Long-term depression in the rat hippocampus as a memory model: Interrogating the role of protein synthesis in NMDA- and mGluR-dependent synaptic plasticity

Bachelor thesis in biomedicine 30 hp

Sameh Mohammad
a07sammo@student.his.se
Biomedicine, 3rd year

Supervisors:
Abdul-Karim Abbas
Abdul-Karim.Abbas@neuro.gu.se
karim.abbas@physiol.gu.se

Holger Wigström
holger.wigstrom@mednet.gu.se

Synaptic plasticity group
Department of Medical Biophysics
Institute of Neuroscience and Physiology
Sahlgrenska Academy
University of Gothenburg
SE-405 30 Göteborg
Sweden

Examinator:
Kjell-Ove Holmström
kjell-ove.holmstrom@his.se

School of Life Sciences
Skövde University
BOX 408
SE-541 28 Skövde
Sweden

Abstract

Long-term potentiation (LTP) and depression (LTD) are important forms of activity-dependent synaptic plasticity believed to play a role in memory at the cellular level. It has previously been described that synthesis of new proteins is needed to maintain LTP longer than a few hours. Other reports argue that sufficient proteins for stable LTP are already available. The present study aims to examine the role of protein synthesis in LTD, the presumed mirror mechanism of LTP.

Experiments were carried out in hippocampal slices from young (12-45 days) and old (12-18 weeks) Sprague-Dawley rats. Extracellular techniques were used to study synaptic responses in the Schaffer-collateral-commissural pathway. Plasticity was induced electrically by low frequency stimulation (2-3 trains at 1 Hz for 15 min) or chemically by brief exposure to certain glutamate receptor agonists (NMDA at 20 μM for 3 min or DHPG at 100 μM for 10 min). Whole slice protein synthesis was quantified by assessing ^{3}H-leucine incorporation.

Stable LTD (> 8 h) was be obtained by either electrical or chemical activation. Protein synthesis inhibitors anisomycin (40 uM) and cycloheximide (100 uM) both failed to influence the magnitude of LTD. Moreover, no age difference was found, in terms of stable LTD in both young and old rats under inhibition of protein synthesis. The potency of the inhibitors was found to be high, depressing synthesis down to a few percent. It is concluded that sufficient proteins for generating stable LTD are normally present in the brain, implying a large safety-margin for cellular memory.

Abbreviations

ACSF: artificial cerebrospinal fluid
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR: AMPA-receptor
AP: action potential
AP5: D-(-)-2-amino-5-phosphonopentanoic acid
CA: cornu ammonis
CHX: cycloheximide
CNS: central nervous system
DHPG: (*R*,*S*)-3,5-Dihydroxyphenylglycine
DG: dentate gyrus
DMSO: dimethyl sulfoxide
DPCPX: 1,3-Dipropyl-8-cyclopentylxanthine
EC: entorhinal cortex
EPSP: excitatory postsynaptic potential
fEPSP: field excitatory postsynaptic potential
iGluR: ionotropic glutamate-receptor
mGluR: metabotropic glutamate-receptor
LFS: low-frequency stimulation
LTD: long-term depression
LTP: long-term potentiation
MF: mossy fiber
NMDA: *N*-methyl-*D*-aspartate
NMDAR: NMDA-receptor
PNS: peripheral nervous system
PP: perforant path
PRP: plasticity related proteins
PSI: protein synthesis inhibition / inhibitor
SC: schaffer collaterals
STD: short-term depression

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Introduction

The complex structure and function of the human brain are often analyzed indirectly via animal models. The rat brain has frequently served as such a model and comprises an essential tool for studying the pathological processes associated with Alzheimer's disease, Parkinson's disease, stroke, head trauma and prolonged epilepsy. Several brain diseases are associated with impaired memory. The fact that lesions or resection of the hippocampus prevent the acquisition of new episodic memories has launched a great interest in the hippocampus as a "memory center". The simple laminar pattern of neurons and neural pathways in the hippocampus enables the use of extracellular techniques to record neuronal signals for virtually unlimited periods in vivo and for several hours in vitro. The hippocampal slice preparation has been of major importance to the study of cellular memory. Since slices can retain their essential neuronal circuitry they can be used for studying the events involved in "synaptic transmission" between neurons (Bear *et al.,* 1996). It is possible also to apply pharmacological agents that can be rapidly washed in and washed out.

The central nervous system

All animals have a nervous system. It is the main system for controlling the total body functions related to survival. Humans have about one hundred billion neurons in their nervous system. In vertebrates (such as humans), the nervous system can be divided into the central nervous system (CNS) and peripheral nervous system (PNS). The CNS consists of the brain and the spinal cord. The brain consists of three major parts: the forebrain, the cerebellum and the brain stem (Figure 1). Each part in turn has different subparts with different structures and functions. The forebrain is made up of the cerebrum and the limbic system. The limbic system is made up of many structures of the brain such as the amygdala, hypothalamus, hippocampus, and several other nearby structures. The limbic system plays a major role in many functions of the brain, from sexual behaviour to memory.

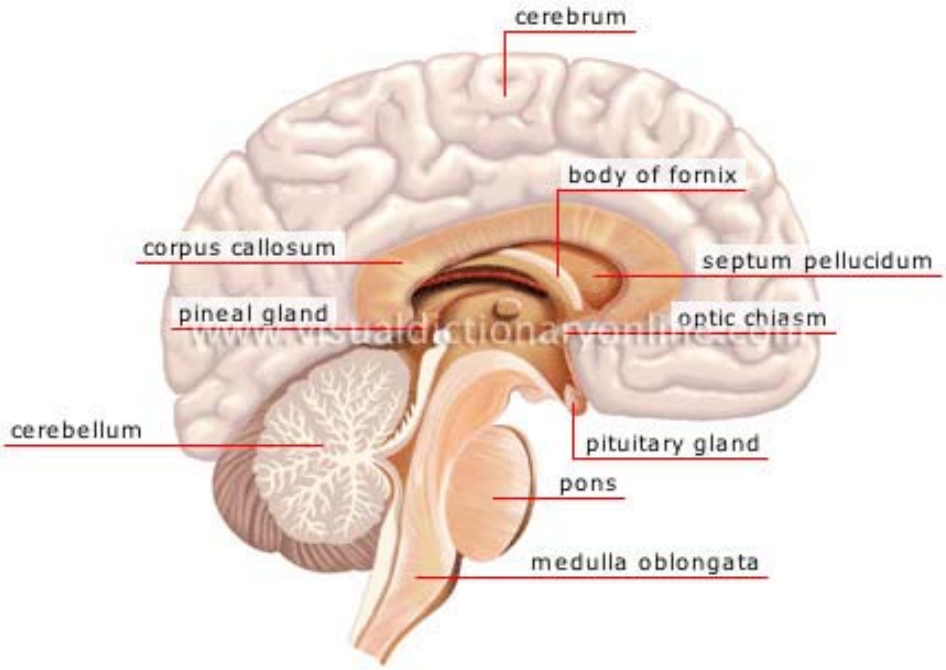

Figure 1. A schematic picture illustrating different parts of the human brain.
(Adjusted from: http://visual.merriam-webster.com/human-being/anatomy/nervous-system/central-nervous-system_1.php, August 25th 2010)

The hippocampus

The hippocampus is perhaps the most studied structure of the brain. This is because it has a simple anatomical structure as well as due to the critical role it plays in formation and retrieval of many forms of memory (e.g. spatial learning and episodic memory). Hippocampus is made up of two parts located symmetrically on each side of the medial temporal lobe of the brain (Andersen *et al.*, 2007; Figure 2). It is divided into several regions, the main areas being: cornu ammonis 1, cornu ammonis 3 (CA1, CA3) and dentate gyrus (DG). The term "hippocampus proper" generally refers to the pyramidal cell regions CA1 and CA3 whereas "the hippocampal formation" refers to the hippocampus proper plus the granule cells of DG and the subiculum. The subiculum is located at the end of the CA1 region (Andersen *et al.*, 2007). The CA1 is the largest and most studied part of the hippocampus and this study is focused on this part.

The structure and function of the rat brain is similar to that of the human brain and therefore a proper place to study memory (Thompson, 2000; Afifi and Bergman, 2005). The input and output to and from hippocampus go via a transition area of cortex proper, the entorhinal cortex (EC). The hippocampus is also connected to other parts of the brain, such as the hypothalamus and thalamus, through the fornix, a major pathway that leads the signals in longitudinal direction with respect to the hippocampus (Afifi and Bergman, 2005).

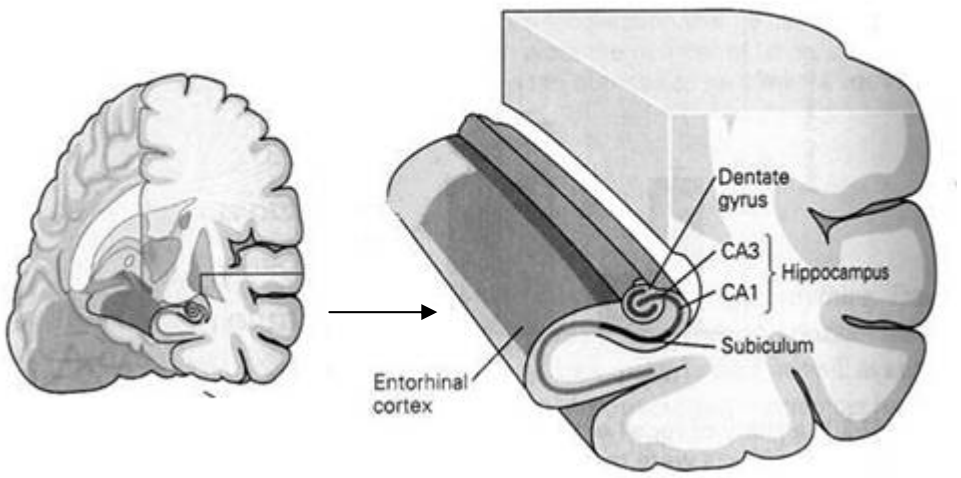

Figure 2. A schematic crosscut of the medial temporal lobe illustrating hippocampal formation: the hippocampus, dentate gyrus and subiculum.

Hippocampal circuits

The entorhinal cortex (EC) transmits excitatory signals to the granule cells of DG as well as to the pyramidal neurons of CA3 via the axon fibers of perforant path (PP). From the granule cells of DG, the connection continues through the mossy fibers (MF) and reaches the pyramidal cells of CA3. The latter then form synaptic connections through their axons with the CA1 pyramidal cells by means of the Schaffer collaterals (SC) and with the contralateral hippocampus via the Associational Commissural pathway (AC). The pyramidal cells in turn, extend their axons that connect to the EC via the subiculum. Overall, there are three key paths that carry out the loop: the perforant pathway, the mossy fiber pathway and the Schaffer collateral pathway (Shepherd, 1990; Figure 3).

The perforant path-to-dentate gyrus-to-CA3-to-CA1 was called the trisynaptic circuit by Per Andersen, who noted that thin slices could be cut out of the hippocampus perpendicular to its long axis, in a way that preserves all of these connections. This observation was the basis of his lamellar hypothesis, which proposed that the hippocampus can be thought of as a series of parallel strips, operating in a functionally independent way (Andersen *et al.*, 1971).

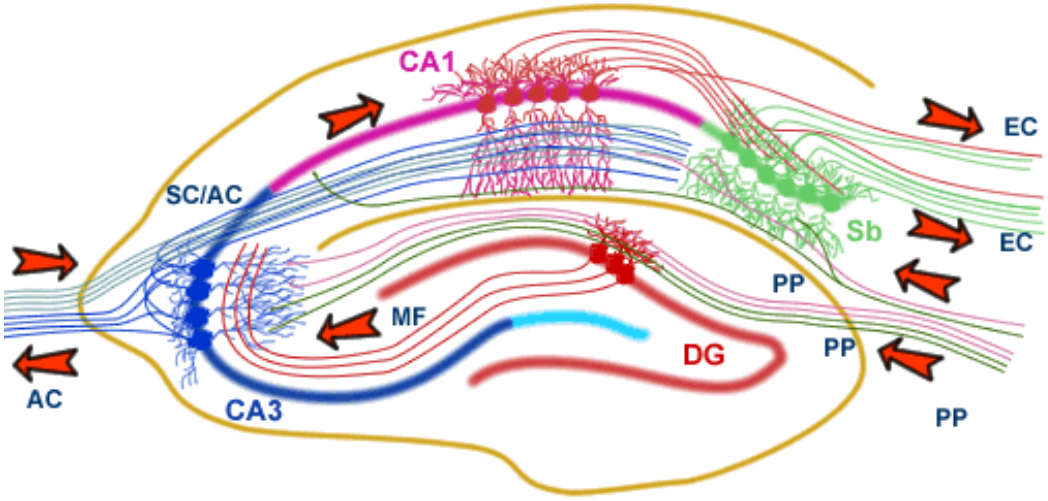

Figure 3. Hippocampal circuits. MF: mossy fibers; SC: Schaffer collateral; AC: association commissural; EC: entorhinal cortex; DG: dentate gyrus; PP: perforant path. (Adjusted from: www.neuropsychoanalyse.wordpress.com/category/moderne-psychoanalyse, August 20th 2010).

Neuron

The nervous system is made up of nerve cells or neurons together with glial cells. It is this combination of cells together with the fact that neurons can transmit and process information in form of electrical or chemical impulses that are responsible for the remarkable control functions of the brain (Thompson, 2000). Depending on the location and function of the individual neurons, these can vary substantially in appearance. Nevertheless, they all have some functionally important elements in common such as the soma (cell body), the axon and the dendrites.

The soma possesses most of the organelles of the neuron including the nucleus and is the main source of synthesis of many important components (Lännergren *et al.*, 1998; Thompson, 2000). The plasma membrane of the soma has receptors on its surface and often receives information from other neurons (Marieb and Hoehn, 2010). A large number of dendrites arise from the soma and have branched extensions that can receive signals from other neurons and transport them to the soma. At the dendrites there are spiky ends known as spines which have a close contact with other neurons. The axon, in most types of neurons is a single extension, generally branching extensively into many thin fibres that conduct and lead the signals away from the soma to other neurons. The axon begins in the axon hillock and ends at the axon terminals where the signal is transmitted further to other neurons (Figure 4).

The information within the neuron is transferred in form of electrical impulses while information from one nerve cell to another in the brain is transferred in form of chemical signalling molecules, neurotransmitters. The connection through which the chemical signals passes from the axon terminal of one neuron to the dendritic spine of another neuron is called synapse (Thompson, 2000; Marieb and Hoehn, 2010).

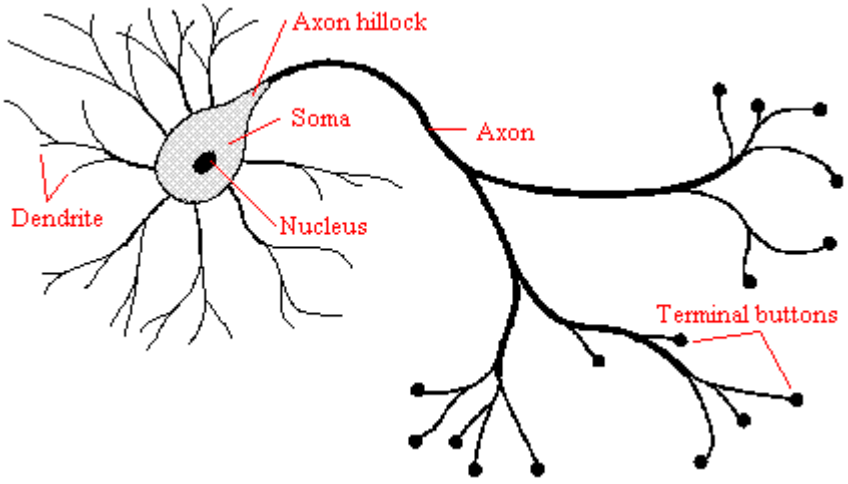

Figure 4. Schematic representation of neuron cell with its three defined regions: the cell body, the dendrites and the axon. (Adjusted from: http://www.aass.oru.se/~tdt/ann/index.html, August 20th 2010)

Action potential (AP)

When there is no signal being transmitted, the nerve cell is usually referred to as at resting potential with a charge of -60 to -80 mV in relation to the outside of the cell membrane. This resting potential is maintained by ionic currents driven by concentration gradients and passing through ion channels in the cell membrane. Some important gradients are maintained by the actively driven Na^+-K^+-pump. The pump transports Na^+ out of the cell and K^+ into the cell, keeping Na^+ concentration high outside the cell and the K^+ concentration high within. However, neurons are highly sensitive and can trigger an electric impulse which travels along the length of its axon when it is adequately stimulated. This kind of response is called an action potential (AP) and does not differ regardless of the type of stimuli or what causes it. A neuron that produces an action potential is often said to "fire".

When a cell is stimulated, there is a change in the permeability of the cell membrane leading to an initial positive-going deflection of the membrane potential, referred to as depolarization. When the depolarization reaches above the threshold, i.e., big enough to stimulate the opening of the voltage-gated Na^+-channels, it causes a large, transient depolarization, the action potential at which the membrane potential goes from -50mV to +30 mV immediately. This is followed by repolarization, in which the Na^+ channels close and voltage gated K^+ channels open, allowing the K^+ to rapidly diffuse out. Both these events quickly bring the membrane voltage back to the resting value. The cell then returns to being "zero" on the outside and negative on the inside and the K^+ channels close (Campbell and Reece, 2005; Marieb and Hoehn, 2010). Sometimes during the repolarization, the level of the membrane potential drops momentarily below the normal resting potential and this is referred to as the after-hyperpolarization (Bear et al., 1996). Both Na^+ and K^+ channels are constantly leaking ions in and out respectively, but despite this, the concentration gradient will not reach equal concentrations on both sides of the cell thanks to the ATP driven Na^+-K^+-pump which ejects three Na^+ from the cell and transfers two K^+ back into the cell (Thompson, 2000; Marieb and Hoehn, 2010).

The structure and function of synapses

Whenever an action potential (AP) occurs, it travels down the axon and generally reaches an end point which is known as terminal where it stops, since APs are rarely transmitted between cells. However, information is still transmitted, and this occurs at the synapses. The synapse (Figure 5) is a tiny gap, at the ending of the terminal, between two neurons. Normally, after a signal is conducted, the information flows from the axon terminal towards the target neuron. The axon terminal, leading away information, is said to be pre-synaptic while the receiving neuron is said to be postsynaptic. The process, in which information is transferred at a synapse, is known as synaptic transmission (Campbell and Reece, 2005).

The absolute majority of synapses are chemical. When an AP travels down the axon, it reaches the pre-synaptic axon terminal where the information is converted into a chemical signal. As the AP reaches the terminal, it changes the voltage at the pre-synaptic membrane, causing the voltage gated Ca^{2+}-channels to open and enabling Ca^{2+} in the extracellular fluid to enter trough the channels. The pre-synaptic membrane has many small vesicles gathered near the membrane which contain chemical neurotransmitters acting as chemical messengers. The rush of Ca^{2+} into the terminal causes the synaptic vesicles to fuse with the pre-synaptic membrane (Thompson, 2000; Campbell and Reece, 2005). The neurotransmitter is then released by exocytosis into the 20-50 nm gap between the pre- and postsynaptic membrane called the synaptic cleft.

The main transmitter substance in the hippocampus is the amino acid L-glutamate (Brodal, 1992) which plays a key role in the biochemical processes of memory. In the postsynaptic membrane, the neurotransmitter is converted again into an electrical signal when it binds to specific receptor molecules (Bear et al., 1996; Thompson, 2000; Marieb and Hoehn, 2010). The synaptic potential in the chemical synapses can either be inhibitory or excitatory. In an excitatory postsynaptic potential (EPSP), the diffusing ions consisting of Na^+ and K^+, have a depolarizing effect on the postsynaptic neuron while the inhibitory postsynaptic potential (IPSP) use K^+ or Cl^- (Marieb and Hoehn, 2010).

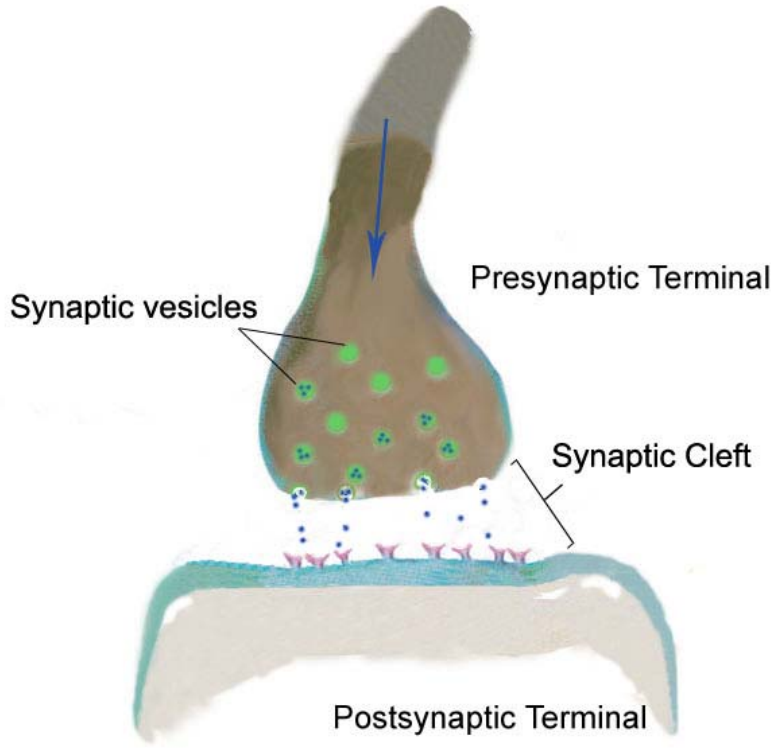

Figure 5. Schematic representation of a synapse. Pre- and postsynaptic components separated by a synaptic cleft where the chemical events occur via neurotransmitter release from the presynaptic terminal and its conjugation with postsynaptic receptors. These events subsequently lead to a cascade of biochemical changes underlying synaptic plasticity.

Glutamate and glutamate receptors

The amino acid glutamate is a major excitatory neurotransmitter in the central nervous system. Glutamate receptors are divided into two major families, ionotropic glutamate receptors (iGluRs) that act through ligand gated ion channels and metabotropic receptors (mGluRs) acting through G-protein coupled receptors. There are three main types of ionotropic receptors: *N*-methyl-*D*-aspartate (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazole- propionic acid (AMPAR) and kainate receptors. However, most of the studies about synaptic transmission are focused on AMPAR and NMDAR. Fast excitatory synaptic transmission seems to be dominated by the AMPAR whereas slower transmission is mediated by NMDAR, although both coexist at the same membrane (Bigge, 1999; Rang *et al.*, 2003). One of the key differences between NMDAR and AMPAR is that the NMDAR is predominantly permeable to Ca^{2+} unlike the AMPAR, which is predominantly permeable to Na^{+} and K^{+}. Another difference is the fact that the release of glutamate from the pre-synaptic membrane unconditionally stimulates a major response in the AMPAR in contrast to the NMDAR which require activation by glutamate together with depolarization of the postsynaptic membrane.

NMDAR are believed to contribute to synaptic plasticity by acting as a triggering mechanism. An additional trigger is present via receptors of the mGluR family (Seeburg, 1993; Bear *et al.*,

1996). In the present study, synaptic plasticity will be considered using both of these trigger systems. In contrast to the iGluRs, which have both a transmitter binding function and an ion channel function in its structure, mGluRs lack the ion channels and instead rely on the modulation of second messengers. Studies have also shown that mGluR can be found in the pre-synaptic membrane as well, controlling the neurotransmitter release.

The mGlu-family contains a total of eight mGluRs (mGluR1-8) and can be divided into three major groups. Receptors in the first group, group I, include mGluR1 and mGluR5 types and are believed to be excitatory because they increase the release of neurotransmitters and raise the efficiency of iGluR and depolarization. The second group, group II contains mGluR2 and mGluR3 while mGluR4, -6, -7 and -8 belong to group III. Both group II and group III receptors reduce the release of neurotransmitters and inhibit the activity of ion channels in the synapse (Alexander and Godwin, 2006).

Synaptic plasticity

Synaptic plasticity can be defined as a more or less persistent change of the synaptic response, either an increase (potentiation) or a decrease (depression), induced by certain stimulation patterns (Bear, 2003). A change in the synaptic efficiency that lasts for a few milliseconds to a couple of minutes is termed short-term synaptic plasticity whereas a synaptic efficiency lasting for several minutes to even days or weeks is known as long-term plasticity (Bear, 2003; Turrigiano, 2008). Synaptic plasticity is believed to be one of the major mechanisms behind learning and memory in many brain regions. There are several types of synaptic plasticity associated with memory, the most important ones known as long-term potentiation (LTP) and long-term depression (LTD).

Studies on the CA1 region of hippocampus have demonstrated the presence of NMDAR- and non-NMDAR-dependent types of synaptic plasticity. The influx of Ca^{2+} through the NMDAR acts as a second messenger involved in several cascades of biochemical events. The intracellular signalling can either trigger LTP or LTD depending on the intracellular level of Ca^{2+} concentration (Johnston et al., 1992; Bliss and Collingridge 1993; Nicoll and Malenka, 1995). LTP and LTD can also be triggered via mGluR activation in an NMDAR-independent manner. While the mechanisms of NMDAR-dependent neuroplasticity have been thoroughly examined the non-NMDAR-dependent forms are still incompletely understood in terms of induction and expression mechanisms. Moreover, substantially less is known about the mechanisms underlying LTD as compared to LTP. The present thesis describes experimental work on LTD, induced by either NMDAR or mGluR activation. For consistency of the presentation, the remainder of this introduction is therefore focused on LTD, considering LTP only when necessary.

Long-term depression

An activity-dependent decrease of synaptic efficiency ranging from 30 minutes up to several hours is generally termed as LTD. Such LTD can be induced by either NMDAR or mGluR depending on the type of receptors activated during the induction. However, other receptor types have been implicated as neuromodulators, being able to influence LTD (Kemp and Bashir, 1997; de Mendonca *et al.*, 1997; Kirkwood *et al.*, 1999; Massey *et al.*, 2001; Huang *et al.*, 2004). There are several ways in which the induction of LTD can be achieved in the pyramidal cells of the CA1 area. The LTD can either be electrically induced, usually via low-

frequency stimulation (LFS, 1-5Hz for 5-15minutes), or it can be chemically induced by application of certain receptor agonists. The electrically induced LTD has the ability to involve only few synapses whereas chemically-induced LTD has the potential to cover a significantly larger population of synapses.

The induction and expression mechanisms of LTD vary depending not only on the type of the stimulated receptors but also on experimental conditions. This variability makes the phenomenon more complex in comparison with LTP. A further complicating factor is the lack of purely one-receptor-type LTD. Instead, several forms of LTD appear to coexist, in a developmental relationship, even at the same synapses. NMDA-dependent LTD, for example, has been demonstrated to require Ca^{2+} entry into the postsynaptic neuron and the activation of serine/threonine protein phosphatase (Mulkey *et al.*, 1994), and is believed to be expressed postsynaptically by reducing expression of postsynaptic AMPARs (Bear and Abraham, 1996; Carroll *et al.*, 1999;). Unlike NMDA receptor-dependent LTD, (*R*,*S*)-3,5-Dihydroxyphenylglycine-induced or DHPG-induced LTD does not require either Ca^{2+} (Fitzjohn *et al.*, 2001) or serine/threonine protein phosphatase (Schnabel *et al.*, 2001) but involves activation of Gαq (Kleppisch et al., 2001), protein synthesis (Huber *et al.*, 2001), p38 mitogen-activated protein kinase (Rush *et al.*, 2002) and tyrosine dephosphorylation (Moult *et al.*, 2002; Zhang *et al.*, 2008).

Activity-dependent mGlu receptor LTD in the CA1 region has previously been reported to depend on the activation of PKC (Bolshakov and Siegelbaum, 1994; Oliet *et al.*, 1997). However, LTD induced by application of DHPG is not blocked by inhibitors of PKC (Schnabel *et al.*, 1999; Rush *et al.*, 2002). DHPG-induced LTD might depend on both pre- and postsynaptic processes because studies using hippocampal slices from young rats suggest that a combination of postsynaptic induction and pre-synaptic expression is involved in mGluR-dependent forms of LTD induced by synaptic stimulation (Bolshakov and Siegelbaum, 1994), or in adult rat induced by DHPG (Tan *et al.*, 2003).

Long-term depression and memory

Apart from the problem of understanding how synaptic plasticity in general leads to the storage and recall of information in neuronal networks, there is considerable uncertainty regarding the physiological role of LTD. It has been proposed that LTD, working together with LTP, underlies storage of memory (see for example Heynen *et al.*, 1996). However, it is still unknown whether LTD plays an independent role by underlying certain distinctive types of information storage or merely enhances the signal-to-noise ratio. Alternatively, LTD may function as a means to erase stored memory engrams (i.e. a forgetting mechanism). The latter is more easily understood with depotentiation, an LTD-related phenomenon which is induced in a similar way as LTD but causes elimination of previously established LTP (Wagner and Alger, 1996).

The role of protein synthesis in synaptic plasticity

It is conceived in the literature that the possible roles for newly synthesized proteins include: successively replacing degraded proteins, increasing the levels of existing proteins, or expressing novel or alternatively spliced forms of proteins (Steward and Schuman, 2001). All of these have been implicated in the phase differentiation of LTP. Protein synthesis inhibitors have thus been used to define an early protein synthesis-independent phase of potentiation

lasting ~1-3 h and a late protein synthesis-dependent phase (Frey *et al.*, 1988; Otani *et al.*, 1989; Frey *et al.*, 1993; Huang *et al.*, 1994;; Huang and Kandel, 1996; Osten *et al.*, 1996). This idea applies to LTP at the Schaffer collateral/ commissural input to area CA1 pyramidal cells (Frey *et al.*, 1988; Nguyen *et al.*, 1994) and at the perforant path input to the dentate granule cells (Krug *et al.*, 1984; Otani *et al.*, 1989). Although such temporal differentiation is not fully observed with LTD there is evidence for a dependency of LTD on protein synthesis, involving early and/or late phases.

It has been proposed that synaptically-induced mGluR-LTD (Huber *et al*., 2000; Huber *et al.*, 2001; Park *et al.*, 2008) as well as chemically induced mGluR-LTP (DHPG-LTD) (Raymond *et al.*, 2000; Hou and Klann, 2004; Waung *et al.*, 2008) are dependent on protein synthesis and are blocked by inhibitors of translation. A role of protein synthesis in mediating the conversion of short-term depression (STD) into mGluR-LTD has also been considered (Neyman and Manhahan-Vaughan, 2008). In addition, the expression of NMDA-dependent homosynaptic LTD has been reported to involve protein synthesis (Kauderer and Kandel, 2000; Manahan-Vaughan *et al.*, 2000; Sajikumar and Frey, 2003). However, more recent works have shown controversial results concerning the protein synthesis dependency of this LTD (Huber *et al.*, 2000) as well as synaptically-induced mGluR-LTD (Moult *et al.*, 2008; Gladding *et al.*, 2009).

Aim

It has previously been shown that LTP in the CA1 region of hippocampal slices from young rats (2-3 weeks) can be maintained for several hours following induction under conditions of protein synthesis inhibitor (PSI) (Abbas et al., 2009). It was accordingly proposed that sufficient and necessary proteins are available at the induction time in these young animals, which are generally good learners. Whether this situation can be verified with other forms of synaptic plasticity such as various forms of LTD is a major issue of the current project. Moreover, the question whether there might be a difference in the protein synthesis dependence among different age groups was addressed as well. The currently studied LTD includes NMDAR dependent as well as mGluR dependent types, induced by either electrical stimulation or chemical activation.

Methods

General

Experiments are carried out in submerged hippocampal slices using electrophysiological techniques with capability for studying two independent pathways in each slice. The brain-slice technique provides for well controlled stimulation and recording as well as easy exchange of extracellular solutions. To increase performance, a multi-chamber system has been developed at the Department of Medical Biophysics, allowing 4 slices to be studied in parallel. Electric stimulation as well as solution exchange can be run via computer control in a fully automated manner. Data are analyzed for presentation by specially designed computer software.

Animal handling and preparation of brain slices

All animal handling and subsequent procedures to prepare brain slices conform to the guidelines of the Swedish Council for Laboratory Animals and are approved by the Local Ethics Committee of University of Gothenburg. Male and female Sprague-Dawley (Charles River Laboratories, Germany) rats aged 12-21 days, 21-45 days and 12-18 weeks were used. Young pups were housed with their dams until the experiment. The rats were maintained on a 12 h dark/light cycle and had access to food pellets and water ad libitum. After decapitation under initial isoflurane (Baxter Medical AB, Sweden) anesthesia, the brain was rapidly removed and immersed in ice-cold dissection buffer composed of (in mM): 119 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 26 $NaHCO_3$, 1.0 NaH_2PO_4, and 10 D-glucose) bubbled with 95% O_2, 5% CO_2. The hippocampus of one or both sides was dissected out and transverse slices, 400 μm thick, were prepared by a tissue chopper. The slices then were transferred gently to a submersion holding chamber containing the same dissection solution. The slices were left at room temperature to recover for at least 90-120 min. Slices were then gently transferred to a submersion-type recording chamber continually perfused with 31°C oxygenated ACSF (simular to the dissection solution, but with $CaCl_2$ and $MgCl_2$ concentrations at either 2.5 and 1.3 or 1.5 and 1.3, respectively, depending on the type of experiment) oxygenated by 95% O_2, 5% CO_2 (artificial cerebrospinal fluid, ACSF).

Electric stimulation and recording

For extracellular field potential recordings, a single slice was maintained in the recording chamber between nylon net and a set of parallel nylon threads attached to a U-shaped platinum wire. The chamber consisted of a circular well of a low volume (1-2 ml) and was perfused continuously with oxygenated ACSF at a flow rate of 1.5-2 ml/min. Stimulating and recording electrodes (Figure 6) were positioned via micromanipulators and placed in the slice under visual guidance by an upright "Nikon" microscope equipped with a 3.5x objective and a 20x ocular which was used to identify the CA1 region of the hippocampus.

The Schaffer-collateral-commissural synaptic pathway to CA1 pyramidal cells of the hippocampus is used. Field excitatory postsynaptic potentials (fEPSPs) were normally recorded from the dendritic layer by a microelectrodes pulled from thin-wall glass capillaries (o.d. 1.5 mm, i.d. 0.86 mm, Warner Instruments, USA) and filled with 1 M NaCl to obtain 2-5 MΩ resistance. Normally, AMPA-receptor mediated EPSPs were recorded. Two monopolar tungsten stimulating electrodes (World Precision Instruments, USA) were placed on either side of the recording electrode to provide for stimulation of two separate synaptic pathways (S1 and S2). Negative, constant current pulses, 100 μs, 10-50 μA, were alternately delivered to the two stimulating electrodes, the interval between successive stimuli ranging between 5 and 10 s (10 and 20 s for each pathway) depending on type of experiment. LTD was induced by either (1) LFS paradigm which usually involved two trains of LFS, each of 900 pulses at 1 Hz for 15 min, to fully saturate the LTD (Kemp and Bashir, 1997) or (2) chemically, by applying NMDA at 20 μM for 3 min or DHPG at 100 μM for10 min.

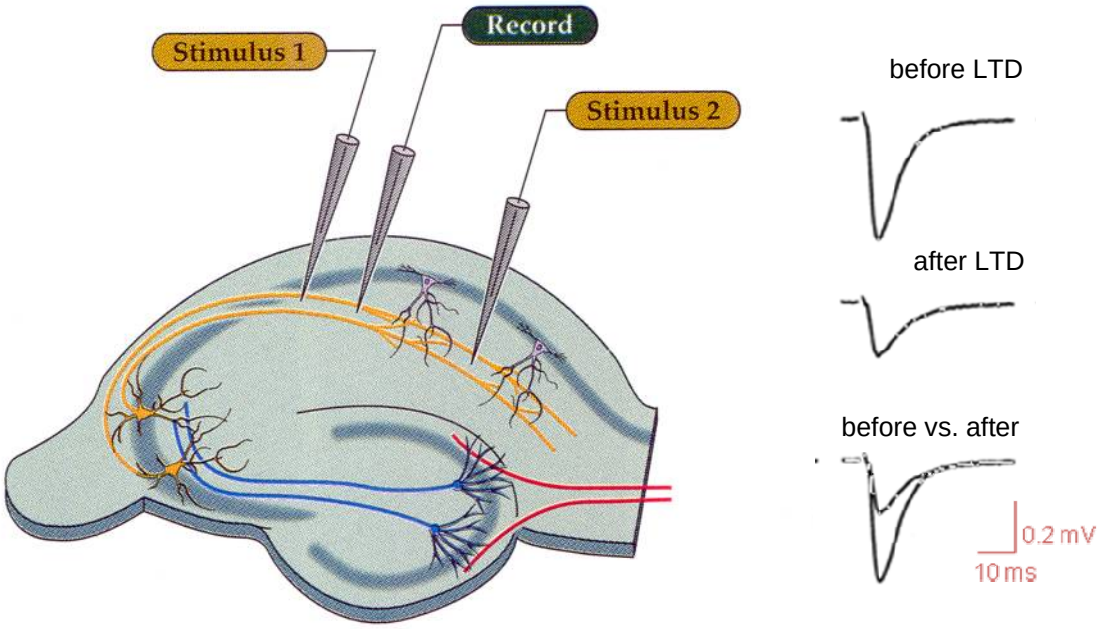

Figure 6. Stimulation and recording electrodes inserted in CA1 region. Test stimulation is performed with 20-50 µA current pulses, once per 10 or 20 seconds. Synaptic LTD is induced in one pathway (S1; test) and compared with another pathway (S2; control) or pharmacological LTD is induced in both pathways and compared to fEPSPs recorded before induction. Protein synthesis inhibitors are applied in one group of experiment; the results are compared with experiments without the inhibitor. Typical synaptic potentials before and after induction of LTD are shown on the right panel.

Perfusion and slice maintenance

Slices were constantly perfused by oxygenated ACSF at 1.5-2 ml/min for each experimental chamber using a peristaltic pump. The solution was normally recirculated in order to save solution and so reduce the needed amount of test drugs. In attempts to optimize experimental conditions, the treatments were balanced between test and control slices (in terms of number of slices used for each treatment).

Drugs were made up as stock solutions (10-1000 x final concentration) in H_2O for D-(-)-2-amino-5-phosphonopentanoic acid (AP5), NMDA and anisomycin and in dimethyl sulfoxide (DMSO) 99% v/v. for cycloheximide (CHX). DMSO at final concentration of 0.1-0.4% had either only a slight, or no, effect on synaptic transmission hence it was added to the solution of the control group as vehicle to balance any slight effect it might have on LFS- or chemical-LTD. These stocks were diluted in artificial CSF to achieve their desired final concentrations and were applied by switching the perfusion from control ASCF to drug-containing ACSF.

To avoid "contamination" of the synaptic plasticity (once induced) with small amounts of NMDA receptor-dependent LTD induced by mere test stimulation, experiments were routinely performed in the presence of the NMDA receptor antagonist AP5 (50 µM). This compound was applied in the bath at least 40 min before delivering DHPG and usually maintained for the entire duration of the experiment (>10 h). In LFS-LTD experiments, it was applied immediately after the end of last LFS train to avoid further NMDAR dependent decay due to the ongoing stimulation.

Data analysis

Signals were amplified, filtered, digitized and transferred to a "PC clone" computer for on-line and off-line analysis by specially designed electronic equipment and in-house developed computer software. EPSPs were measured using an early time window of 1-2 ms duration positioned just after the presynaptic volley, thereby avoiding contributions of postsynaptic firing. The area under the curve was used as a measure of EPSP size. Alternatively, the EPSP can be quantified by a slope measurement, shown to give similar results (Dozmorov *et al.*, 2003). The area measurement was generally preferred in the present study due to less noise and better immunity to volley-EPSP overlap.

Readouts from experiments

Responses of the test pathway (S1) were expressed relative to the preinduction baseline in case of chemically induced LTD and relative to responses of the control pathway (S2) in case of electrically induced LTD. The amount of LTD was estimated by measuring the response size during 5 min intervals positioned at certain times, e.g. even hours, after the induction of LTD. Values are expressed as mean (in time course plots) or mean ± SEM (bar diagrams) for the slices in a defined group. Statistical comparisons used unpaired or paired Student's *t*-test. Values of less than 0.05 were considered to be significant.

Increasing performance by running parallel experiments

Four slices can be run in parallel in a multi-chamber setup. Each of the 4 chambers provides 2 stimulating electrodes and one recording electrode. Each electrode is controlled by a mechanical 3D micromanipulator operated by hand. Electrode positioning, which is essential to performance, is performed under visual guidance using an upright "Nikon" microscope. The microscope can be easily moved between the chambers by a horizontal swinging movement about an upright pivotal axis. The multi-chamber system is equipped with a perfusion multiplexer, making it possible to select one bottle of solution out of 4 alternative ones. The multiplexer uses a collection of pinch valves on both inlet and outlet sides. Two 4-channel peristaltic pumps are used to actively pump the solution(s) into and out of the chambers.

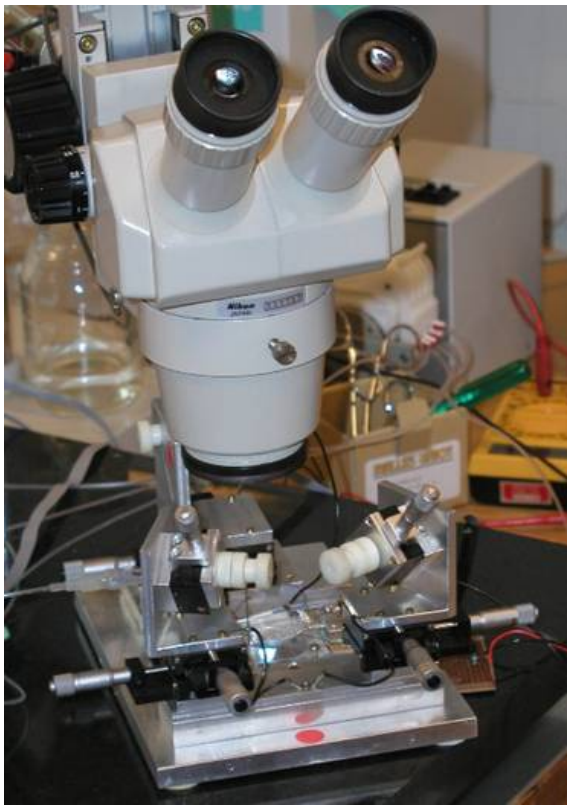

Figure 7. Recording chamber and upright microscope. One chamber among a multichamber setup for running 4 experiments is shown here. The chamber is equipped with 2 stimulating and one recording electrode and is perfused via a multiplexer consisting of a bank of pinch valves.

Investigation of protein synthesis inhibitors by leucine incorporation

Hippocampal slices were prepared as described above. The slices were transferred immediately after sectioning to a 8-well multiwell plate with mesh inserts (Corning Incorporated, Corning, NY, USA) Each well, containing 2 slices, was filled with 2 ml of ACSF (2 mM $MgCl_2$ and 2 mM $CaCl_2$) and gassing with 95% O_2 and 5% CO_2 was allowed through an opening in the top of the plate cover. The margin of error derived from hippocampal slices weight difference was minimized by assigning subsequently dissected slices in corresponding wells making the weight of slices nearly identical between test and control groups.

The slices were kept at room temperature for at least 90 min. After equilibration, cycloheximide (100 µM) or anisomycin (40 µM) was added to one group of slices while the other group was considered as control. After 10 min of drug pre-incubation, both test and control slices were transferred to another plate filled with 3H-leucine-containing ASCF (1.3 mM $MgCl_2$ and 2.5 mM $CaCl_2$; at final concentration of 3H-leucine of 0.5-1 µCi/ml) placed in a water bath at 31°C for 55 min. The slices were then gently rinsed with ice-cold saline for three times and 0.1 M NaOH was added before putting them in Eppendorf tubes for analysis. After protein purification (Lipton and Heimbach, 1977), leucine incorporation was measured in a scintillation counter (LKB Wallace, 1219 Rackbeta, Finland). Percentage inhibition of leucine incorporation produced by drug treatment was calculated by comparing counts in treated slices with those of control slices.

Results and discussions

Electrically-induced LTD in young rats

Electrically-induced LTD was studied in 12-21-day old rats. A saturated level of LTD was induced by two, and in some slices three, 15 min trains of 1 Hz stimulation (2 or 3 x 900 pulses) provided to one pathway (S1), using the other pathway (S2) as control. During the LFS paradigm, fEPSPs often underwent initial facilitation, which was always followed by a depression below the baseline level. After the second, or third, LFS a long-lasting depression of the synaptic response was obtained. Figures. 8A and B show average recordings from test (red colour) and control pathways (blue colour) depicting LTD under normal condition and under drug treatment. As shown there was no observable difference between drug and no-drug experiments during the whole recording period (10 h).

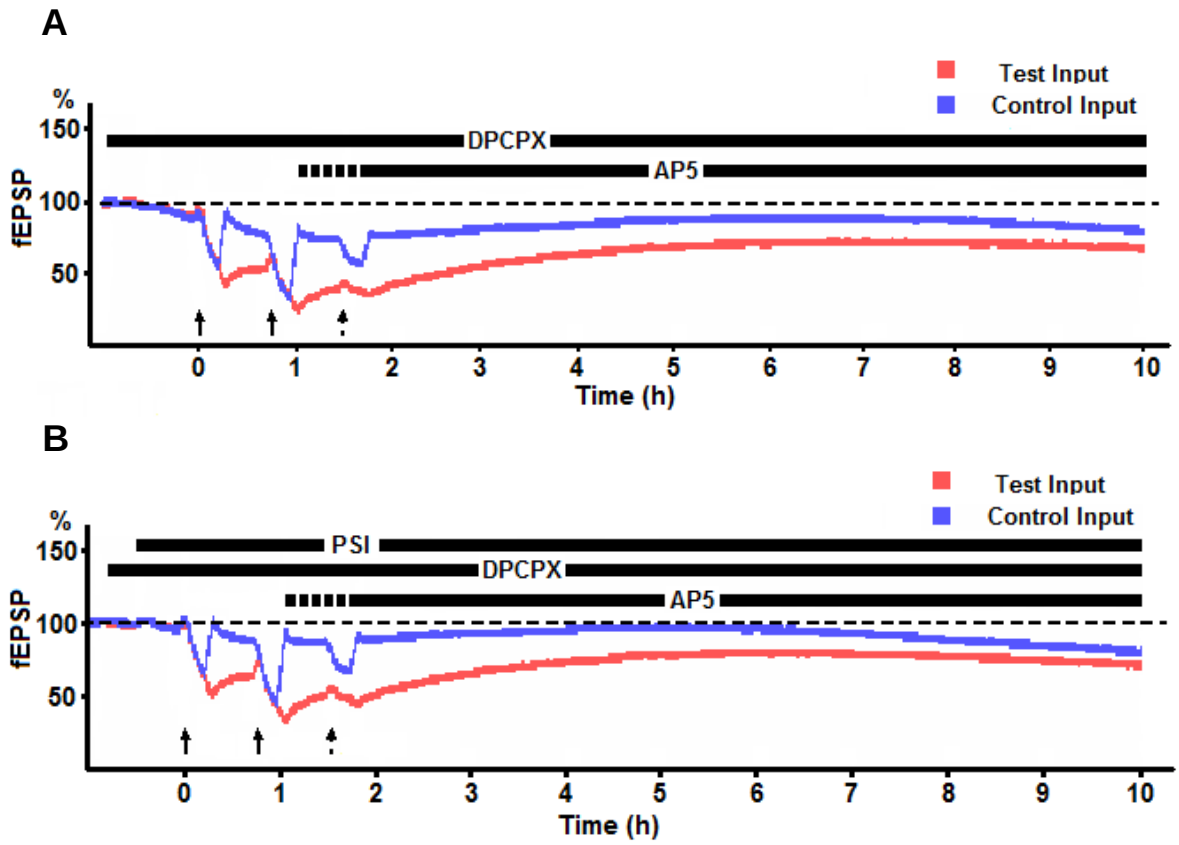

Figure 8. Effect of PSI on LFS-LTD. Successive measurements of fEPSP are shown for successive responses in two separate pathways. **A)** Two or three trains of LFS (arrows) were delivered to one pathway (test pathway; red colour) in presence of 1,3-Dipropyl-8-cyclopentylxanthine DPCPX (50 nM), a selective adenosine A_1 receptor antagonist to prevent an inhibitory effect of adenosine on presynaptic release of glutamate. AP5 was added to the solution immediately after the end of the last LFS train to block further NMDAR-mediated decay that might be induced by the test stimulation. **B)** The same condition as with A but PSI, either cycloheximide (100 µM) or anisomycin (40 µM) was applied 30 min before the first LFS train and kept in the solution throughout the experiment. No observable difference was found with PSI in comparison with non-drug slices. Data are averages of 13 experiments (A) and 10 experiments (B), respectively. The dotted line in both figures represents primary default value of fEPSP.

The bar diagram in Figure 9 quantifies the LTD obtained after drift compensation by calculating the ratio between test and control pathway measurements. Error bars depicting

S.E.M. are included. It can be seen that there was a significant LTD throughout the recording period of 8 h. However, the values were nearly the same for normal and drug-treated slices, implying that the drug treatment had no significant effect on LTD.

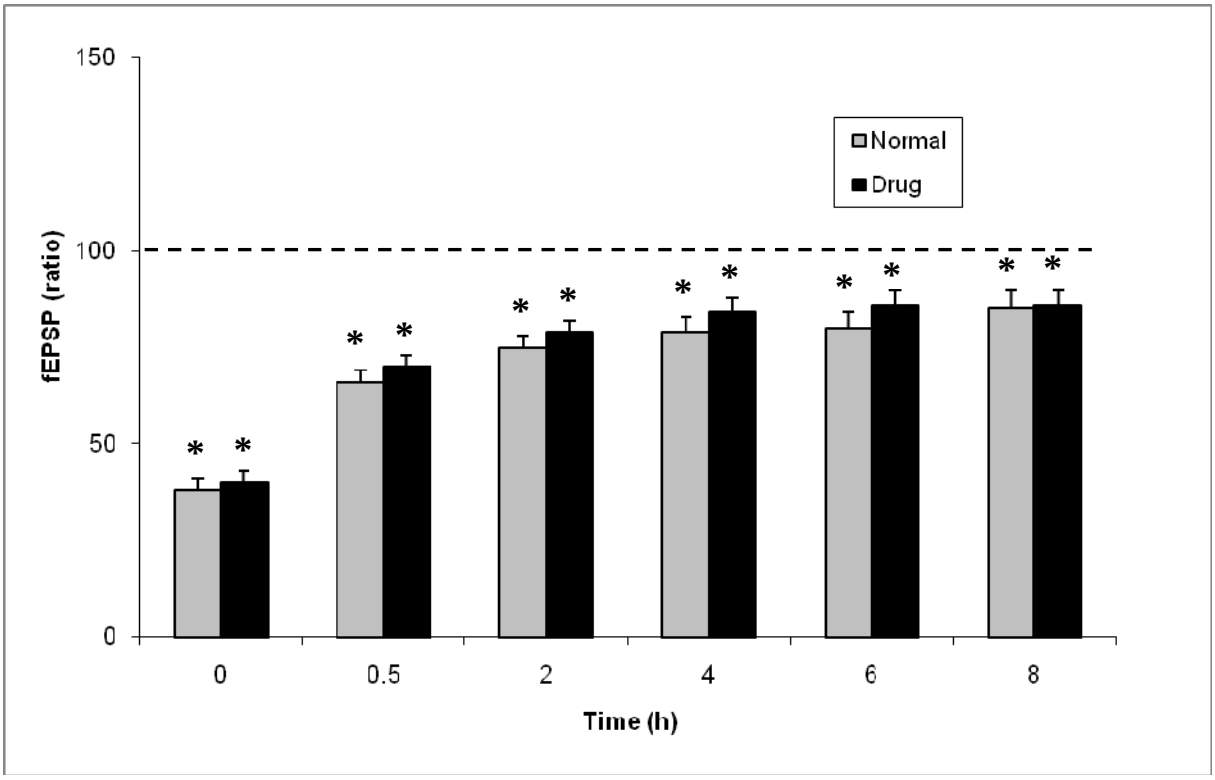

Figure 9. Statistical analysis of LFS-LTD. LTD without (light bars) and with (dark bars) PSI expressed as the percent ratio of test vs. control pathway. LTD was induced by two, and in some cases by three, trains of LFS (arrows) depicted shortly before zero time. A significant depression was obtained 30 min following the last LFS and maintained throughout the recording time (8 h after LFS). No statistical difference between the groups was observed. Results are expressed as mean ± SEM (n is 13 for drug group and 17 for normal group); asterisks indicate $p < 0.05$.

NMDA-LTD in old animals

The stimulus protocols generally used to induce LTD in young animals are relatively ineffective in inducing LTD in adult animals (Wagner and Alger, 1995; Abraham *et al*., 1996; Kemp and Bashir, 1997; but see Heynen *et al.*, 1996; Staubli and Ji, 1996). However, it has been demonstrated that brief application of relatively low concentrations of NMDA can induce an "NMDA-LTD" in both young and old rats, which has many similarities to electrical stimulation-induced LTD and appears to share common induction and expression mechanisms (Lee *et al.*, 1998; Beattie *et al*., 2000). Furthermore, it has also been shown that this type of LTD is blocked by AP5 (Lee *et al.*, 1998; Kamal *et al.*, 1999; van Dam *et al.*, 2002) indicating that the NMDA exerts its effect via NMDAR.

Hence, the effect of PSI on relatively old rats (12-18 weeks) was investigated. Perfusion of the slices with 20 μM NMDA for 3 min initially caused a marked decrease in fEPSP slope in about 60% of slices. However, other slices (10%) needed another 3 min perfusion to yield a prominent decrease (see Mockett *et al.*, 2007) while about 30% of slices did not exhibit any degree of synaptic depression. The responses recovered slowly and progressively until a

stable fEPSP slope about half the baseline were obtained at 30 min after the drug washout, see figures 10A and B.
The depression caused by the NMDA was most likely due to a synaptic plasticity rather than due to an excitotoxic effect of NMDA. Firstly, the NMDA concentration as well as duration used was well below that required to elicit excitotoxicity under normoxic conditions (Hartley and Choi, 1989; Pizzi *et al.*, 1996). Secondly, there was no observable change in the fiber volley was found during the entire recording period (data not shown). This finding indicates that the same number of presynaptic axons was recruited by baseline stimulation before and after NMDA treatment.

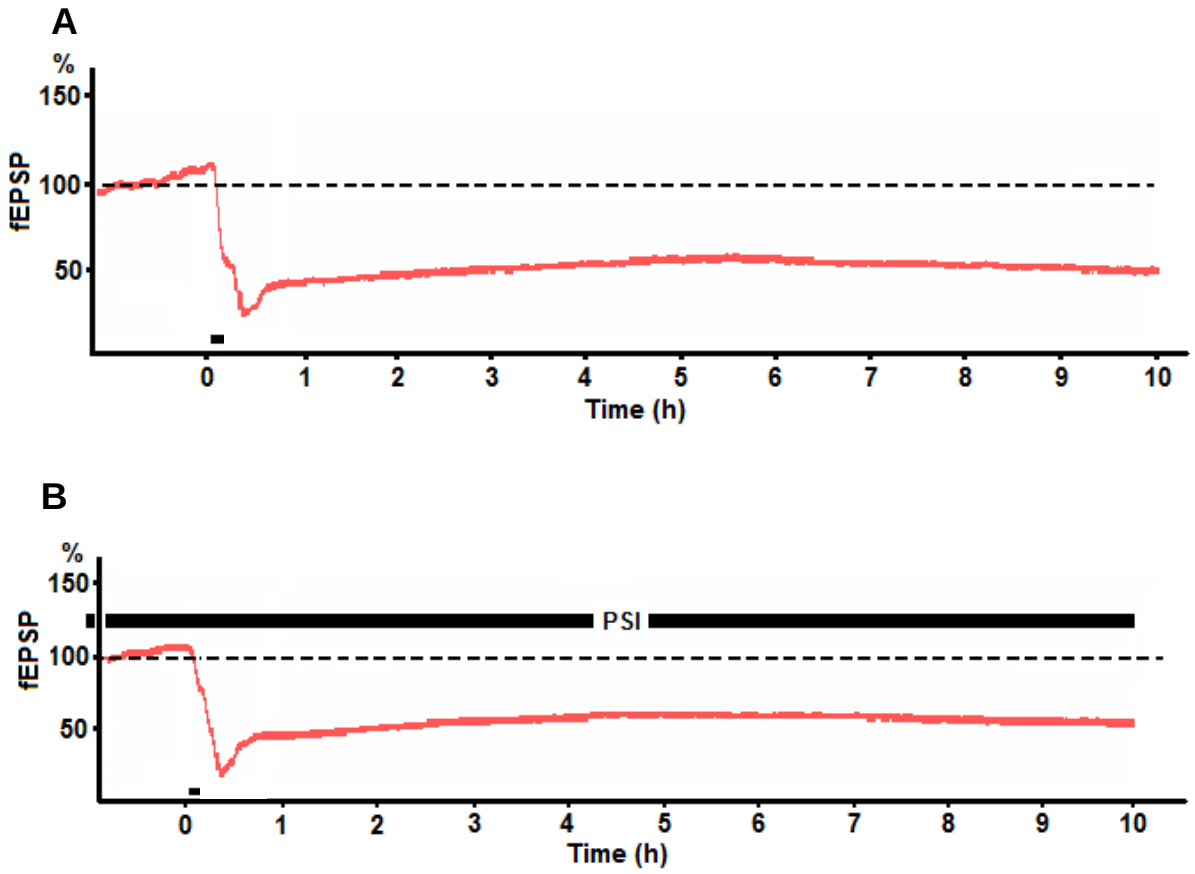

Figure 10. Effect of PSI on NMDA-LTD. Successive measurements of fEPSP are shown for successive responses in a single pathway. **A)** One, sometimes two, puffs of low concentration (20 µM) of NMDA (horizontal bar), delivered after 60 min of stable synaptic response, induced a robust depression of fEPSP maintained for up to 10 h (n = 6). DMSO, at final concentration of 0.01-0.4%, was added as vehicle. **B)** Application of cycloheximide (100 µM) 60 min before the NMDA puff(s) did not cause a difference in LTD obtained by NMDA in comparison with the normal group (A) (n = 8). The dotted line in both figures represents primary default value of fEPSP.

Again, application of PSI did not cause an effect on LTD as compared with normal slices in the studied age group of rats (Figures10, 11). Taken together with the results in Figures 8-9, the data suggest that there was no developmental difference with regard to protein synthesis dependency of NMDAR dependent LTD. As shown here, such dependency was lacking in both young (21 days) and old (12-18 weeks) rats.

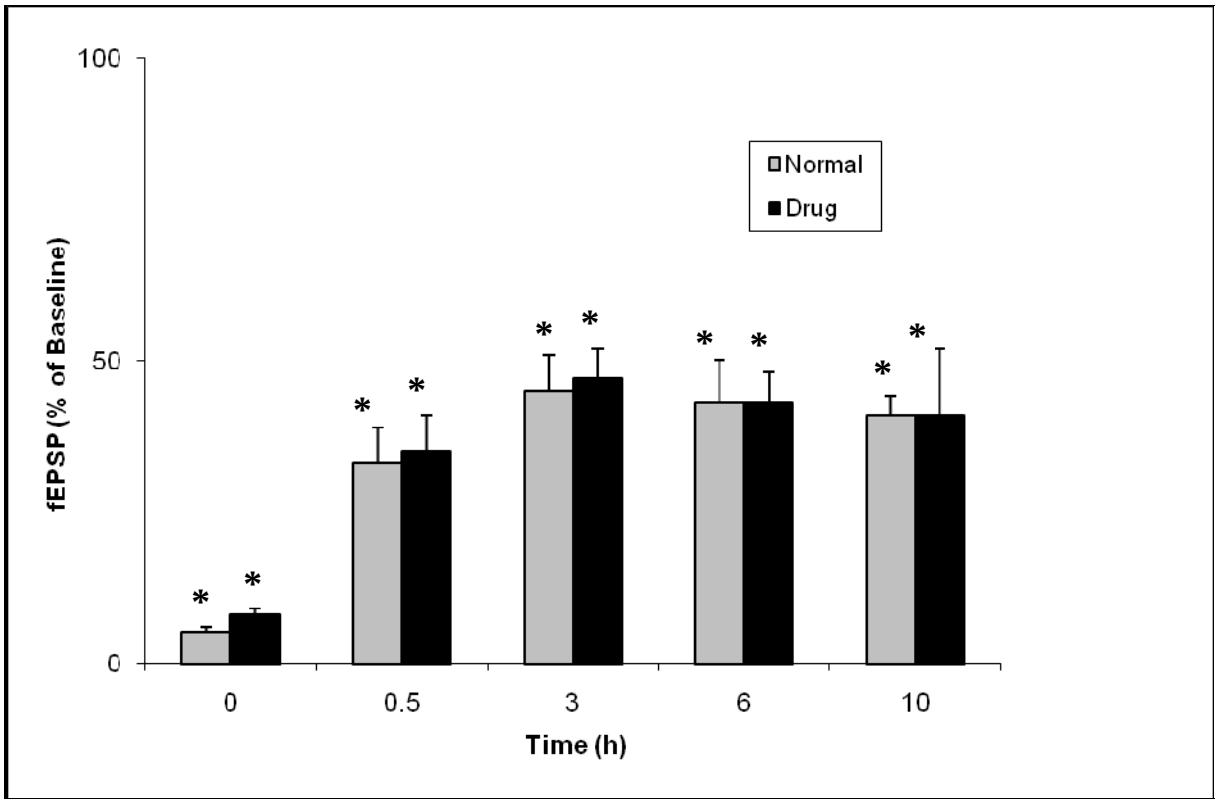

Figure 11. Statistical analysis of NMDA-LTD. LTD is expressed as fEPSP values relative to the pre-induction baseline. A significant LTD was observed for up to 10 h in both normal group of slices (light bars; n = 6) and drug-treated ones (dark bars; n = 8). Cycloheximide (100 µM) was delivered 60 min before the application of NMDA and kept in the solution throughout the experiment. There was no significant difference between LTD values in the drug-treated slices as compared to the normal group. Data are depicted as mean ± SEM; asterisks indicate $p < 0.05$.

It is thought that a small rise in postsynaptic intracellular Ca^{2+} is needed for the induction of LFS-LTD, leading to activation of protein phosphatases and subsequent dephosphorylation of synaptic proteins, for example AMPA receptors (Lee *et al*., 1998; Lee *et al.*, 2000). A similar mechanism of postsynaptic Ca^{2+} is considered to be responsible for chemically-induced NMDA-LTD although the primary inducing stimulus is different. Despite the difference in induction the two LTDs share several common characteristics and they occlude each other, implying that induction of one form of LTD prevents the induction of the other form (Lee *et al.*, 1998). This suggests that it is reasonable to consider these LTDs induced by different paradigms as similar entities.

DHPG-LTD in young animals

It is generally believed that DHPG-LTD (i.e. LTD induced by the mGluR agonist DHPG-LTD) and synaptically-induced mGluR-LTD involve similar expression mechanisms, because the two forms of LTD occlude each other (Huber *et al.*, 2001). Furthermore, it is evident that induction of both DHPG-LTD and synaptically-induced mGluR-LTD employ similar signalling cascades involving MAPKs and protein tyrosine kinases (Rush *et al.*, 2002; for review see Gladding *et al.*, 2009). This corroborates the concept that DHPG-LTD is a valid experimental model for the investigation of molecular and cellular mechanisms underlying synaptically induced mGluR-LTD (Gladding *et al.*, 2009).

The next step was to investigate mGluR-LTD with respect to protein synthesis dependence. A specific group I mGluR agonist, R,S-DHPG was delivered to two groups of slices obtained from 21-45 days old rats. The first group was conducted under normal conditions whereas the second one was conducted under protein synthesis inhibition. DHPG was found to induce a significant, though gradually decaying depression, of fEPSP (LTD) for up to 4 h. In similarity with data on NMDAR dependent LTD, no effect of PSI was observed in treated slices in comparison with the normal group (Figure 12).

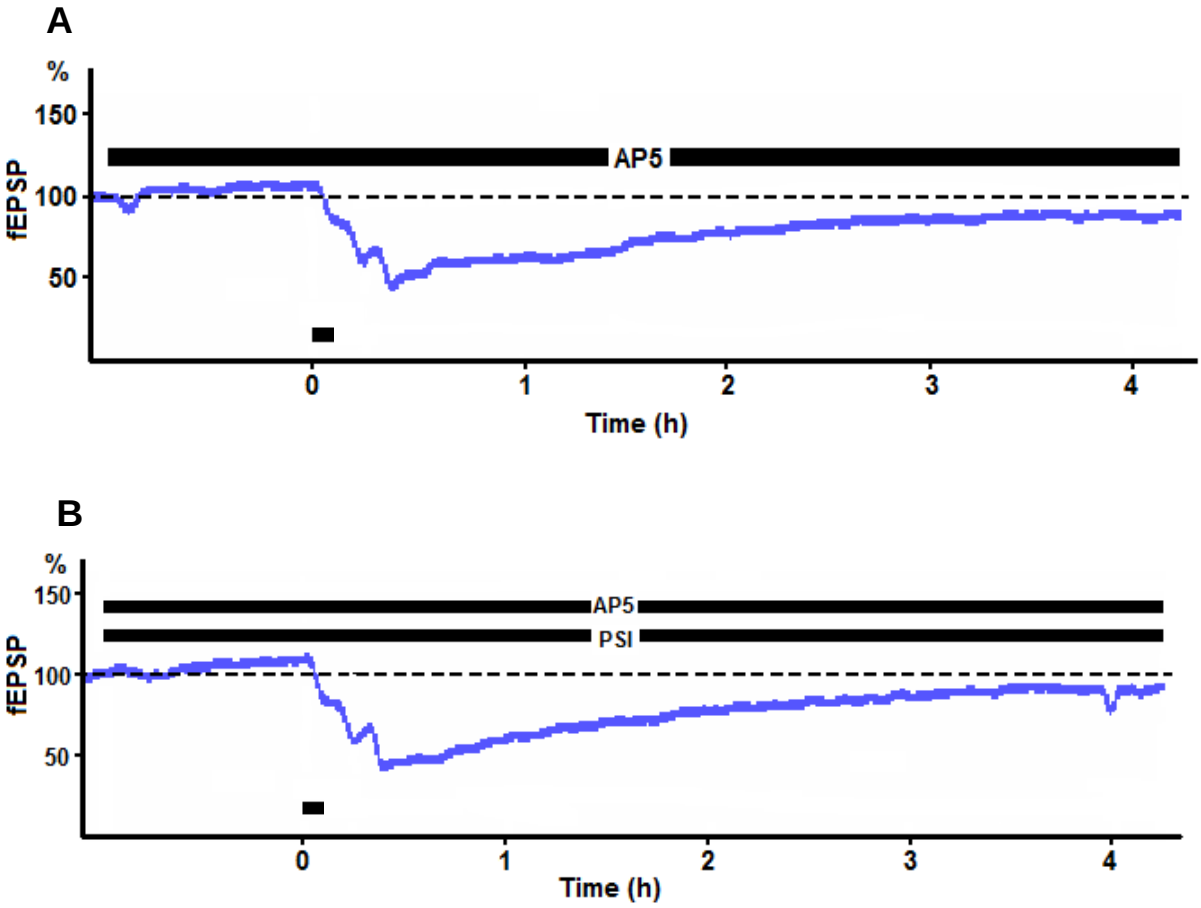

Figure 12. Effect of PSI on DHPG-LTD in young animals. Successive measurements of fEPSP are shown for successive responses in a single pathway. **A)** DHPG (100 µM; 10 min) was added after at least 60 min of baseline recording to induce LTD in a group of slices not treated with PSI. AP5 was applied from the beginning of experiments to prevent any contribution of NMDAR in this form of LTD (n = 6). **B)** Cycloheximide (100 µM) was added to the solution from the start of the experiment without obtaining an observable effect on the induced LTD (n = 6). The dotted line in both figures represents primary default value of fEPSP.

Statistical analysis has revealed a significant depression of fEPSP at different times following DHPG application compared to normalized baseline level in both groups of slices (Figure 13). However, no significant difference between the two groups was found, indicating that PSI had no effect on this form of LTD.

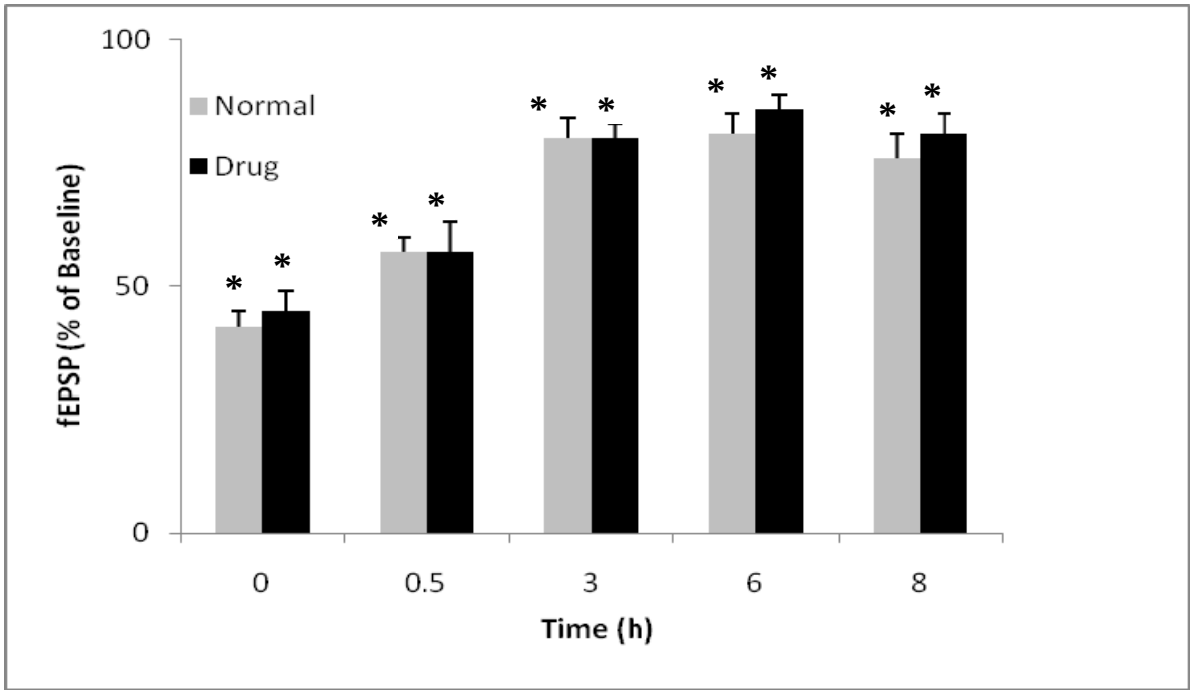

Figure 13. Statistical analysis of DHPG-LTD in young animals. LTD is expressed as fEPSP values relative to the pre-induction baseline. Significant LTD was observed with recording times up to 8 h. Still, there was no significant difference between the normal group of slices (light bars; n = 10) and the cycloheximide-treated group (dark bars; n = 10). Asterisks indicate $p < 0.05$.

DHPG-LTD in old animals

As the results with young animals revealed no effect of cycloheximide on the LTD induced by DHPG i the question whether older animals might show sensitive LTD under the condition of protein synthesis inhibition was addressed. Another group of rats aged between 12- to 18-week was used in this type of experiment. However, the low magnitude of LTD observed in young rats led us to modify the conditions under which DHPG to induce more stable and constant LTD. It has previously been shown that lowering the extracellular Ca^{2+} concentration can enhance the DHPG-induced LTD in adult animals, suggesting that the effect of DHPG on synaptic transmission may be due to presynaptic changes mediated via postsynaptic mGluRs (Watabe et al., 2002). Therefore 1.5 mM was used instead of 2.5 mM Ca^{2+} concentration in the ACSF for these tests, demonstrating a significant LTD. The data showed that there was no difference in magnitude of LTD obtained under low Ca^{2+} concentration when comparing experiments with and without the cycloheximide (Figure 14). It is intriguing that these results were actually obtained with recording times up to 14 h (not illustrated).

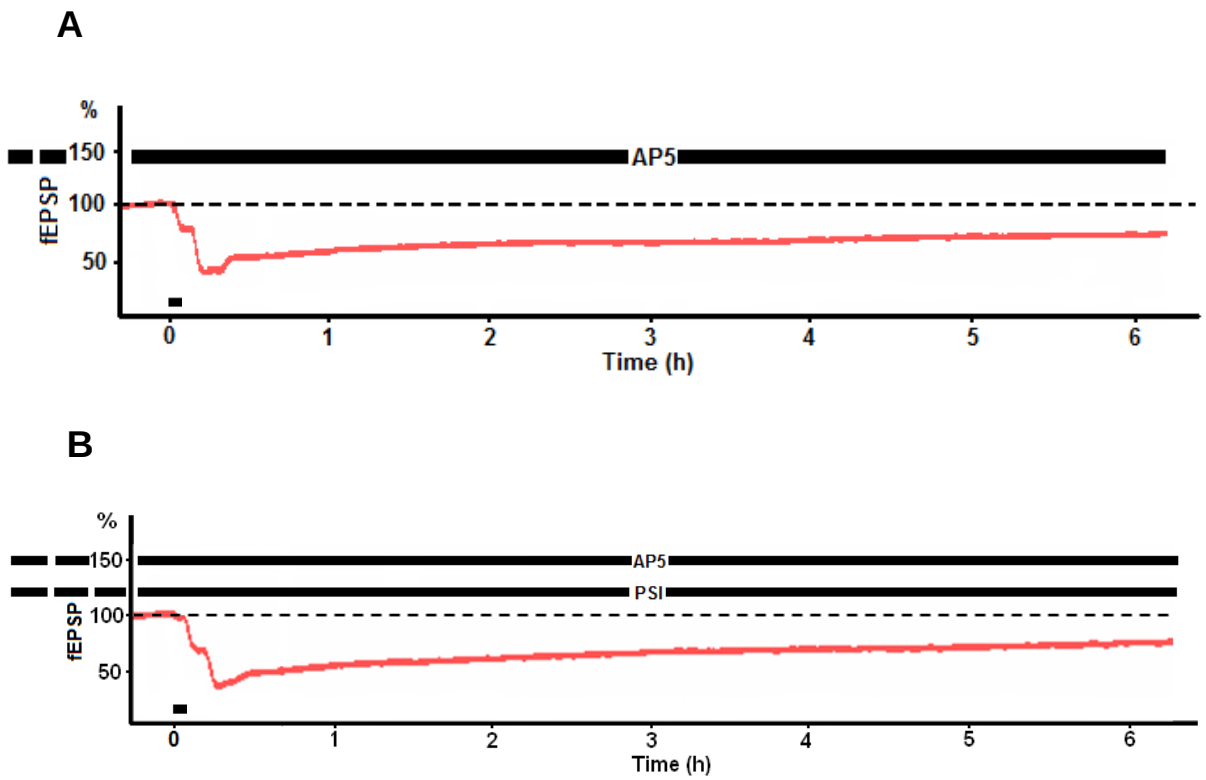

Figure 14. Effect of PSI on DHPG-LTD in old animals. Successive measurements of fEPSP are shown for successive responses in a single pathway. **A)** Under conditions of low Ca^{2+} concentration (1.5 mM) 100 µM of DHPG delivered for 10 min induced long-standing LTD in a normal group of slices. AP5 was applied from the beginning of the experiments to prevent stimulation of NMDAR, hence to obtain a pure mGluR-LTD (n = 8). **B)** The same experimental conditions as in A with exception of adding cycloheximide (100 µM) 60 min before the application of DHPG and kept in the solution throughout the experiments (n = 8). Results were similar in drug and no-drug experiments. The dotted line in both figures represents primary default value of fEPSP.

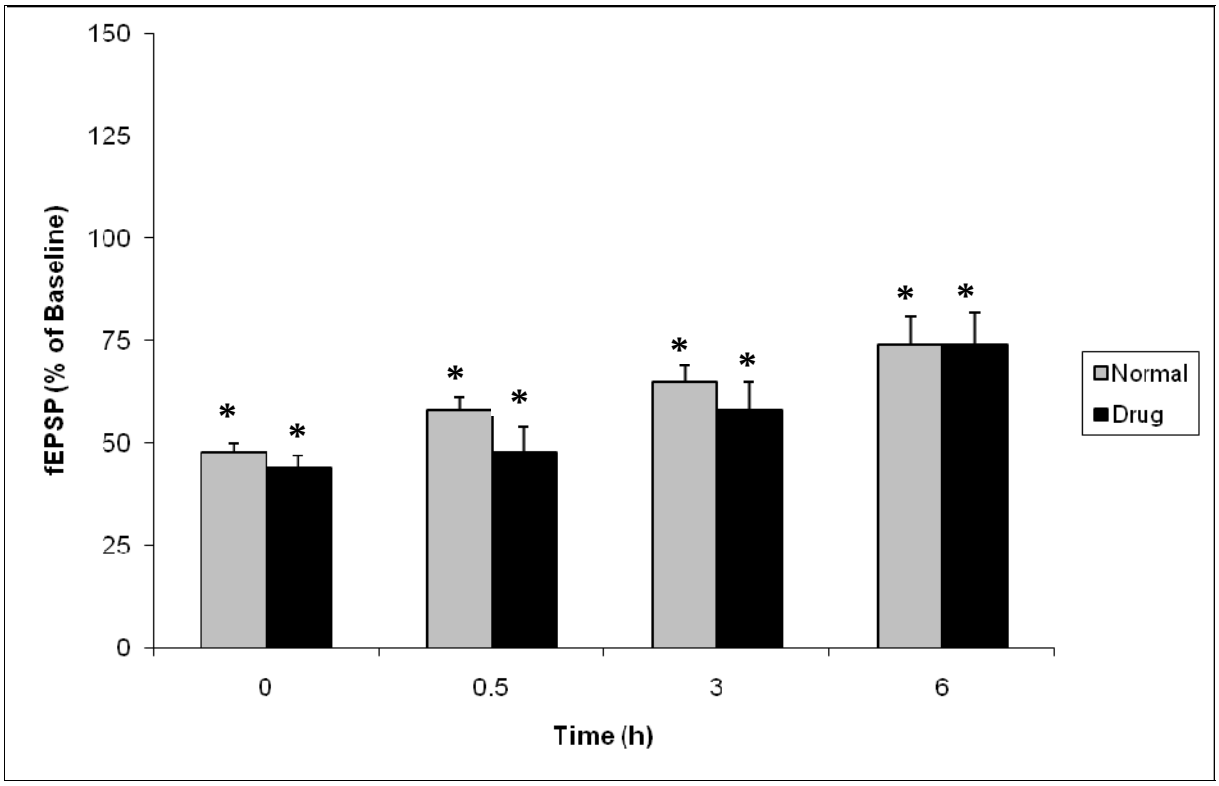

Figure 15. Statistical analysis of DHPG-LTD in old animals under lower Ca^{2+} concentration. LTD is expressed as fEPSP values relative to the pre-induction baseline. There was a significant depression throughout the recording time in both normal (without CHX; grey bars) and drug groups (black bars). When comparing cycloheximide-treated and drug-free groups (n=8 in both cases) there was no significant difference. Asterisks indicate $p < 0.05$.

Potency of the PSIs

A necessary question to answer is whether the lack of effect of PSIs on the different forms of LTD is a "real result" or merely an "artifact" due to a low potency of the drugs used. The level of protein synthesis inhibition by anisomycin and cycloheximide were therefore measured directly by tritiated leucine incorporation into the proteins of whole slices (Lipton and Heimbach, 1977). During incubation with 3H-leucine, slices were maintained under similar conditions as in the electrophysiological experiments.The results demonstrated that both anisomycin and cycloheximide effectively blocked protein synthesis measured in this manner. With the same concentration of drugs as used in LTD experiments (40 μM and 100 μM, respectively) the level of remaining protein synthesis in treated slices was only a few percent of the control value, see Figure 16.

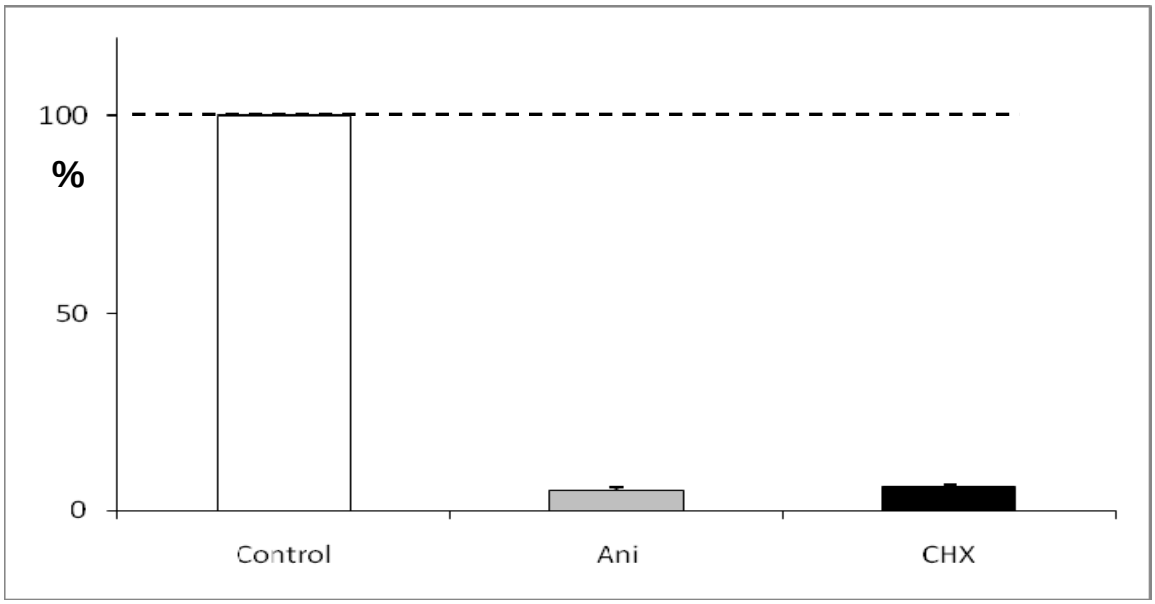

Figure 16. Potency of PSIs anisomycin and cycloheximide as determined by radioactive assay according to Lipton and Heimbach (1977). Anisomycin (40 µM) reduced protein synthesis down to 5.2±1.0 of normal value (n=7+7). The corresponding value for cycloheximide (80-100 µM) was 6.3±0.4% (n=12+12). Bars indicate mean ± SEM. The dotted line represents a value of 100% protein synthesis.

Conclusions

All body proteins are subject to a continuous remodelling process involving synthesis, modifications and breakdown (turnover). This process ensures continuous repair and maintenance of the quality of proteins, in addition to production of the essential ones. Decreased protein synthesis rates could either lead to a reduction in the amount of proteins or a reduced quality of some of them. If the synthesis of certain regulatory proteins is inhibited this may even cause higher levels of the controlled proteins. Still, this scenario does not seem likely in view of the observed high potency of the drugs (anisomycin and cycloheximide) used here to inhibit protein synthesis.

Little is still known about the proteins most crucial for setting up activity-dependent synaptic plasticity such as LTP and LTD. Regardless of which proteins are crucial, the present data suggest that the available supply under basal conditions is sufficient for induction and expression of fully fledged LTD, NMDAR dependent as well as mGluR dependent, in young animals as well as in old ones. These results, which are in line with prior ones on LTP (Abbas *et al.*, 2009), indicate a large safety-margin for generating long-term synaptic plasticity in hippocampus. This feature might be related to the urgent need for a well-functioning memory mechanism in probably all animal species.

Under conditions of severely impaired protein synthesis, as might occur at high age or during certain brain diseases, there might be a shortage of the necessary plasticity-related proteins. Studies as the present one can help to pinpoint the critical factors and so may assist in developing treatments for impaired memory. To further improve the available models, preapplication of drugs for much longer times may be needed, realizing that baseline content of Plasticity related proteins (PRPs) would only then be suppressed enough to interfere with mechanisms of synaptic plasticity.

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