-lives of a set of proteins supposed to be important for LTP (for references see Papers III, IV). As predicted, 4 h application with cycloheximide while protein synthesis during induction events was relatively intact (Fig. 4, Paper III) was able to block LTP stabilization, indicating that triggered protein synthesis during a presumed "critical time window" is unlikely crucial for LTP stabilization.

6.4. Long-lasting "E-"LTP or beyond dichotomy?

Given the traditional definition of L-LTP as dependent on triggered protein synthesis, neither the lack of effect of PSI on LTP "the negative data" (Paper I, III) nor the effect on long pre-TBS cycloheximide effect on LTP "the positive data" (Paper III) entail that an "L-LTP" was intact or blocked, respectively. Hence, provisionally accepting the temporal distinction of LTP phases as true, it remains to investigate whether the recorded LTP, in our slices, was an E-, or an L-LTP (despite the logical contradiction this reasoning contains).

Although, it is controversial whether PKA is involved in E-LTP gating, in the switching of E-LTP into L-LTP or in the maintenance of L-LTP (Huang et al., 1994; Blitzer et al., 1995; Abel et al., 1997; Winder et a., 1998; Otmakhova et al., 2000; Grønli et al., 2014), it is widely held that PKA is involved in the persistence of LTP and inhibiting PKA, or its upstream cAMP signaling pathway activated by dopamine receptors, leads to an effect on LTP similar to that induced by PSIs. Hence, PKA activation was inhibited. However, neither the dopamine D1/D5 antagonist nor the cAMP synthesis inhibitor led to an effect on LTP recorded for 7 h (Fig. 6; section 5.9). These findings confirm that, under our experimental conditions, PKA is neither crucial for early, intermediate nor for the late component according to the traditional timescale-based distinction (Paper IV). One implication that can be drawn is that the reported PKA-dependency of the early component can be "bypassed" or compensated by other multiple pathways. This is consistent with the idea that several convergent and redundant signaling pathways can induce LTP expression and early maintenance. The results are also in accordance with several other reports that show that cAMP inhibitors do not preclude LTP expression in mature animals (Yasuda et al., 2003). This "resistance" of LTP to kinase inhibitor is also shown by inhibiting other kinases destined for the late component of LTP (e.g. MAPK: Opazo et al., 2003; Capron et al., 2006; Steward et al., 2007). A second implication is that what we observe in our slices is an LTP that does not involve protein synthesis processes for its persistence as curtailing one presumed important signal transduction cascade mediating them did not affect LTP stabilization. However, one may argue that these findings do not rule out that PKA might be actually activated due to HFS and consequently it could "gate" the L-LTP that in its timescale is beyond the observed 7-8 h. If that was true, it would not change the conclusion that LTP stabilization with a time range longer than that attributed to E-LTP is achievable without the requirement for either protein synthesis or one of its upstream regulators (Paper IV, for further implications).

6.5. Accelerating the proteins turnover by chemical manipulation

Depleting the protein pool by long pre-application of cycloheximide is restricted to proteins with relatively short half-life. We therefore tried a set-up where the half-lives of long-lived proteins were accelerated by oxidative stress, using hydrogen peroxide (Paper III, IV). Despite hydrogen peroxide application immediately following LTP induction revealed that the effect on LTP mainly was due to a synaptic transmission decrease (Figs. 6, 7, Paper III), addition of cycloheximide caused a further LTP decay that was not due to effects on the synaptic transmission (Fig. 7, Paper III). To verify the hypothesis that the effect of peroxide on LTP was due to modification of a set of proteins, which results in acceleration of its turnover, radiolabeled assay revealed a significant decrease in the [^{3}H]leucine incorporation rate 3 h following the peroxide application (Fig. 8, Paper III; section 5.2.5). Overall, the data validate the proposal that accelerating the proteins turnover rate, including proteins with longer half-lives, immediately following LTP induction together with compromising the capacity for their replenishment was implicated in the consequent hampering of LTP stabilization.

6.6. Turnover protein dynamic and protein synthesis-independent LTP stabilization

Together with the above described findings and an extensive review of the literature led us to introduce a tentative model that tries to explain both the positive and negative findings with regard to the effect of PSIs on LTP time-course (Paper IV). The model concedes that the degree HFS changes the protein turnover determines whether or not PSI blocks LTP. In other words, if the degradation rate of an essential set of proteins for LTP generation increases, protein synthesis would be required to replenish them and to restore proteins to normal levels after the effect of HFS on nerve activity ceases. Thus, if the activity-dependent protein degradation is low, PSIs might have no effect unless the inhibition interval covers the half-lives of these necessary proteins (Paper III), i.e. when PSI's effect has more chance to match the half-lives of that set of proteins. This proposal seems partly consistent with the idea that molecules which may be utilized for signal transduction reflect also fluctuation of molecule concentrations defined by their temporal repertoire and depleting them by PSIs may explain the sensitivity of synaptic plasticity and memory for PSIs (Routtenberg and Rekart, 2005). The findings that enhancing general degradation following LTP induction together with protein synthesis inhibition that precludes the replenishment of the degraded proteins influences LTP maintenance are in accord with the model. However, the responsive translational process to the HFS-induced perturbation of the synthesis/degradation balance (e.g. Hershko and Tomkins, 1971; Goldberg and St. John, 1976; Etlinger and Goldberg, 1977) is not as straightforward as an increase over baseline level, a decrease reaching protein amount below baseline, or fine-tuning of the replenishment process are all plausible. Thus, triggered protein synthesis is not ruled out from the model but its significance in LTP stabilization is doubtful. We aimed to test the tentative model which concedes that HFS might probably lead to ATP-mediated disturbance in translation/degradation processes and the degree of this disturbance is determined, at least partly, by the pattern of stimulation. We used a proteasome inhibitor MG-115 which has the property that at low concentration it targets the 20S component of the UPS, i.e. the ATP-independent component. The

initial experiments[4] reveal that low concentration (10 µM) of the drug applied around the LTP induction events enhances the magnitude of LTP induced by weak protocol (Fig. 7). Although it is early to conclude, as neither the effect of such concentration on LTP induced by strong protocol nor targeting the S19 component, i.e. the ATP-dependent component, have not yet been investigated, we can presume that under this form of stimulation partial ATP-dependent protein synthesis and degradation is preserved. Adding to these partially preserved processes an already intact ATP-independent degradation would result in higher net protein degradation. Thus, blocking the ATP-independent component of degradation could improve LTP generation; possibly because they are relatively intact during HFS in comparison to the depressed ATP-dependent mechanisms (Paper IV).

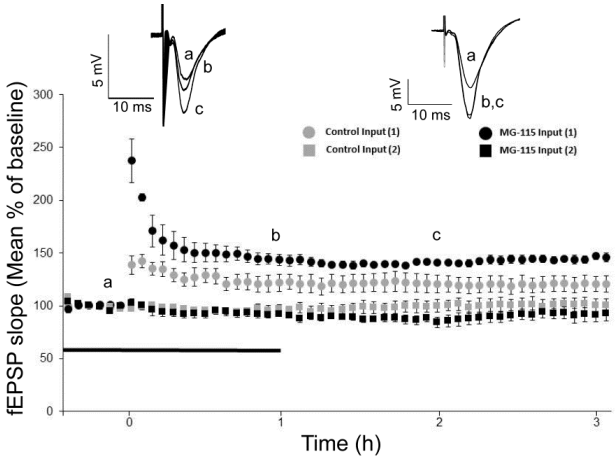

Fig. 7. *MG-115 improves the LTP induced by "weak" stimulation protocol*. LTP was induced using 50 pulses at 100 Hz frequency in two sets of slices. Each point represents the average of four evoked responses (4 min). In one group of slices MG-115 (10 µM) was applied starting 30 min before the induction of LTP on one pathway to 60 min following LTP induction (horizontal bar). The values of the fEPSP slope (±SEM) in the tetanized pathway 3 h after LTP induction are 147±3 in the presence (black circles; $n = 5$) and 121±7 in the absence (grey circles; $n = 6$) of the drug ($p < 0.05$, Student's t test). Sample fEPSP traces from individual experiments are shown in the insert for the potentiated pathways from drug-free slices (thick traces) and drug-treated slices (thin traces). They were recorded just before the LTP induction and at 1 h and 2 h after LTP induction.

[4] In this set of experiments, male C57BL/6 (6-10 weeks old) mice were used and the procedures were carried out in accordance with National Institutes of Health regulations for the care and use of animals in research and with the agreement of the ethics committee of the University of Mons, Belgium. Animals were deeply anesthetized by ether and decapitated. The dissecting, pre-incubation and incubation ACSF solution used in mice experiments contains: 124 mM NaCl, 4.4 mM KCl, 26 mM, $NaHCO_3$, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 10 mM glucose. All solutions during experiments were oxygenated by O_2 95% and CO_2 5% and their pH was at 7.4 value. At least 90 min was allowed for slice to be pre-incubated in interface condition at 31 °C before starting the recording. Two bipolar cluster electrodes were used to stimulate two distinct bundles of Schaffer collaterals. LTP was induced by 50 pulses at 100 Hz frequency yielding less magnitude in comparison to LTP induced by strong protocol (4 trains of 100 Hz, 1 sec tetani, 1 min inter-train interval; data not shown). MG-115 is dissolved in DMSO for stoke preparation at 10 mM concentration and appropriate volumes are stored at -80 °C until they are used for each experiment. Stoke is dissolved in ACSF for final concentration of 10 µM and the solution is protected from exposure to light by covering the bottle with aluminum foil.

Generally, it is supposed here that the main effect of PSI is not to block triggered protein synthesis but to skew protein metabolism toward degradation. This is obvious when long pre-incubation PSI was able to impede the maintenance of LTP (Paper III). However, there remains further work to establish the definitive range of the protein half-lives that are crucial for LTP stabilization *in vitro*. The model also tries to put forward a plausible explanation why PSIs, as usually documented, blocked LTP when they were applied for a short time-window around LTP induction. According to the slow reversibility findings, the "short" application interval does not in fact represent a short inhibitory effect. This explains, at least partly why PSIs washed out immediately before tetanization also block LTP (e.g. Deadwyler et al., 1987; Calixto et al., 2003). The findings by Ris et al. (2009), though failed to be replicated (Villers et al., 2012; Paper III), revealing that the degree of PSI-mediated decay in LTP is proportional to the application time interval of PSI, are consistent with this interpretation (see Paper IV, for the implications).

However, the evidence that ongoing rather than triggered protein synthesis is crucial for LTP stabilization does not tell how LTP can be stabilized, given that such proteins are with no significance if they were not posttranslationally modified, i.e. activated (functional). Unfortunately, none of the studies that insisted the idea that triggered *de novo* protein synthesis directly tested how it serves persistence of LTP or memory engram, given proteins have limited half-lives in the range of days, at best. Nor have any explained how such proteins might serve LTP persistence if they were still in inactive forms. Answering such a question would require a complementary model (e.g. the possibility that protein kinases which are considered to merely activate translational and transcriptional processes may subserve LTP stabilization under conditions of protein synthesis inhibition, if, and only if, the inhibitory effect does not cover the half-lives of one or more of them). If such proposal were true, it can be thought that the "triggered" protein synthesis is a byproduct of converging signaling pathways on one hand, and of accelerated protein turnover that is augmented by protein modifications, on the other.

6.7. Functional significance

Beside the importance of how protein synthesis plays a role in physiological memory, there are several neurological and neuropsychiatric conditions that have been shown to be associated with disturbances in protein synthesis and/or degradation. These proteinopathies include diseases such as lateral amyotrophic neuropathy, autism, status epilepticus, and Alzheimer's disease (e.g. Carnevalli et al., 2006; Chevalier-Larsen and Holzbaur, 2006; Kwon et al., 2006; Kim et al., 2007; Bingol and Sheng, 2011; Gkogkas et al., 2013). Furthermore, stroke, which is associated with deficient memory, is accompanied with increase in the highly reactive, free radical species. It has been proposed that protein modification by the free radicals (e.g. Berlett and Stadtman, 1997; Dean et al. 1997) may underlie the memory deficit associated with proteinopathic conditions**.** This proposal can be generalized to involve a wide-spectrum of proteinopathies in several brain regions as it is supported by Parkinson's disease and Alzheimer's disease animal models (e.g. Butterfield and Stadtman 1997; Kouchi et al., 1999; Castegna et al., 2002; Holtz and O'Malley, 2003; Kim et al., 2007) as well as brains of Alzheimer's disease patients (Kim et al., 2007). Given that synaptic plasticity is a strong candidate underlying learning and memory, the implications of this work might be profound. If one considers that memories are fixed structural entities (say "traces") then protein turnover would constitute a major constraint for preservation the identity of the trace (e.g. Crick, 1984). In such case, the search for "memory molecules" that cope with such challenge (e.g. bistable autophosphorylated kinase,

persistent PKMzeta, prion-like protein, clustered engram, etc.) is justified. However, if memories are always updated and restructured (e.g. Nader, 2003; Miyashita et al., 2008) then such problem would not be prominent because the process is also continuous ascertaining reconstruction. Likely, in everyday life, there is neither fixed, unchangeable memories nor loosely formed ones, rendering both preserving and dynamic processes of protein synthesis and breakdown finely tuned through time. When such physiological "order" of protein synthesis/degradation is chronically disturbed, it is expressed as pathological manifestations. Briefly, the presented model implies that what is more crucial for LTP stabilization is the degree of response of a set of proteins to the LTP-induction protocols which in turn modify their half-lives rather than triggered protein synthesis (Paper IV). Thus, dynamic, i.e. turnover rates, rather than fixed, i.e. "triggered" synthesis have presumably predictive power for the time-course of LTP, and for its sensitivity, or "resistance" for interruption. Nevertheless, although this work is neurobiological in essence, it may contribute in the bridging the gap between low-level reductionist processes and high-level clinical situations.

7. General Conclusions

To conclude, PSIs seem likely to decrease the lifespan of synaptic plasticity rather than to mechanistically differentiate temporal phases. Furthermore, the temporalization of LTP, and perhaps LTD (Mohammad, 2010), has likely hampered our understanding of the general mechanisms underlying synaptic plasticity stabilization. One major issue addressed in this thesis informs a possibility of LTP stabilization independent of triggered protein synthesis but dependent on the availability of constitutive proteins. However, neither the identity of the set of available proteins which are essential for stabilization nor the mechanisms by which such proteins underlie stabilization are disclosed. Thus, there remains a further step that shifts these descriptions presented in this work into building a testable hypothesis which can directly explain how long lasting stabilization is obvious under certain conditions but not in the overwhelming reported studies. Such questions might have no one answer, therefore, careful examination of the experimental context, i.e. the relationship between manipulation and outcome, the relationship that the CNS and machineries of learning are highly sensitive to, might be worth investigating.

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