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Mechanism of inhibition of LTP induction by preconditioning stimulation in the rat dentate gyrus *in vitro*.

by

Barbara Gisabella, Laurea

A dissertation submitted for the degree of Doctor of Philosophy at the
University of Dublin, Trinity College, Dublin 2, Ireland.
This research was conducted in the Department of Physiology in the Faculty of Health Sciences.

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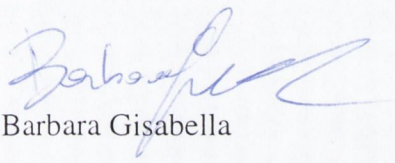

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To my Mother, light of my life

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Summary

The modulation of synaptic plasticity (LTP) by prior synaptic activity represented by preconditioning stimulation (weak HFS) was investigated in the medial perforant pathway of the dentate gyrus *in vitro*. The inhibition of LTP by preconditioning stimulation represented by weak HFS has been observed previously in CA1 (Huang et al, 1992). The results reported in this thesis have verified that similar inhibition occurs in the medial perforant path of the dentate gyrus. In addition, a possible model for the mechanism of inhibition of LTP by preconditioning stimulation has been proposed. Previous synaptic activity resulting in a change in the capabilities of synapse to undergo subsequent plasticity has been referred to as metaplasticity.

In chapter 3, the 'window period' of the inhibition of LTP was described by preconditioning stimulation. The preconditioning stimulation inhibited subsequent HFS-induced LTP, if applied 10 and 20 min, but not 2 or 45 min prior to the HFS.

In chapter 4, the involvement of receptors in the mechanism of inhibition of LTP by preconditioning stimulation was investigated. The application of NMDAR or mGluR antagonists (D-AP5, MPEP, EGLU and MCPG) during weak tetani was found to prevent subsequent LTP inhibition.

The use of different types of priming from weak tetani in the medial perforant path of rat dentate gyrus was also investigated. In order to determine whether lower frequencies of stimulation than 50 Hz result in a subsequent inhibition of LTP, preconditioning stimulation was applied in the form of prolonged LFS (1 Hz, 900 stimuli) terminating 20 min prior to the standard HFS. LFS was not found to inhibit subsequent LTP induction. Several pharmacological agents acting at the same receptors involved in the inhibition of LTP by weak HFS were also applied directly in the hippocampal slices. For instance, in order to investigate the priming effect on LTP induction through the group II mGluR, hippocampal slices were perfused with DCGIV, a specific group II mGluR agonist. DCGIV inhibited LTP.

In chapter 5, the involvement of second messengers chain, such as protein kinases (PKA, PKC) and the p38 -MAP kinase, responsible for the inhibition of LTP by preconditioning stimulation is described.

Publications

Abstracts

Gisabella, B., Anwyl, R., Rowan, M.J. (2001). Mechanism of inhibition of LTP induction by preconditioning stimulation. *Society for Neuroscience* Abstracts 272 (31st Annual meeting).

List of abbreviations

°C	Celsius degree
AMPA	α-Amino-3-hydrox-5-methyl-4-isoxazolepropionate
AMPAR	AMPA receptor
ACSF	artificial cerebral spinal fluid
Bis-I	Bisindolylmaleimide I
cAMP	cyclic AMP
CNS	central nervous system
CaMKII	Ca^{2+}/calmodulin-dependent kinase
D-AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
DAG	diacylglycerol
(S)-3,5-DHPG	(S)-3,5-Dihydroxyphenylglycine
DCGIV	(2S,2'R,3'R)-2-(2',3'-Dycarboxycyclopropyl)glycine
DMSO	dimethylsulfoxide
EPSP	excitatory post-synaptic potential
EGLU	(S)-α-ethylglutamate
GABA	γ-aminobutyric acid
$GABA_A$	GABA A receptor
h	hour(s)
HFS	high frequency stimulation
Hz	Hz
H-89, HCl	N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl
IP_3	inositol triphosphate
LTP	Long-term potentiation
LTD	Long-term depression
LFS	Low -frequency stimulation
mGluR	Metabotropic glutamate receptor
MAP kinase	mytogen-activated protein kinase
M	molar
ml	millilitre(s)
mM	millimolar

μM	micromolar
μm	micrometer
msec	millisecond
mV	millivolt
MΩ	megaohm
MCPG	(+)-α-Methyl-4-carboxyphenylglycine
MPEP	2-methyl-6-(phenylethynyl) pyridine hydrochloride
NBQX	6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione
NMDA	N-Methyl-D-aspartic acid
NMDAR	NMDAR
PKA	cAMP-dependent protein kinase (Protein kinase A)
PKC	Protein kinase C
PP	Protein phosphatase
PTK	Protein tyrosine kinase
PD98059	2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one
STP	short -term potentiation
SE	standard error
sec	second(s)
SB203580	4-(4-fluoropheny)-2-(4-methylsulfonilphenyl)-5-(4-pyridil) imidazol

1 Introduction

1.1 Preface

Many higher organisms have the ability to form new patterns of behaviour though experience. The acquisition of new behavioural responses is referred to as learning, which is fundamental to the survival of animals (Abraham and Tate 1997).

It is now widely believed that the nervous system is made up of individual neurons, and that the functional characteristics are a result of integrated activity through specialized neuronal contacts, called synapses. Ramon y Cajal first put forward the hypothesis that learning involves changes in the strength of central synapses (1893). 60 years later Donald Hebb (1949) provided a working model of the mechanism through which changes in synaptic efficacy may encode information in the nervous system. He argued that the strength of the connections between a pair of neurons might be altered as a result of persistent activity. In 1973 Bliss and Lømo provided the first experimental evidence in support of the above hypothesis. Thus a brief high frequency stimulation of the perforant path produces an increase in the excitatory synaptic potential in the granule cells, which can last for hours, and even, under some circumstances, for days or weeks. They called this facilitation long-term potentiation (LTP).

The brain seems to be highly 'plastic', in that both its structure and its moment-to-moment operation can be changed in response to experience. In other words, experience-dependent effects are dependent critically upon gene-environment interaction (Bateson, 1996). Once development is complete, the mature central system rarely responds to an individual event in the same stereotyped way every time that event occurs. It is a complex device whose patterns of activity can be altered by experience in a myriad of different ways so as to optimise its future response to events. In other words, it can learn.

Recently there has been an increasing understanding of the properties and mechanisms underlying LTP of synaptic efficacy, putative learning and memory mechanisms in the mammalian brain.

One body of work is trying to explore its expression in behaving animals during various type of learning. A wider group of neuroscientists have focused on understanding its underlying mechanisms. A critical step in achieving this was the development of in *vitro* brain slices, which permit much better visual control of electrode placement, full experimental control of the ionic environment of the cells and synapses of interest, the ability to wash drugs in and out of the recording chamber, intracellular and patch recording and a number of other advantages over experimentation *in vivo* (Morris, 1996).

1.2 The Memory Engram

The human brain has approximately 100 billion neurons and 1000 times that many synapses. Since the demonstration by Ramon y Cajal that networks of neurons communicate with each other at specialised junctions, called synapses, it has frequently been suggested that information is stored in the brain as changes in the efficacy of synapses. The location of memory storage, or memory engram, is therefore believed to be the synapse.

Hebb consolidated these ideas by proposing that synapses are modifiable as a joint function of pre- and/or post-synaptic activities. "If cell 'A' persistently or repeatedly takes part in firing cell 'B', then some growth-process or metabolic change takes place in one or both cells such that cell A's efficiency in firing cell B is increased" (Hebb, 1949). Such modifiable neuronal connections have become known as Hebb synapses. The first Hebb synapses to be identified in the mammalian brain were in the hippocampus, an area critical for the process of memory consolidation (Milner, 1972) and spatial memory (O'Keefe and Nadel, 1978).

1.3 Hippocampal formation

The hippocampal formation is believed to play an important role in learning and memory. The idea that the hippocampus is important for the formation of memory emerged from studies of animals with brain lesions and from clinical works with human patients (Scoville and Milner, 1957). Scoville & Milner first demonstrated that lesion of hippocampus completely wiped out short-term memory (1957). In fact, the essential role of the hippocampus in memory was established by a clinical case in which a patient with hippocampal damage following global ischemia revealed a bilateral lesion involving the entire the CA1 area of the hippocampus (Zola-Morgan et al., 1986). The use of imaging technologies, such as magnetic resonance imaging and positron electron tomography, have lead to the identification of the importance of hippocampal activity in the memory process (Squire et al, 1990; 1992). In monkeys, lesioning the CA1 and the CA2 cells have been found to result in impaired memory performance (Zola-Morgan et al, 1992).

The hippocampal formation is comprise of a series of adjacent cortical regions including the dentate gyrus, the Ammon's horn (the hippocampus proper, which is itself divided into three subdivisions: the CA3, the CA2, and the CA1), the subiculum, the presubiculum, the parasubiculum, and the enthorhinal cortex (Amaral, 1993).

The highly laminar nature of the hippocampus and dentate gyrus enable them to be used as an ideal model for neuroanatomical and electrophysiological studies. Also, their relationship to the rest of the brain suggests how it may be take part in the generation of memory process.

The unusual shape of the human hippocampus, which resembles that of the sea horse, led to its most common name (in Greek *hippo* means "horse" and kampos "sea monster"). The hippocampus is also sometimes called Ammon' s horn due to its resemblance to a ram's horn (the Egyptian god Ammon had ram's horns).

From a histological perspective, the hippocampus is nicely laminated, with both the neuronal cell bodies and the zones of connectivity arranged in orderly layers. It is a cylindrical structure localized beneath the cortex in the median area of the hemispheres and is composed of 2 folded sheets of neurons that are highly interconnected (fig. 1.1). The first

sheet contains 3 adjacent cortical areas: the CA fields, the subicular complex and the entorhinal cortex. The second sheet, the dentate gyrus, is not continuous with the CA layer but bends around it. Information flows through a loop of neurons or *synaptic circuit* of the hippocampus (fig. 1.1), starting from the neocortex into and out of the hippocampus.

1.3.1 Principal neurons

The principal neurons in the dentate gyrus are the *granule cells*, and in the hippocampus they are the *pyramidal neurons* (the CA3 and the CA1).

(1) Dentate granule cell

The granule cells are small cell bodies (about 10 μm in diameter), spherically shaped and densely packed in the *granular layer*. In the rat this has a U-shaped structure. Granule cells are monopolar neurons because the dendrites emerge only from the top or apical portion of the cell body. The dendritic tree extends from the granule layer into the *molecular layer* where they receive synaptic connections from several sources. The axons, also called *mossy fibers*, originate from the basal portion of the cell body and give rise to an extensive set of collaterals (generally less than 0.2 μm thick), which provide input to the polymorphic neurons of the hilus (Henze et al., 2000). From the hilus, the mossy fibers join onto other mossy fibres to form a mossy fibre bundle that exits the hilus and enters *stratum lucidum* of the CA3 where connections are made with the pyramidal cells of this area.

(2) Pyramidal cells in the CA3 region

The pyramidal cells in the CA3 are considerably larger than those found in the CA1 region. Large, sometimes branched dendrite spines, called excrescences, are located on the proximal parts of the dendritic tree and form synaptic contacts with the mossy fibres originating from the dentate gyrus.

(3) Pyramidal cells in the CA1 region.

The cells bodies of the hippocampal pyramidal neurons are arranged in a layer called *stratum pyramidale* that forms a curved sheet that is 3-6 cells thick. They are called multipolar neurones due to their elaborate dendritic trees extending perpendicularly to the cell layer in both directions. The apical dendrites extend from the apex of the pyramidal cell body and traverse 3 strata: the *stratum lucidum*, the *strata radiatum* and the *stratum lacunosum-moleculare*. In each of strata, dendrites receive different type of synaptic contacts.

(4) Interneurones

Golgi-studies of Ramon y Cajal (1911,1968) and Lorente de No (1934) have firmly established the notion of several heterogeneous classes of non-principal neurons, generally called interneurons.

The majority of these type of neurons are GABAergic and therefore inhibitory (Woodson et al., 1989), with a restricted axon plexus that lack spines and locally restricted target regions.

The pyramidal basket cell is the prominent class of interneurons in the dentate gyrus, and the cell bodies of these neurons are specifically located between the granule cell layer and the polymorphic cell layer. Interneurons with cell bodies in or near the hilus are of 3 types: axo-axonic cells, basket cells, and bistratified cells. Axonic cells exert a strong control over the action potential as they synapse onto the initial segments of the CA1 pyramidal neurones and granule cells. Basket cells synapse onto the somata of pyramidal neurons, making multiple contacts and forming what looks like a "basket" into which the soma sits and innervates the proximal dendritic region. Bi-stratified cells make contacts onto apical and basal dendrites of pyramidal neurons (Turner et al., 1998).

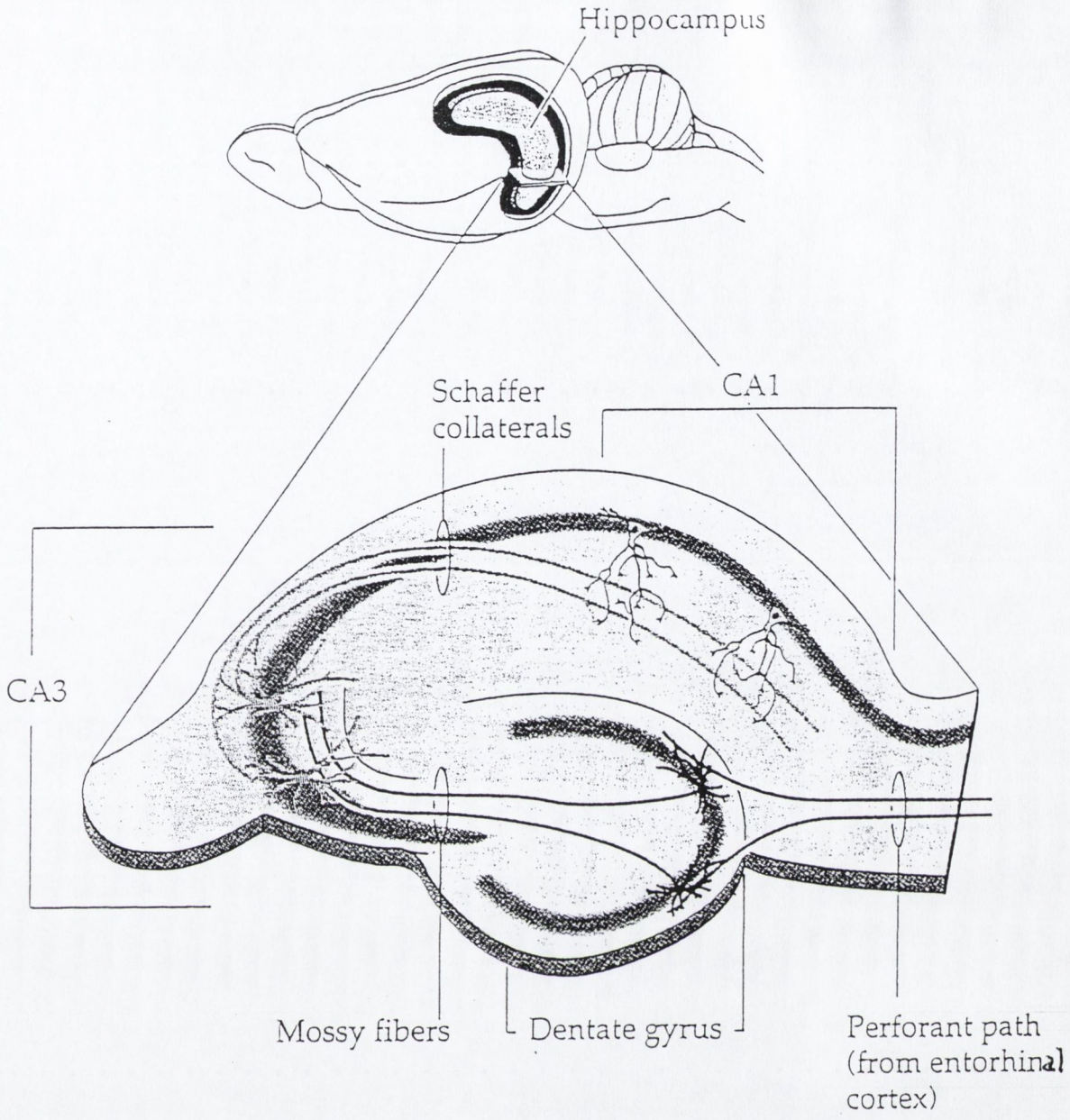


Fig. 1.1 **Schematic diagram of rat brain with part of the cortex removed to show the hippocampus (hip) beneath.**

The figure below represents a transverse hippocampal section showing the trisynaptic circuit. Information flows unilaterally from the subiculum (not shown) along the perforant path to the dentate gyrus (DG). Mossy fibres from DG project to CA3 region where they synapse on the pyramidal cells. Pyramidal cells send their schaffer collateral fibres to the CA1 region. The output from CA1 is largely to the entorhinal cortex, both directly and via the subiculum.

1.3.2 Circuitry models and function of the hippocampus

The hippocampus seems to function in multiple behavioural roles and is integrated into a number of CNS circuits, particularly sensory and limbic circuit.

Projections from the neocortex (these inputs include particularly sensory afferents from olfactory and visual cortices) arrive in the parahippocampal cortex and the perihirnal cortex, they project onto the entorhinal cortex and finally onto the hippocampal synaptic circuit. Thus, the flow of information through the rat hippocampal region is through a loop of neurons called also synaptic circuit. In the synaptic circuit of the hippocampus there is a neuronal network forming synapses: the spiny stellate neurones in the entorhinal cortex project along the perforant path (through the perforant fibres) to the dentate gyrus to form the first synapse. There are about 1 million granule cells in the dentate gyrus, and these project along so called mossy fibres to approximately 330,000 pyramidal cells in the CA3 region of the hippocampus (Amaral et al., 1990). The CA3 schaffer collateral fibres in turn project to the CA1 fields. Finally, the pyramidal cells of the CA1 field send axons out of the hippocampus to the entorhinal cortex, both directly and via the subiculum. There are also direct inputs from the entorhinal cortex to CA1 and CA3. Thus the information can be returned to the neocortex via the subiculum and entorhinal cortex; this also means that the hippocampus is widely innervated and it has high connectivity, facilitating the processing of information to many brain areas.

1.3.3 Synaptic transmission at synapses

During the learning process, sensory stimulation is changed into digital information (action potentials) via our ears, eyes, skin, nose etc and reaches the central nervous system through the peripheral neuronal fibres. When action potentials reach the presynaptic terminals, they trigger the release of neurotransmitters into the synaptic cleft. From the cleft, neurotransmitters bind to specific receptors localised in the post-synaptic membrane of other neurons inducing a change in the postsynaptic membrane potential. If enough excitatory neurotransmitters bind to the receptors of the postsynaptic membrane, excitatory

postsynaptic potentials (EPSP) are induced that are large enough to generate action potentials that can travel to the next terminal and trigger the release of neurotransmitters. This process travels from neurone to neurone in the circuit of the neuronal network.

1.3.4 Synaptic plasticity in the hippocampus

1.3.5 Long -term potentiation (LTP)

One form of activity-dependent synaptic plasticity that has been investigated extensively is long-term potentiation (LTP). LTP is an induced phenomenon that was first observed in the hippocampus of rabbits in vivo (Bliss and Lømo in 1973, Bliss & Gardner-Medwin, 1973). Subsequently, it was characterised in the hippocampal slice preparation (Schwartzkroin and Wester, 1975). Both the preparations continue to be utilised today.

In many regions of the brain such as for instance CA1, dentate gyrus and CA3, when excitatory synapses are electrically stimulated at high frequency for brief period, for example 100 Hz for 1 second, the strength of those synapses increases – this is LTP. Considering LTP as a long-lived increase in synaptic strength is the most popular model for the cellular process that may underlie information storage within neuronal system.

LTP can persist for many weeks (for hours in hippocampal slices in vitro and even months in vivo), and it can be elicited by patterns of synaptic activity similar to those that occur naturally in the brain of freely behaving animals. It can be induced in all three synapses in the tri-synaptic circuit, and in other areas of the mammalian nervous system (Morris, 1990).

LTP occurs in many pathways of the brain, such as the amygdala and cortex, not just the hippocampus and dentate gyrus, where it was first observed (Martin et al., 2000).

The fact that LTP requires both presynaptic activity and postsynaptic depolarisation has lead it to be described as being 'Hebbian-like' (Collingridge, 1992). Hebb argued that a set of synaptically connected cells in a neuronal network could store associative memories if strengthening occurs in both presynaptic activity (input to the synapse) and sufficient convergent excitatory input to fire the postsynaptic cell ('Hebb rule') (Lisman et al., 2001). All of the three major synaptic pathways can undergo LTP; however, the potentiation has

different properties and perhaps a different mechanism at different sites (Brown et al, 1990 and Zalutsky et al., 1990). In the CA1 region and the dentate gyrus, LTP has Hebbian characteristics because both postsynaptic pyramidal neuron and presynaptic neurons are required for its activation. In the CA1 area, LTP seems to have the property of associativity, in contrast the CA3 area is not Hebbian, and it is not associative (Chattarji et al., 1989).

Thus, in the CA1 area and the dentate gyrus, LTP is characterized by the properties of input-specificity, cooperativity and associativity. Input-specificity means that only those inputs activated at the time of the tetanization will display LTP (Anderson et al., 1997 and Lynch et al., 1977).

Cooperativity refers to the existence of a certain intensity threshold for the generation of LTP; weak tetanic activation of a relatively small number of afferents is filtered out. Thus, a number of afferent fibres must be active at the same time, and for a minimum period of stimulation, if a long-lasting potentiation is to be induced; stimulation of only a few fibres is not sufficient to induce LTP (Riedel et al., 1996, Bliss and Lynch, 1988). Consequently, induction requires association between the pre- and post-synaptic events (presynaptic activation and postsynaptic depolarisation), and only synapses active inside a certain time window with respect to the postsynaptic activity are potentiated (Gustafsson et al., 1990). Associativity means that a weakly tetanised input can express LTP when other independent afferent fibres, converging to the same target cells, are simultaneously stimulated by a strong tetanus (Recanses, 1995).

1.3.5.1 Cellular mechanism of LTP induction

Induction of LTP refers to the transient events that serve to trigger the formation of LTP (Sweatt, 1999).

In the hippocampus there are two main distinct forms of LTP. The first type, a homosynaptic (non-associative) LTP, occurs in synapses from the mossy fibers onto the CA3 neurons (Harris and Cotman, 1986; Kauer et al., 1988). It can be induced only by high frequency stimulation of presynaptic terminals, and the strength of regulation required is

not influenced by depolarisation of the post-synaptic membrane. The second type, which is the subject of the present work, is an associative form at the perforant path-dentate gyrus and Schaffer collateral CA1 synapses. It is an NMDA receptor-dependent form of LTP, and it can be obtained by strong high frequency stimulation and also by weak tetani coupled with depolarisation of the postsynaptic membrane (Barrionuevo and Brown, 1983; Kennedy, 1989).

A. Excitatory amino acid receptors involved in the synaptic transmission

Glutamate is believed to be the neurotransmitter at the principal synaptic junctions of the hippocampus, producing rapid neuronal excitation (White et al., 1977). In 1978, Dunwiddie tetanised perforant path fibers while bathing the dentate gyrus slice in APB (a glutamate receptor antagonist). On washing out APB, there was no LTP, thus suggesting that glutamate neurotransmission is necessary for LTP in dentate (Teyler and DiScenna, 1987). Glutamate plays a role in neuronal survival and refinement of neuronal connections during brain development, as well in the synaptic plasticity underlying learning and memory. Thus, over-activation of glutamate receptors leads to neurodegeneration, and this phenomenon, called excitotoxicity, has been found to occur in major areas of brain pathology.

Glutamate receptors can be classified in two groups: ionotropic receptors, which contain integral cation specific ion channels, and metabotropic receptors, which are G protein coupled receptors and modulate the production of second messengers. In the latter group, the signal may be transduced to other intracellular messengers, such as inositol triphospate or cyclic AMP. The ionotropic glutamate receptors are further sub-classified according to their interaction with the non-physiological glutamate analogs: kainite (KA), alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), and NMDA (N-methyl-D-aspartate). Kainate and AMPA receptors are referred to as the non- NMDA receptors (Collingridge & Bliss, 1987 and Plátenník et al., 2000).

During normal synaptic transmission (when the stimulation is low and insufficient to induce LTP), the excitatory postsynaptic potential (EPSP) is composed of fast and slow (late) components (Blake et al, 1988; Herron et al, 1985). The AMPA receptor seems to be responsible for the fast component, and this has been demonstrated through the application of CNQX (6-cyano-7-nitroquinixaline-2, 3-dione), an AMPA receptor blocker that leaves only the slow EPSP intact. Thus, AMPA receptors open and desensitise rapidly in response to an agonist. The slow component (receptors takes less than 2 ms to open) is caused by ion flux (mostly calcium) through the NMDA receptors. Application of AP5, and the NMDA receptor blocker, inhibits this slow component leaving only the fast one (Kleshchevnikov, 1998).

B. NMDA receptors and LTP induction

The dual-voltage and chemical–gating properties of the NMDA receptor suggests that it may be involved as a coincidence detector in associative learning and in the modulation of LTP/LTD.

NMDA receptors consist of one or more NR1 subunits, together with NR2A-D subunits and probably the recently identified subunit NR-L/X1 (Seeburg, et al, 1993). Five separate genes encode for the first 5 different subunits. NR1 subunit can also be divided into a large N-terminal region, a core region including four trans-membrane domains (TMDs), and C-terminal extension. It is generally agreed that the N-terminal region is extracellular and that the C-terminal region is intracellular. NR1 appears to be essential for functional activity and requires co-expression with at least one NR2 subunit to form the NMDA receptors. The fundamental properties of the NMDA receptor, such as voltage dependent-Mg^{2+} block, the Ca^{2+} permeability, and regulation by phosphorylation and channel kinetics are modulated by NR2A, NR2B, NR2C, and NR2D subunits.

C-terminal contains several potential sites for phosphorylation by PKC, based on the consensus sequence for PKC (Kennelly and Kreb, 1981). In *vitro* experiments have demonstrated that the NR1 subunit can be phosphorylated by PKC to potentiate receptor function (Kutsawada et al., 1992).

The NMDA receptor appears to be activated by L-glutamate, L-aspartate and the specific agonist NMDA (N-methyl-D-aspartate) (Patneau et al., 1990). Glycine has been shown to act as an essential co-agonist at the NMDA receptors (Kleckner et al., 1988). The NMDA receptors can be blocked competitively by (D)-2-amino-5- phosphonopentanoic acid (D-AP5) and (R)-3-) 2-carboxiypiperazin-4-yl) propyl-1-phosphonic acid ((R)-CPP), and non-competitive by 5-methyl-1-10, 11-dihydro-5H-dibenzo [*a, d*] cyclohepten-5, 10-imine (MK-801). Interestingly certain agonist at mGluRs can also act as antagonists of the NMDA receptors. Significant disagreement is evident in the literature regarding the involvement of mGluRs in the NMDA receptor-dependent long-term potentiation. Several works have demonstrated that the mGluR antagonist (MCPG) blocks high frequency stimulation-induced long-term potentiation at the Schaffer collateral CA1 synapse. Other groups have reported that they cannot block long-term potentiation under apparently identical conditions. In hippocampal neurons Contractor et al. show that MCPG at concentrations used to block mGluRs, also reduced the NMDAR mediated current if glycine concentration was very low and at very high the NMDA concentration (Contractor et al., 1998).

At resting potential potentials, the NMDA channels are blocked by a voltage-dependent Mg^{2+} block (Nowak et al., 1984). During basal synaptic transmission, the inhibitory postsynaptic potential (IPSP) hyperpolarizes the cell and greatly enhances the Mg^{2+} block before a significant number of the NMDA receptors are in an open state. Thus, under normal experimental conditions (i.e. stimulation insufficient to elicit LTP), they seem to contribute little to baseline the EPSP. This is due to their voltage-dependence and slow time course of activation. Glutamate binds both AMPA and NMDA receptors, however, since extracellular Mg2+ blocks the majority of NMDA receptors, the EPSP is mainly mediated by AMPA receptors. The NMDA receptors can work as a cellular 'coincidence detector'. This means that the channel opening requires simultaneous occurrence of glutamate binding and postsynaptic membrane depolarisation in order to remove the voltage-dependent Mg^{2+} block from the channel.

Therefore, the depolarisation produced by the high frequency stimulus, the binding of glutamate and summation of AMPA receptor mediated EPSPs are sufficient to remove Mg^{2+} from the NMDA channels to allow Ca^{2+} and Na^{+} to enter the postsynaptic cell. In turn, the additional depolarisation and binding of glutamate open more NMDA receptors.

This may highly increase Ca^{2+} concentration in the dendritic spines, leading to the generation of a cascade effect, initiating the LTP process. Thus, during high frequency stimulation, the contribution of the NMDA receptor in synaptic transmission is radically changed.

Postsynaptic elevation of Ca^{2+} is a fundamental element to triggering LTP, and the possible Ca^{2+} sources seems to be NMDA receptors, voltage-dependent Ca^{2+} channels (not for the NMDA-dependent LTP) and intracellular stores. NMDA receptors function as ligand-gated calcium channels which may be activated by Ca^{2+}/calmodulin-dependent kinases. In addition, Ca^{2+} influx through the NMDA receptor–channels causes activation and translocation of protein kinase C and activation of tyrosine kinase Src (Vaccarino, et al., 1987).

Gene knockout experiments have confirmed the central importance of the NMDA receptor. Mice lacking the NR1 subunit manifest deficiencies and can even die within a day of birth. Mice lacking NR2 subunit show reduced NMDA synaptic currents and reduced LTP in the Morris water maze (Larkman and Jack, 1995). An important discovery by Collingridge was that the selective antagonist, AP5, blocks the induction of LTP in the CA1 area in *vitro* (Collingridge et al., 1983).

NMDA receptors have been found highly enriched in dendrites in the CA1 area and in the dentate. They are, however, also present in the mossy fiber terminals, in the CA3 region (Monaghan et al., 1985). This differential distribution is linked with the fact that LTP induction in the mossy fiber synapses is not associative and is not blocked by NMDA receptors blockers. LTP at the mossy fiber CA3 synapses is therefore probably presynaptically induced and maintained. Evidence exists that postsynaptic depolarisation has no effect on LTP induction (Nicoll et al., 1988), and also that postsynaptic injection of a calcium chelator does not result in LTP being blocked (Zalutsky and Nicoll, 1990). Using calcium-free media, Ito et al. (1991) blocked presynaptic transmission LTP induction.

As mentioned before, contrary to what happens at the mossy fiber synapses in the CA3 area, LTP elicited in perforant path-granule cell synapses in the dentate gyrus, at associational-commissural synapses in area the CA3 area and at Schaffer-commissural synapses in the CA1 area is NMDAR-dependent. In *vivo* and in *vitro* experiments have demonstrated that in these regions LTP induction may be blocked by presence of the

noncompetive NMDAR antagonists, MK-801, CPP and phencyclidine, and by antagonists acting at the allosteric glycine site (Bashir et al., 1990).
Activation of the NMDAR through the bath application of the exogenous the NMDA induces STP, but not LTP (McGuiness et al., 1991).

The regulation of the NMDA receptor channel-opening is quite complex, as in addition to the recognition site for glutamate, the receptor contains a site for the co-agonist glycine, a site for the Mg^{2+} block located inside the ion channel, modulatory sites for polyamines, proton and redox active reagents and Zn^{2+} (Yoneda et al., 1991; Sucher et al., 1996). Reducing agents, such as dithiothreitol (DTT), can potentiate the NMDA receptor channels, while oxidizing agents are inhibitory (Aizenman et al., 1989).

Recent studies have given insight into the upregulation of the NMDA receptor by Src family tyrosine kinases, which bind to scaffolding proteins in the NMDA receptor complex. Src acts in signalling cascades that link G-protein-coupled receptors with protein kinase C via the intermediary cell-adhesion kinase beta. This signalling to the NMDA receptors is required for long-term potentiation in the CA1 region of the hippocampus (Ali et al., 2001).

Recently, it has been shown that NMDA receptors are linked to intracellular cytoskeletal and signalling molecules via the PSD-95 protein complex. Thus, the intracellular face of the receptor seems to be associated with various cytoskeletal components, among them the 'synaptic organisers' PDZ domain–containing proteins, such as PSD-95 (SAP90) and PSD-93 (chapsyn 110). This family of postsynaptic density (PSD) proteins, termed Shank, binds via the PDZ domain to the C-end of the NMDA subunit via the PSD-95-associated protein GKAP (guanylate kinase-associated protein). A GKAP splice variant that lacks the Shank-binding C terminus, results in inhibition of synaptic localization of Shank in neurons. In addition, to its PDZ domain, Shank contains a proline-rich region that binds to cortactin and a SAM domain that mediates multimerization. Thus, shank may function as a scaffold protein in the PSD, cross-linking the NMDA receptor/PSD-95 complexes and coupling them to regulators of the actin cytoskeleton (Naisbitt et al., 1999; Rossum et al., 1999).
Interestingly, these proteins may also link the receptors to other downstream-signalling molecules, such as the neuronal nitrix oxide syntase (nNOS) that is activated through Ca^{2+}

/calmodulin by the NMDAR. The nNOS molecule also contains a PDZ complex and interacts with PSD-95 and PSD-93. In this way, the activation of nNOS via the NMDA receptor was prevented by inhibiting PSD-95 expression (Brenman et al., 1996 and Sattler et al., 1999). In addition, NMDARs not only directly bind to signalling molecules (calmodulin, the CAMKII), but also is indirectly complex with other molecules, such as K^+ channels, Ca^{2+} pumps and Na^{2+} channel α subunit via interaction with actin-binding and the PDZ.

There is also an interaction between glutamate receptors and the cytoskeletal proteins, which include PDZ-containing proteins, actin and tubulin and associated proteins. These cytoskeleton-associated proteins can directly interact with NMDA receptor channels (they do not interact through C-termini), providing an alternative targeting mechanism. The synaptic-activity-controlled balancing of monomeric, dimeric and polymeric forms of actin and tubulin may underlie the changes in spine shape. Interestingly these continuous adaptations could be relevant for physiological events, such as learning and the formation of memory.

NMDAR activity may also be regulated by MAP kinase. One proposed model to explain the interaction between the NMDAR and MAP kinase is the following. After the activation of the NMDAR and of L-type-voltage-sensitive calcium channels, calcium ions bind calmodulin (that acts as a Ca^{2+} sensor). Calmodulin is translocated to the nucleus and activates nuclear CaM kinase IV, which then phosporylates response element binding protein (CREB) at Ser133. Experimental evidence indicates that CREB phosphorylation in hippocampal neurons by electrical stimulation or depolarisation could be partially prevented by antisense oligonucleotides targeted to CaM kinase IV (Bito et al., 1996).

In PC12 cells and hippocampal neurones, Impey (Impey, et al., 1998) demonstrated that CREB phosphorylation in response to depolarisation in addition to calcium requires also the Ras/ERK/RSK pathway and protein kinase A (PKA). The sequential activation of ERK and Rsk2 by Ca^{2+} leads to the phosphorylation and transactivation of CREB. In this mechanism Ca^{2+}-induced nuclear translocation of ERK and Rsk2 to the nucleus require PKA activation. This may explain why PKA activity is required for Ca^{2+}-stimulated CREB-dependent transcription. LTP in hippocampal slices from CREB mutants (lacking CREB) decayed to baseline 90 min after tetanic stimulation. This shows that the full expression of the late phase of long-term potentiation (LTP) and L-LTP-associated CRE-mediated transcription requires ERK activation, suggesting that the activation of CREB by

ERK plays a critical role in the formation of long lasting neuronal plasticity (Bourtchuladze et al., 1994).

From the work of Impey and Bito we can deduce that CaMK IV may be important in rapid (up to 2 mins) CREB phosphorylation. The Ras/ERK/RSK pathway can play a role in the more sustained response; however, there is no experimental evidence for that.

Therefore with slower kinetics, calcium may activate Ras/ERK/RSK pathway and the NMDA receptor may couple directly to the MAP cascade via CaM kinase II and SynGAP protein (Synaptic GTPase activating protein) that are physically associated with the receptor. PKA is then responsible for the translocation of ERK and RSK kinases to the nucleus, where they respectively phosphorylate Elk-1 and CREB. These last factors may contribute to a cascade of events resulting in the activation of gene transcription (Bourtchuladze et al., 1994 and Impey et al., 1996).

C. AMPA receptors and LTP induction

AMPA glutamate receptors (AMPA-Rs) mediate the majority of rapid excitatory synaptic transmission in the brain and play a role in the synaptic plasticity underlying learning and memory. AMPA receptors are heteromeric complexes of four homologous subunits (GluR1-4), encoded by four separate genes, which differentially combine to form a variety of AMPA receptor subtypes, with different functional and structural properties. For instance, high calcium permeability is obtained in the absence of the GluR2 subunit. Thus, subunits control permeation and desensitisation, which can be monitored as a function of relative expression level and subunit composition. Multiple AMPA receptor subtypes coexist within the same neuron. It has been shown that AMPAR channels with both low and high calcium permeability can be found within the same retina ganglion neuron (Zhang et al., 1995). Alternative splicing enhances the diversity further. Each of the four genes can encode one of the two splice variants termed 'flip' and 'flop' domains, an α- helical structure on the side opposite the ligand binding gorge, which forms channels with different conductance properties (Dingledine et al., 1999).

In the hippocampus, most AMPA-Rs are composed of GluR1/GluR2 or GluR2/GluR3 subunits. GluR1/GluR2 receptors are added to synapses during plasticity with an interaction between GluR1 and group I PDZ domain proteins; while at synapses that already have AMPA-Rs, GluR2/GluR3 receptors replace existing synaptic receptors continuously and require interactions of GluR2 with N-ethylmaleimide-sensitive fusion protein (NSF) and group II PDZ domain proteins. The regulated addition and continuous replacement of synaptic receptors can stabilize long-term changes in synaptic efficacy and may serve as a general model for how surface receptor number is established and maintained. These subunits have a large extracellular amino-terminal domain, three transmembrane domains and an intracellular carboxy-terminal domain. Recently, it has become clear that for GluRs, two discontinues extracellular domains of GluR subunits contribute to the formation of their ligand-binding site. There is in particular a fusion protein consisting of two short segments termed S1 and S2 (S1-S2 monomer), bridged by a linker to the discontinuous extracellular domain, that is capable of binding GluR ligands (Gregg et al., 2001).

AMPA receptors are localized at excitatory synapses; however, they are never found on adjacent inhibitory synapses enriched in GABA (A) receptors. AMPA and the NMDA receptors are localised at the postsynaptic membrane in a scaffolding organelle called the postsynaptic density (PSD). In addition, to glutamate receptors, the PSD also contain many signalling molecules modulating synaptic transmission at synapses. The physiological function of the activated AMPA receptor is to mediate postsynaptic depolarisation by Na^+ influx, whereas the NMDA receptor due to the Mg^{2+} -voltage dependent block is mainly inactive. Only high frequency stimulation, removing the Mg^{2+} block with a sufficient depolarisation, can activate the NMDA receptor to mediate both Na^{2+} and Ca^{2+} influx. Although NMDA receptors are essential for the initiation of LTP, while expression of LTP is mediated primarily by AMPA receptors.

Evidence exists that protein tyrosine kinase Lyn can physically associate with AMPA receptors and be activated independently of Na^{2+} (or Ca^{2+}) influx through the channel, and results in activation of mytogen-activated protein kinase (MAPK) (Hayashi et al., 1999).

A synaptic PDZ domain-containing protein, called GRIP (glutamate receptor interacting protein), has been identified that mainly interacts with the C termini of AMPA receptors.

GRIP is a PDZ domain-containing protein family that has seven PDZ domains and no catalytic domain. GRIP appears to serve as an adapter protein that links AMPA receptors to other proteins and may be fundamental for the clustering of AMPA receptors at excitatory synapses in the brain. Another AMPA receptor-binding protein, called ABP, interacts with GluR2 and GluR3 subunits. Both GRIP and ABP proteins have proposed roles during long-term potentiation and long-term depression in the delivery and anchorage of AMPA receptors at synapses (Shi et al., 2001 and Dong et al., 1997).

GluR2 and GluR4c interact with NSF, which is an ATPase involved in various membrane fusion events, such as inter-Golgi protein transport and exocytosys of synaptic vesicles. In the CA1 area of the hippocampus this protein appears to be essential for AMPA receptor turnover at the synaptic membrane, acting as a chaperone for (re) insertion of new or recycled AMPA receptors into the plasma membrane. Furthermore, F-actin contributes in localizing AMPA receptor clusters to synapses, which means that, prior to induction of LTP; some synapses do not have functional AMPA receptors, whereas after LTP induction, there may be recruitment of AMPA receptors to the synapse from some intracellular pool ('silent synapse' hypothesis). Experiments in *vitro* and in *vivo* show that PICK1 (protein interacting with C kinase), a PDZ domain-containing protein, interacts with the C termini of AMPA receptors. In neurons, PICK1 specifically colocalizes with AMPA receptors at excitatory synapses and induces clustering of AMPA receptors. The distribution of the AMPA receptor and its rapid recycling in and out of synaptic membranes has been documented (Engert et al., 1999; Lusher et al., 1999).

AMPA receptors are controlled by various proteinkinases (Smart, 1997). AMPA receptors can be potentiated by calcium/calmodulin kinase II (CaMKII), PKA, PKC (Knapp et al., 1990, Wang et al., 1994). Experiments in cultured neurons showed that PKA can potentiate native AMPA (GluR1) due to an increase in channel open probability. Ser-831 in GluR1 seems to be phosphorylated by PKC and Ser845 is a probable PKA target (Greengard et al., 1991, Roche et al., 1996).

Phosphorylation of AMPA receptor by CaMKII and PKC was also produced by electrical stimulation patterns that induce LTP, and this correlated temporally with the increased AMPA receptor-mediated responses during LTP. Support for this comes from work carried out in mice lacking the α subunit of CaMKII, which seem unable to show LTP or LTD. The, AMPA-R phosphorylation catalysed by CaMKII is correlated with the activation and autophosphorylation of CaM-KII, because the CaM-KII general inhibitor KN-62 blocked it

(Barria et al., 1997). It appears likely that crosstalk and feedback loops between multiple molecules signalling (PKA, PKC, CaMKII, etc.) produce prolonged activation of CaMKII. After LTP induction in the CA1 area, which is initiated by NMDA-receptor stimulation and subsequent Ca^{2+} influx, CaMKII phosphorylates the native AMPA receptor, verifying a postsynaptic locus of LTP expression. CaMKII phosphorylates Ser-831 in the GluR1 subunit; however, there is no analogous site in GluR2 (Mammen et al., 1997). Experiments in transgenic mice confirmed this hypothesis (Zamanillo et al., 1999), and reports indicate that after the induction of LTP there is an enhancement of AMPA –receptor responsiveness that develop over approximately 30 min. AMPA receptor channel unitary conductance is increased in about 60% of the cells that are potentiated (Davies et al., 1989; Thomas et al., 2000).

Experiments showed that is possible to obtain a rundown of basal AMPA receptor-mediated current in hippocampal neurons by disrupting the interaction between PKA (localised in the PSD complex) and its anchoring protein AKAP (A kinase anchoring protein). Interestingly AKAP seems to interact also with the NMDA receptors (Westphal et al 1999).

Selective ligands are indispensable as tools for the elucidation of the physiological role of AMPA receptors and as leads for the development of therapeutic agents. Over the last decade a wide variety of such ligands have been developed and studies on the structure-activity relationships of these compounds have contributed to our understanding of the mechanisms involved in AMPA receptor activation and blockade. AMPA is the preferred agonist binding to AMPA receptors. 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2, 3-dione (NBQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (F), tetrazole-substituted decahydroisoquinolines, LY293558 and LY2944886 are AMPA receptors specific antagonists (Honore et al., 1988; Bleakman et al., 1996).

Many analogs of AMPA have been synthetised that exibit potent agonist properties, among them the carboxy derivative (RS)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl) propionic acid (ACPA), the phenyl derivate, (S)-2-amino-3-(hydroxy-5-phenyl-4-isoxazolyl) propionic acid ((S) APPA), certain neurotoxins, such as domoic acid from dinoflagellates and the β-N-methylamino-L-alanine (Ebert et al., 1996, Wahl et al., 1996, Schiffer et al., 1997).

The synthesis and anticonvulsant activity of the novel 7,8-methylenedioxy-4H-2,3-benzodiazepin-4-ones 3a-e, which is structurally related to GYKI 52466, are reported (De Sarro et al., 1998).

Currents through AMPA receptors can be up- or down-regulated by compounds that allosterically modulate receptor kinetics through binding sites distinct from that for glutamate. Ito at al. discovered that aniracetam selectively enhances AMPA current by binding to a site distinct from that for glutamate. Cyclothiazide is a very potent modulator that effectively blocks AMPA receptor desensitisation, and it seems to be less effective than other modulators, such as aniracetam and benzoylpiperidine compounds. Another modulator is the benzothiadiazide IDRA-21, which has proven to have efficacy in enhancing synaptic responses; however, it requires threshold concentrations of at least 100 microM to be active in vitro (Phillips et al., 2002).

D. mGlu receptors and LTP induction

The induction of LTP has been proven to require the influx of Ca^{2+} through the NMDA receptors under most experimental conditions (Bliss and Collingridge, 1993; Malenka 1994). However, because an activation of the NMDARs alone results in decremental short-term potentiation (Kauer, et al., 1988), the question arises as to which other mechanisms may generate long-term processes in plasticity. Likely candidates for such mechanisms are the involvement of G-protein-coupled metabotrobic glutamate receptor (mGluRs). The existence of mGluRs was first postulated in 1985, when biochemical studies demonstrated that L-Glu stimulates inositol trisphospate (IP3) production in the CNS (Sladeczek et al., 1985 and Nicoletti et al., 1986). Nakanishi's group have proven the molecular existence of a G-protein coupled glutamate receptor by expressing cloning of cDNA encoding the rat mGluR1a (Masu et al., 1991).

These receptors belong in origin to a family of heptahelix G-protein-coupled receptors (GPCRs), called the family 3 GPCRs, which mainly include all mGluRs subtypes, Ca^{2+} - sensing (CaS), and $GABA_B$ receptors. These receptors comprise two main domains, separated in most cases by a cysteine- linker and a large extracellular domain, containing the N-terminal agonist-binding site, an intracellular domain and seven-closed linked

hydrophobic transmembrane domains. The carboxy terminus is thought to be intracellular. The second intracellular loop has been shown to play a critical role in the receptor-G-protein interaction. More specifically in mGuR1 and mGluR5, its C-terminal part is critical in determining PLC coupling (Franke et al., 1990, Alaluf et al., 1995, Knöpfel et al., 1995). Despite the distance separating the glutamate binding site and the transmembrane region, these domains of the receptor may interact dynamically together upon agonist binding

The question as to whether mGluRs and the NMDA receptor are structurally linked bears consideration. A recently finding reports that Shank protein binds to Homer proteins, which form multivalent complexes binding proline-rich motifs in group 1 mGluRs and inisitol triphosphate receptors, thereby coupling these receptors in a signalling complex. Because Shank also functions as part of the NMDA receptor-associate PSD-95 complexes in the PSD, it plays a role in the signalling mechanism of both mGluRs and the NMDA receptors (Tu et al., 1999).

Moreover, it has been reported that PKC phosphorylation sites may also participate in the potentiation of mGluR5 responses by the NMDA receptor in hippocampal slices. Because previous studies demonstrated that also mGluR5 could potentiate the NMDA receptor, there is therefore a reciprocal positive feedback interaction between mGluR5 and the NMDA receptor with important implications in synaptic plasticity (Nicoletti et al., 2001).

Until now, eight subtypes mGluRs (mGluRS1-8) have been cloned and assigned to three different groups according to their sequence homology, pharmacology characterisation, and coupling to second messengers pathways.

(1) Group I mGluR

Group I mGluRs (mGluR1 and mGluR5) are localised in the peripheral parts of PSD and contribute to the regulation of synaptic plasticity. This group of receptors is couple to phospolipase C (PLC), and binding of glutamate results in breakdown of phospatidylinositol 4,5-biphosphate into inositol 2,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG activates PKC, which is important for LTP maintenance, and IP_3 releases Ca^{2+} from internal stores (ICSs), which providing an alternative/additional source for Ca^{2+} in the synaptic compartment (Nakanishi, 1994; Conn and Pin, 1997).

Cerebellar Purkinje cells seem to be enriched with mGluR1 receptors, which play a key role in motor learning and motor coordination. The expression of mGluR5, as confirmed using mGluR5 antibodies, is found in many brain areas and is predominantly located at the level of dendrites (specially in the CA1 area) and cell bodies of neurones (in the PSD). mGluR1 is mainly present in the dentate granule and the CA3 pyramidal cells. In the hippocampus they can thus contribute to the induction of LTP. Both these receptors are implicated in neurodegenerative processes, and their antagonists are protective against excitotoxic neuronal death (Nicoletti et al., 1999; Shigemoto et al., 1997).

The interaction of group I mGlu receptors with Homer proteins (or Vesl) has recently been demonstrated. Homer proteins bind to a distal, proline-rich region of the long C-terminus of the mGlu1/5 receptor (Kato et al., 1998). Homer is divided in 3 subtypes: homer1, homer2 and homer3, which can give rise to many different homer protein isoforms (Fagni et al., 2000; Soloviev et al., 2000).
In addition, to their anchoring role, Homers may also act as 'chaperones' affecting the level of mGluR cell surface expression and clustering of these receptors and thus the rate at which mGlu receptors are internalised. Thus in neurons, Homer proteins are capable of oligomerizing (the so called 'long form') and so are involved in the clustering of mGluR1 and mGluR5, whereas Homer 1a antagonised and reverses, such assemblies. This type of Homer (1b, 1c, 2b) seems also to modulate cellular responses to mGluR5 receptor activation by affecting the ability of this receptor to couple to N-type Ca^{2+} and M-type K^{+} channels (Kammermeier et al., 2000). Homer proteins could therefore make an important contribution to the long-term changes in synaptic plasticity though their precise role at the moment is not very clear.

(2) Group II mGluR

Group II mGluR (mGluR2 and mGluR3) are coupled to the inhibition of adenylate cyclase and decrease forskolin-stimulated cAMP production. For example, activation of the mGluR3 agonist, N-acetylaspartylglutamate (NAAG), inhibits adenylyl cyclase and reduces the cAMP-mediated second-messenger cascade. Therefore, it has demonstrated that the administration of NAAG in the dentate gyrus may lead to LTP inhibition (Paul et al., 2000).

It has also been shown that presynaptic inhibition is negatively regulated by PKC and cyclic-AMP-dependent processes (Cartmell et al., 1992; Kamiya et al., 1997).

Group II mGlu receptors can be differentiated by their sensitivity to L-2-amino-4-phosphonobutanoic acid (L-AP4), which acts as an agonist at group III mGlu receptors (Pin et al., 1995).

Immunolabelling of mGluR2 has shown the presence of this receptor in neuropil of the CA1 stratum lacunosum moleculare, the inner layer of the stratum lacunosum moleculare in the CA3 area, and in the middle one-third of the molecular layer (medial perforant path) in the dentate gyrus. Immunohistochemical studies at electron microscope level have revealed a high density of mGluR2/3 in the dendrites and neuron somas and axonal structure in the hippocampus, cerebral cortex and caudate putamen. There is also evidence of high level of mGluR2/3 mRNA in granule cells of the dentate gyrus and also the use of immunostaining antibodies showed glial staining with the antibodies to mGluR2/3 in the hippocampus (Ohishi et al., 1993, Petralia et al., 1996).

The use of selective agonists for this class of receptor revealed the widespread effects of mGluR2/3 localised at presynaptic level. The role of mGluR mediating presynaptic inhibition in the CA1 region was established in studies in which (1S,3S)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3S-ACPD), called also 'trans-ACPD', was shown to reversibly depress excitatory synaptic transmission (Sladeczek et al., 1993, Baskys et al, 1991, Desai et al., 1991). Evidences of a presynaptic locus of these receptors come from studies in the basolateral amygdala, the locus ceruleus, and the striatum, where the use of mGluR2/3 agonists, such as trans-ACPD or L-APB inhibited synaptic transmission (Dube et al. 1997, Pisano et al., 1997, Rainnie et al., 1992).

Moreover, paired–pulse facilitation, an indicator of presynaptic modulation of transmitter release, was enhanced by mGluR agonist acting on mGluR2/3 in the CA1 region, in mossy fibres CA3, and in the frontal cortex (Baskys et al, 1991; Gereau et al., 1994; Burke et al., 1994; Kamiya et al., 1996; Manzoni et al., 1997). The mGluR–mediated inhibition of excitatory transmitter release is not likely to be mediated via action of PKC or PKA, because inhibitors of these kinases do not inhibit the (2S,1R,2R,3R)-2-(2,3-dicarboxycyclopropil)glycine (DCG-IV)–induced presynaptic inhibition in the dentate gyrus. Therefore the presynptic inhibition was proposed to be via a direct suppression of a presynaptic Ca^{2+} conductance or an activation of K^+ channels and direct inhibition of the probability of transmitter release (Anwyl 1999; Cartmell et al., 1992, Swartz and Bean 1992; Sladeczek et al., 1993).

It is has been theorised that in the CA3 region the activation of presynaptic mGluR2/3 requires a high level of glutamate release by high frequency activity, leading to a rapid inhibition of transmitter release (Scanzini et al., 1997). It is probable that, in this area, normal levels of glutamate release is evoked by low frequency activity do not activate mGluR2/3 receptor because these receptor are localised relatively far from synapses and the affinity of glutamate for mGluR2/3 is ~10μM, higher than for AMPA receptors (Shigemoto et al., 1997; Scanzini et al., 1997).

(3) Group III mGluR

Group III mGluR includes mGluR4, mGluR6, mGluR7 and mGluR8. The activation of these receptors also leads to the inhibition of forskolin-stimulated cAMP production.

The members of the group III mGluRs are potently activated by L-AP4, and may have the function of autoreceptors.

Shigemoto et al (1999) demonstrated that subtype mGluR4a, mGluR7a, mGluR7b and mGluR8 of this group of receptors are expressed in the hippocampus. Immunoreactivity for group II and III mGluRs was predominantly localised to presynaptic elements, whereas that for group I mGluRs was localised to postsynaptic elements.

In *situ* hybridisation studies have demonstrated high levels of mGluR4 mRNA in the cerebellar granule cells, which form synapses with Purkinje cells. mGluR6 is exclusively expressed in the outer zone of the inner nuclear layer of the retina, playing an important role in the synaptic transmission between photoreceptor cells and ON-bipolar cells in the visual system (Akazawa et al., 1994).

It has been suggested that mGluR7 mediates inhibition of transmitter release at glutamatergic synapses in some brain regions. mGluR7 mRNA seems to be highly expressed in the cerebral cortex, olfactory bulb and in the cell body of the cerebellar Purkinje cells. Immunoreactivity for mGluR7a demonstrated its presence also in the CA1 and the CA3 region and in the medial perforant path in the dentate gyrus. Expression of mGluR8 seems to be mainly in the lateral perforant path of the dentate gyrus and the CA3 area (Shigemoto et al., 1997).

1.3.5.2 Pharmacology and physiology of mGluRs and role in LTP

Initial evidence implicating mGluRs in LTP came from experiments using the semi-selective antagonists aminophosphonopropionate (AP3) and aminophosphonobutanoate (AP4) (Izumi et al., 1991, Reymann and Matthies, 1989). In these experiments perfusion of AP3 inhibited the late phase of LTP, an effect not mediated by the NMDA receptors since AP3 caused only a weak block of NMDA receptor mediated currents. AP4, which is also a potent class III mGluR antagonist, also blocked maintenance of LTP. Further support for the role of mGluRs in LTP has come from the use of the mGluR1/2 selective agonist trans-ACPD. In the CA1 region, brief application (20 min) of trans-ACPD induces slowly a developing long lasting potentiation of the synaptic response, which is occluded by prior saturation with tetanically induced LTP (Bortolotto and Collingridge, 1992, 1993). Also, in the dentate gyrus trans-ACPD induced LTP (O'Connor et al., 1995). In addition, trans-ACPD can enhance the degree of potentiation when paired with tetanic stimulation (McGuiness et al., 1991).

The use of (RS)-α-methyl-4-carboxyphenylglicine (MCPG), a selective mGluR antagonist has further established the role of mGluRs in LTP. MCPG was found to block both HFS and ACPD induced LTP, probably via inhibition of ACPD-induced phosphoinositide hydrolysis, in adult hippocampal slices (Bashir et al., 1993; Bortolotto et al., 1994).
In addition, experimental evidence has shown that MCPG inhibits LTP in the dentate gyrus of freely moving rats (Riedel and Reymann, 1993), inhibits spatial learning (Riedel and Reymann, 1994; Richter-Levin et al., 1994), and prevents the LTP related increase in postsynaptic AMPA sensitivity. Thus, inhibition of LTP by MCPG was found in studies in the CA1 in vitro, in the dentate gyrus in vivo and in vitro, in the frontal cortex and in the cerebellar granule cells (Bashir et al., 1994; Bortolotto et al., 1995; Wang et al., 1995; Richter –Levin et al., 1993; Vickery et al., 1997; D'Angelo et al., 1998). Controversy exists, however, concerning the effects of MCPG, because opposite results have been obtained from other works in the CA1 region, the dentate gyrus *in vivo* and the visual cortex (Chinestra et al., 1993; Manzoni et al., 1994; Selig et al., 1995; Harata et al., 1996; Martin et al., 1997).

A 'molecular switch' hypothesis has been proposed for the inconsistencies between these studies. Bortolotto and colleagues showed that MCPG only blocked tetanus-induced LTP

in the CA1 in naïve slices, i.e. if activation of mGluRs had not previously occurred, such as with previously induced LTP. Thus, activation of mGluRs sets a protein kinase-dependent 'switch', after which the pathway remains in a conditioned, MCPG –insensitive state. If the switch has been set, prolonged low frequency stimulation (LFS) may reverse the switch, removing the effect of previous mGluR activation, and then MCPG can block the induction of LTP (Bortolotto et al., 1994). Also, the mGluR antagonist LY341495, which inhibits mGluR1/8, results to reverse the molecular switch (Kingston et al., 1998).

A second explanation for the inconsistent effects of MCPG is that mGluRs may have only a modulatory effect on LTP induction. Alternatively, it has suggested that these receptors may be involved in the generation of LTP induced by a particular form of stimulation. Thus, MCPG could block LTP induced by HFS, but not by theta-burst-stimulation (Brown et al., 1994).

Finally, another hypothesis proposed that MCPG blocked the induction of LTP by acting also as an agonist on group I mGluRs. Thus its block was reversed with the use of a specific group I mGluR antagonist, MCCG, in the CA1 region (Breakwell et al., 1998). Similar evidence that MCPG acts as an agonist was found in the amygdala, where the AMPA receptor mediated current was reduced by a presynaptic action (Keele at al., 1995).

To test the involvement of mGluRs in LTP, several investigators have generated mutant mice that express, for instance, no mGluR5 but normal levels of other glutamate receptors. LTP in mGluR5 mutants was partially reduced in the CA1 and dentate gyrus of the hippocampus, due to a reduction of the NMDA receptor mediated –component of the synaptic response, whereas LTP remained intact in the mossy fiber synapses on the CA3 area (the NMDA -independent pathway). Mutant mice were also significantly impaired in two different spatial learning tasks that are known to depend on an intact hippocampus, i.e. the acquisition and use of spatial information in both the Morris water maze and contextual information in the fear–conditioning test (Lu et al., 1997). In further studies LTP reduction was also found in mutant mice lacking mGluR1 in the CA1 region of the hippocampus (Aiba et al., 1994).

There is also experimental evidence for the involvement of group II/III mGluRs in the NMDA receptor-independent form of LTP in the CA1 area of rat hippocampus. The use of

high concentration of the group II-selective mGluR antagonists (2S)-α-ethylglutamic acid (EGLU), prevented NMDA receptor-independent LTP. Moreover, high concentration of the group III-selective mGluR antagonist, (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), blocked NMDA receptor-independent LTP (Grower et al., 1999).

1.3.5.3 Role of Ca^{2+} in LTP

The induction of LTP was shown to require the postsynaptic influx of Ca^{2+} through the NMDARs with subsequent activation of calcium dependent processes (Jahr et al., 1987). Injection of Ca^{2+} chelators into the postsynaptic cell prevented the induction of LTP, and potentiation is induced when the level of postsynaptic calcium is artificially raised using the photolabile Ca^{2+} chelator nitr-5 (Lynch et al., 1983, Malenka et al., 1988).

The results of the Ca^{2+} imaging studies led to the conclusion that tetanic stimulation produced LTP via a postsynaptic Ca^{2+} transient component in the dendritic area near the activated synapses. AP5 was shown to block this transient component, leading to the thought that the restricted diffusion of calcium following entry through the NMDA channels is the basis of synapse specificity (Regeher et al., 1990).

As previously stated, induction of LTP in the CA1 area requires elevation of the free intracellular Ca^{2+} in the postsynaptic neuron. There are three main Ca^{2+} sources during LTP induction which are dependent on the tetanisation strength: entry into the dendrites by permeation through the NMDA receptor gated channels, entry into the soma through voltage operated calcium channels (VOCCs) and release from intracellular stores, i.e. IP3- and ryanodine sensitive ICSs, after activation of group I mGluRs. Activation of VOCCs appears to be critical for LTP only if it is induced by strong tetanisation at 200Hz or higher frequencies. In addition, the block of Ca^{2+} released from ICSs with a selective Ca^{2+} -ATPase inhibitor thapsigargin was absent if applied during tetanisation, but not if given 30 min afterward, or with robust LTP generated by a single triple 100Hz tetanus (Wilsh et al., 1998).

Probably during LTP induction, the three main Ca^{2+} sources interact with each other; this is dependent on the tetanisation strength. Thus, during weak tetanisation the rise of Ca^{2+} for LTP induction is due to the activation of the NMDA Rs and group I mGluRs, but not by L-

type VDCCs. In these conditions the application of group I mGluRs antagonists results in LTP impairment. In contrast, during strong tetanisation, there is a strong depolarisation and the rise of the Ca^{2+} entry is fed through the NMDARs, VDCCs and Ca^{2+} from ICSs. Blocking one of these two last sources of Ca^{2+} there is no effect in LTP induction (Wilsh et al., 1998; Schiegg et al., 1995).

Experimental evidence has demonstrated the different spectrum of action of mGluRs in plasticity potentiation or inhibiting different types of Ca^{2+} channels. mGluRs have been shown to inhibit voltage –gated Ca^{2+} channels, such as N-type, L-type and P/Q channels. Occasionally reports describe the potentiation effect of mGluRs agonists on N-type and L-type voltage-gated Ca^{2+} channels. Interestingly, mGluRs could also have ability to sense physiological changes in the levels of extracellular Ca^{2+}, i.e. increase of external Ca^{2+} may activate mGluRs (Anwyl, 1999).

1.3.5.4 Expression of LTP: postsynaptic or presynaptic locus?

The synaptic locus of expression of LTP is a matter of strong debate among researchers in the field. A presynaptic expression of LTP may result from an increase in presynaptic transmitter release induced by a variety of mechanism. The existence of a postsynaptic locus for the expression of LTP suggests that there may be changes in the postsynaptic cell, leading to increased sensitivity of the cell to neurotransmitter. Moreover, the role of glial cells in reducing the uptake of glutamate, giving higher availability of glutamate at the receptors, may be taken in consideration as an extrasynaptic alternative locus for LTP expression (Bliss et al., 1993).

It is not completely verified if the increase of glutamate in the extracellular space after LTP induction is due to an increased release from the tetanised neurons or from other areas, such as glial cells. There is evidence supporting the role of the glial cells to decrease glutamate uptake rather then increase in release. The presence of presynaptic group II / III mGuRs and other evidence, such as the presence of presynaptic inhibitory glutamate receptors, using different agonists, may lead to the possibility of a presynaptic locus of expression of LTP. Also, paired –pulse facilitation, an indicator of presynaptic modulation of transmitter release, was enhanced by group II / III mGluRs agonists, in the CA1, mossy fibre-the CA3 and frontal cortex (Bliss et al., 1993, Bashir et al., 1994, Kamiya, et al.,

1997, Burke et al., 1994). In addition, the inhibition of mGluRs through ACPD did not show any postsynaptic changes (holding current, membrane potential, block of K^+ conductance, etc.) (Bashir et al., 1994, Burke et al., 1994, Glaum et al., 1992).

On the other hand, in this complex debate there is also strong evidence for a postsynaptic locus of LTP expression. It has been shown that applying ionophoretically AMPA receptor ligands, the sensitivity of the CA1 neurons increase slowly after LTP induction, providing evidence for a postsynaptic change. Experiments in vitro also shows LTP associated with increased AMPA binding (Sergueeva et al., 1993, Tocco et al., 1992).

Moreover, there is in the postsynaptic membrane, an intricate functional relationship between many of the neuronal protein phosphorylation systems, which allow "cross-talk" between distinct signals to take place in various brain cells. Therefore, the properties of protein phosphorylation systems at postsynaptic level may allow these regulatory systems to influence the expression of events taking place on a microsecond scale (e.g., neurotransmitter release), and or events lasting for hours and days (e.g., LTP).

1.3.5.5 Maintenance of LTP

The mechanism involved in the maintenance of LTP is complex. There is an intricate functional relationship between different proteins and second messengers, which allow "cross-talk" between distinct signals and can therefore contribute to the maintenance of LTP. Protein synthesis seems to be important, for at least the later stages of LTP since anisomycin, an inhibitor of protein synthesis, blocks late LTP in area the CA1 of hippocampus. It has also been proved that activation of class I mGluRs trigger *de novo* protein synthesis from existing mRNA promoting the persistence of LTP. The newly synthesised proteins promoted LTP stability are demonstrated by the synaptic priming experiments. In addition, it has been reported that a phase of dentate gyrus LTP in anaesthetised rats may involve transcription-independent protein synthesis. Interestingly, because proteins are locally synthesised (for instance in close proximity to the sites of mGluR activation), providing a means for rapid synapse-specific stabilisation of LTP, without requiring complex macromolecular trafficking (Frey et al., 1988, Raymond et al., 2000, Otani et al., 1989).

LTP can exist in short-term (early LTP) and long-term form. Whereas, the short-term forms appear to require modifications of pre-existing proteins, and the contribution of several

kinases, the long- term forms require RNA, protein synthesis in association with the establishment of new synaptic connections. Experiments with mutant mice deficient for Pim-1, a proto-oncogene which is induced rapidly after tetanisation to produce a nuclear and dendritically localised protein kinase, lost the ability to establish enduring LTP (Grant et al., 1994, Konietzko et al., 1999, Geinisman et al., 1996).
Quantitative analyses of synapse in the CA1 region and in the dentate gyrus have also shown that the long duration of synaptic enhancement, may be due to structural synaptic modifications, such as an increase of in the number of asymmetrical axodendritic synapses (Geinisman et al., 1996).

Although, several reports indicates that phosphatases and protein kinases, such as protein kinase C (PKC), CaM kinase II and cAMP-kinase (PKA) could contribute in the late phase of LTP maintenance.

1.3.5.5.1 Protein kinase C (PKC)

Protein kinase C (PKC) is a multigene family of at least ten isoforms, nine of which are expressed in brain (alpha, betaI, betaII, gamma, delta, straightepsilon, eta, zeta, iota /lambda).
The involvement of protein kinase (PKC) in the induction and maintenance of LTP has been tested by administering specific inhibitors during tetanisation.
Several studies indicate that LTP maintenance requires a sustained activation of PKC during the first hours following HFS (Klann et al., 1991), but the nature of this persistently active PKC is not clear. PKC is activated in a reversible manner by attachment to membrane phospholipid in the presence of Ca^{2+}. A small amount of diacylglycerol (DAG), a minor component of the cellular lipids, increases the affinity of this enzyme for Ca^{2+} and phospholipid. When DAG is transiently formed in the membrane, it permits the activation of PKC, and then is translocated from the cytosol to the membrane (Kawahara et al., 1980). In the hippocampal CA1, translocation of protein kinase C (PKC) activity from cytosol to membrane and subsequent phosphorylation of growth associated protein (GAP)-43 have been demonstrated to be critical events for the maintenance phase of LTP (Van Der Zee et al., 1997). Phorbol esters compete with DAG for the same binding site and activate PKC in

a similar fashion, increasing the enzyme's affinity for Ca^{2+}, causing translocation of PKC to membranes. Thus, it has been shown that transient application of phorbol ester activates PKC and increases presynaptic neurotransmitter release inducing an enhancement of synaptic transmission at synapses in the hippocampus in a manner that is similar to that of LTP (Ruttenberg et al., 1986, Malenka et al., 1986).

Other findings report that direct injection of PKC into hippocampal pyramidal cells induces LTP, indicating a postsynaptic role of PKC in LTP induction (Hu et al., 1987). Oleic acid, an unsaturated fatty acid, such as arachidonic acid (AA), also activated PKC enhancing LTP in hippocampal neurones (Linden et al., 1986). AA may also contribute synergistically with DAG to the translocation of PKC inducing an irreversible association of PKC with the membrane, due to the fact that AA reacts with the membrane resulting in a more hydrophobic form of PKC (Bramham et al., 1994, Linden et al., 1986).

Another mechanism of PKC activation that does not depend on second messengers, involves cleavage of the regulatory catalytic domains, generating an independent, constituently active catalytic domain, protein kinase Mzeta (PKMzeta). The conversion of PKC to PKMzeta seems be to increased in the cytosol during the maintenance phase of LTP, while the multiple PKC isoforms are transiently activated in the induction phase of LTP (Naik et al., 2000, Sacktor et al., 1993, Osten et al., 1996).

Several studies showed the block of LTP using PKC inhibitors during tetanus, such as polimixin B (Reymann et al., 1988a), H-7, K-252b (Reymann et al., 1988b), mellitin (Lovinger et al., 1987), K252a (Matthies et al., 1991), sphingosine (Malinow et al., 1988), staurosporine (Derry et al., 1990). However, there is criticism of these studies due to the non-specificity of the drugs, due to wide spectrums of action.

In the CA1 region, the induction of LTP is blocked by injection of specific inhibitors of PKC, such as $PKC_{19\text{-}31}$, a region of the regulatory domain of the PKC family, that represent a pseudo substrate antagonist of PKC activity (Malinow et al., 1989). However, contradictory results indicated that the PKC inhibitor H-7 did not inhibit LTP and by co-activation of two pathways in the presence of the PKC inhibitor staurosporine (Muller, et al., 1988, 1992).

Postsynaptic effects of PKC are likely to involve the modulation of the sensitivity of channels, such as calcium, potassium, chloride channels and receptors. It has been suggested that PKC mediates LTP via a modulation of the NMDA receptors by reducing the Mg^{2+} block (Cheng et al., 1992, Kaczmarek, 1987). In addition, K252b can also block the up-regulation of AMPA response following LTP (Reymann et al, 1990). Moreover, the use of PKCγ mutant mice to test hippocampal LTP showed deficits in LTP. This suggests PKCγ, as a 'key regulatory component and not part of the molecular machinery that produce LTP' (Abeliovich et al., 1993).

1.3.5.5.2 Calcium/calmodulin kinase II (CAMKII)

Calcium/calmodulin kinase II (the CAMKII) is a neuron-specific protein kinase, regulated by Ca^{2+} and calmodulin activity. It is believed to play a crucial role in synaptic plasticity in the hippocampus and is expressed at high level in the forebrain (mRNA for the alpha-subunit of CaMKII is abundant in dendrites of neurons in the forebrain) and cerebellum, making up ~ 2% of total protein in the hippocampus. In addition, it is highly concentrated in both postsynaptic and presynaptic compartments. Elevation of intracellular Ca^{2+} can generate autophosphorylation of the CAMKII at threonine-286 producing Ca^{2+} - independent kinase activity and playing a crucial role in LTP. The CAMKII can then act as a molecular detector, recording the occurrence of a previous Ca^{2+} transient (Otani et al., 1993, Kennedy et al., 1989, Ouyang et al., 1997, Steward, 1997, Benfenati et al., 1992).
It has been demonstrated that tetanic stimulation of the Schaffer collateral pathway causes an increase in the concentration of alpha-CaMKII in the dendrites of postsynaptic neurons. The increase is blocked by anisomycin and it has been detected by both quantitative immunoblot and immunocytochemical techniques (Ouyang et al., 1999). Rymann et al, (1988) showed that calmidazolium, a CAMKII blocker, blocked both the initial and long term increase in EPSP following tetanus. The CAMKII inhibitor, KN-62, also blocked LTP induction. In addition, postsynaptic injection of the membrane–impermeable CAMKII inhibitor peptide, $CAMKII_{273-302}$, and calmodulin-binding peptides, CBP and CBP3, has been demonstrated to block LTP (Malenka et al., 1989).
Extensive biochemical and molecular studies have identified sites of the CAMKII phosphorylation (and also of PKC and PKA) in the C-termini of the GluR1 and 4 subunits.

GluR1 phosphorylation seems to be bidirectionally altered during long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus. The CAMKII seems to be necessary in recruitment and/or clustering of AMPA receptors in the spines and phosphorylation may be required for expression of functional AMPAR in the PDS to form functional synapses (Fukunaga et al., 2000).

The CAMKI via synapsin I, which is a synaptic vesicle-associated phosphoprotein that is located in the presynaptic sites, is shown to modulate neurotransmitter release. Ca^{2+}/calmodulin-dependent protein kinase II, which phosphorylates two sites in the carboxy-terminal region of synapsin I, causes synapsin I to dissociate from synaptic vesicles and increases neurotransmitter release. Conversely, inhibition of neurotransmitter release is triggered by dephosphorylated form of synapsin I, but not the form phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (Benfenati et al., 1992). Taken together, interaction of the CAMKII with cytoskeletal proteins and interaction with scaffold proteins and receptors are potentially important mechanisms in the reorganisation of dendritic morphology and recruitment of glutamate receptors in the spines.
Experiments with the use of targeted mutation of the α-the CAMKII gene provides strong evidence for involvement of the enzyme in LTP, as well as in spatial learning, and explicit memory in the CA1 area of the hippocampus (Silva et al., 1992a, 1992b).
Furthermore, in the CA1 pyramidal neurons, infusion of CaMKII via the recording pipette resulted in an increase of synaptic transmission, greatly diminished by prior induction of LTP. Thus, tetanic stimulation failed to induce LTP. These finding may suggest a simple switch model of LTP in which the CAMKII alone is sufficient to induce LTP (Ledo et al., 1995).

1.3.5.5.3 Protein kinase A (PKA) and cAMP

cAMP is an ubiquitous second messenger, which exerts most of its effect via activation of cAMP-dependent protein kinase (PKA). PKA is composed of regulatory (R) and catalytic (C) subunits. In mice the C subunits are of two gene types: Calpha and Cbeta. It has been demonstrated that mice lacking the Cbeta1-subunit can produce synaptic potentiation by HFS in the CA1 region, but they cannot maintain the synaptic potentiation (Qi et al., 1996).

Additional evidence of their possible role in the LTP maintenance derives from studies using transgenic mice expressing an inhibitory form of the regulatory subunit of PKA where able to express the early phase, but not the late phase of LTP. Other studies reported that mice lacking both types 1 and 8 calcium-activated adenylyl cyclase deficiets in the late phase of LTP (Abel et al., 1997, Wong et al., 1999). These results show that cAMP and PKA are important mediators of the late phase of LTP. cAMP signalling may also underlie gating the CAMKII activation in LTP through protein phosphatase 1 (PP-1). This is explained by the fact that tetanisation trigger cAMP-dependent phosphorylation of inhibitor 1 causing a decrease of PP-1 activity. Since PP-1 in the PSD is contributing to keeping the CAMKII in a dephosphorylated state, the decreased PP-1 activity may increase autophosphorylation of the CAMKII in LTP (Blitzer et al., 1998).

It has also been shown that inhibitors of PKA and of transcription block synthesis of AMPA receptors. AMPA receptor synthesis is increased as a result of PKA-dependent gene transcription, thus PKA may play an important role for the maintenance of AMPA receptors mediated responses and then for the LTP maintenance (Nayak et al., 1998, Frey et al., 1993). One nuclear targets of PKA is the cyclic AMP response element binding protein (CREB). It has been suggested that PKA phosphorylates CREB, activating a cascade of changes in gene expression that may induce LTP. This has been demonstrate through the use of mice lacking the α and δ isoforms of CREB showing a deficit in the late phase of LTP (Bourtchuladze et al., 1994).

In the CA3 region, PKA appears to play a role in both early and late phase LTP. PKA activators, such as SP-cAMPS, were found to induce both early and late phase LTP, while PKA inhibitors blocked LTP. LTP induced by the presence of PKA activators was associated with a change in paired -pulse facilitation, indicating an increase in presynaptic neurotransmitter release (Weisskopf et al., 1994).

1.3.5.6 Retrograde messengers

As stated above, in the CA1 region of the hippocampus, LTP seems to be induced postsynaptically and, at least in part; its expression depends on presynaptic mechanisms. Therefore, a retrograde messenger that is released from the postsynaptic dendrite and diffuses back across the synapse to increase neurotransmitter release has been proposed. There are several candidates that have been considerate as retrograde messengers, such as lipid mediators, including arachidonic acid (Izumi et al., 2000) and platelet-activating factor (Kato et al., 1994), and gases, such as nitric oxide (NO) (O'Dell et al., 1991, Von Bohlen et al., 2002, Bon et al., 2001) and carbon monoxide (CO) (Stevens and Wang, 1993, Zhuo et al., 1993). The involvement of these intercellular messengers in LTP, the relation between LTP and memory and the role of these candidate retrograde messengers in the acquisition and consolidation of memories are still discussed and are a matter of controversy.

1.3.5.7 Protein tyrosine kinases (PTKs)

There are two main groups of PTKs: one is a family of receptors for cellular growth factors, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF); and the other including a family of cytosolic, nonreceptor kinases (src- and fyn-related PTKs). Tyrosine phosphorylation of the NMDA receptors by src-family tyrosine kinases, such as Fyn is implicated in synaptic plasticity (Nakazawa et al., 2001).

Protein tyrosine phosphorylation is thought to play an important role in the regulation of neuronal function. Previous work has shown that protein tyrosine kinase (PTK) inhibitors can inhibit the induction of long-term potentiation. However, how PTK activity may contribute to LTP induction remains elusive.

Directly activating Src in the postsynaptic neuron enhanced excitatory synaptic responses, occluding LTP. Src-induced enhancement of alpha-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) receptor-mediated synaptic responses required raised intracellular Ca^{2+} and NMDA receptors. Thus, Src activation is necessary and sufficient for inducing LTP and may function by up-regulating the NMDA receptors (O'Dell et al., 1991).

Reports demonstrated that tyrosine kinase inhibitors, herbimycin A and lavendustin A, block formation of long-term potentiation in the dentate gyrus in vivo (Abe et al., 1993). Recent evidence has also shown that brain-derived neurotrophic factor (BDNF) is involved LTP, and in particular in the late-phase of hippocampal long-term potentiation (Korte et al., 1998).

1.3.6 Long –term depression (LTD)

There is another form of plasticity that has not been discussed: long-term depression (LTD).

Long-term depression (LTD) comprises a persistent activity-dependent reduction in synaptic efficacy, which typically occurs following repeated low frequency afferent stimulation (Bear and Abraham, 1996, Dudek and Bear 1992, Mulkey and Malenka 1992). Hippocampal LTD has been a subject of particular interest due to the established role of the hippocampus in certain forms of information storage and retrieval. The CA1 LTD expression may also be increased in stressful conditions. This suggests a more complex role for this form of plasticity than the oft-cited hypothesis that it simply serves to prevent synapse saturation, by means, for example, of enabling reversal of long-term potentiation (LTP). One possibility is that LTD may be directly involved in the creation of a memory trace. Alternatively, LTD may prime a synapse in readiness for the expression of LTP, thereby contributing indirectly to information storage. There is increasing evidence that LTD is not mechanistically the reverse of LTP. Although some common processes exist, molecular, biochemical, electrophysiological and pharmacological studies all point to several quite distinct induction and maintenance mechanisms for this form of synaptic plasticity.

The inclusion of LTD in synaptic transmission may have several advantages. Firstly, it helps prevent saturation of LTP that otherwise would prevent further plasticity. Secondly, LTD increasing the signal-to-nose ratio at neighbouring synapses can enhance the effect of LTP. Thirdly, LTD gives more flexibility of the circuit remodelling. Finally, LTD helps

limit the overall degree of postsynaptic excitation that may lead to neurological disorders (Christie et al., 1994).

Lynch et al. (1977) were the first to report a depression of synaptic response in the CA1 region of the hippocampus. This study demonstrated that induction of LTP in one pathway resulted in a reversible depression in the non-tetanised pathway. Other evidence of this form of LTD, termed heterosynaptic LTD, was also demonstrated in the dentate gyrus in vivo (Levy and Steward, 1979).

Another form of LTP that is most widely studied is termed homosynaptic depression, i.e. depression only in the pathway receiving the induction protocol. It was first described when it was shown that low frequency stimulation reversed stable LTP in the Schaffer Collateral CA1 pathway of the hippocampus (Barrionuevo et al., 1980). This phenomenon is now known as depotentiation (DP) and was induced by prolonged low frequency (1Hz, 900 pulses) stimulation and like LTP was found to be input specific (Dudek and Bear 1992, Mulkey and Malenka 1992).

The ability of synapses to undergo homosynaptic LTD has been established in many brain regions other than hippocampus, including the visual cortex (Artola et al., 1990), the striatum (Calabresi et al., 1992), amygdala (Wang et al., 1999), cerebellar cortex (Ito 1989), DG (Levy and Steward et al., 1979, Trommer et al., 1996, Wang et al., 1997), and the CA3 (Bradler et al., 1989, Yokoi et al., 1996).

In the hippocampus there are two distinct forms of long-term depression (LTD) of excitatory synaptic transmission. In the CA1 region, prolonged low-frequency stimulation induces LTD by activating postsynaptic NMDA receptors, which causes a moderate rise in Ca^{2+} concentrations. In mossy fiber synapses of the CA3 region, similar low-frequency stimulation also gives rise to LTD. However, this form of LTD (mossy fiber LTD) does not require activation of the NMDA receptors, but is mediated by activation of presynaptic metabotropic glutamate receptors. Induction of mossy fiber LTD is not dependent on postsynaptic depolarization or activation of postsynaptic ionotropic glutamate receptors, thus it is likely to be mediated by purely presynaptic mechanisms. This conclusion is confirmed by the analysis of mutant mice lacking presynaptic mGluR2, in which mossy fiber LTD is almost absent (Yokoi et al., 1996). Since long-term potentiation at mossy fiber synapses is also induced presynaptically, the synaptic efficacy may be regulated through common mechanisms bidirectionally, which may contribute to neural information processing in the hippocampus (Domenici et al., 1998, Yokoi et al., 1996).

1.3.6.1 Induction of LTD

There are two main methods of LTD induction. In the first, LTD is induced by prolonged low frequency stimulation (LFS; 1~5 Hz, 900 pulses) (Dudek and Bear 1992, Mulkey and Malenka 1992). The second method involves the paring of a brief LFS (1~10 Hz, 20-60 pulses) with a mild postsynaptic depolarisation (-40~-50mV) (Cummings et al., 1996).

1.3.6.2 AMPA receptors and LTD induction

AMPA receptor internalisation during the expression of hippocampal LTD may be regulated by Ser-880 phosphorylation and GluR2 interaction with PDZ domain-containing proteins. Reports indicate that the LTD-induced increase in Ser-880 phosphorylation was blocked by perfusing hippocampal slices with AP5 or the protein phosphatase inhibitor, okainic acid, two agents that known to inhibit LTD (Kim et al., 2001).
Activation of AMPARs is essential in LTD induction, as is demonstrated by the fact that the blockade of the NSF-GluR2 interaction by a specific peptide (pep2m) introduced into neurons, prevented homosynaptic long-term depression (LTD). Therefore there may be a pool of AMPARs dependent on the NSF-GluR2 interaction and LTD expression may involve the removal of these receptors from synapses (Luthi et al., 1999).

1.3.6.3 NMDA receptors and LTD induction

Experiments in vitro and in vivo indicate the role of the NMDA receptor in LTD. In vitro experiments demonstrated that in the CA1 area of the hippocampus, induction of LTD induced by LFS was dependent on the synaptic activation of the NMDA receptors (Dudek and Bear 1992, Mulkey and Malenka 1992). Subsequent researches have described

NMDA receptor-dependent forms of LTD in different region of the brain, such as in the hippocampus (Kemp et al., 1997, 2001) and visual cortex (Kirkwood et al., 1995).

The application of NMDA by itself has been demonstrated to induce lasting synaptic depression (Lee et al., 1998). Also, in vivo experiments show the role of the NMDA receptors in LTD (Thiels et al., 1994).

Moreover, mice lacking the NMDAR ε2 subunit were found to be deficient in LTD induction in the CA1 region (Kutsuwada et al., 1996). However, LTD in the dentate gyrus seems to be the NMDAR-indipendent, because D-AP5 did not block LTD induction in the medial perforant pathway of the dentate gyrus (Trommer et al., 1996, Wang et al., 1997).

1.3.6.4 Metabotrobic glutamate receptors and LTD induction

There is strong evidence for a role of mGluR in the induction of (LFS)-induced LTD in the hippocampus, neocortex, striatum and cerebellum (Anwyl 1999). Furthermore, the use of mutant mice with mGluR2 knockout showed no LTD in the CA3 region, suggesting a role of group II mGluRs at the mossy fibre-the CA3 synapse (Yokoi et al., 1996).

Initial reports in the CA1 area showed an inhibition by MCPG and L-AP3 of LFS-induced LTD in vitro and in vivo (Stanton et al., 1991, Manahan-Vaughan et al., 1996).

In the CA1 there are two forms of LTD: the first form, which is mGluR-dependent and is blocked by mGluR antagonists, such as AIDA and MCPG (NMDAR-indipendent form); and the second form is mGluR-independent and NMDAR-dependent (Oliet et al., 1997).

In the dentate gyrus evidence exists for a role of group II mGluRs in LTD, because both MCPG and the group II mGluR antagonist MCCG have been reported to prevent the induction of LTD (Wang et al., 1997). Participation of group II mGluRs was also shown by the ability of its selective agonists DCDIV and LY354740, to induce directly LTD in the dentate gyrus (Huang et al., 1997). Other evidence for the involvement of mGluRs in one form of LTD came from studies in which perfusion of ACPD induced LTD/DP in the CA1 in young animals (Overstreet et al., 1997). In addition, the group I mGlu receptor antagonist AIDA blocks LTD in the dentate gyrus in vitro (Camodeca et al., 1999).

1.3.6.5 Role of Ca^{2+} in LTD

The trigger for postsynaptically induced, activity-dependent LTD is mainly an increase in postsynaptic Ca^{2+}. It has been shown that LTD induction is blocked by intracellular injection in the postsynaptic neurone of either EGTA or BAPTA [bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate], two different Ca^{2+} chelators (Brocher et al., 1992). Because the postsynaptic rise of Ca^{2+} is implicated in both LTP and LTD, it is assumed that particular properties of the Ca^{2+} signal (temporal, spatial, or magnitude) may determine weather LTP or LTD results. For this reason it has been proposed that a moderate Ca^{2+} influx into the postsynaptic cell is necessary for LTD induction while a strong Ca^{2+} influx is necessary for the LTP induction (Lisman, 1989). In the dentate gyrus, Wang et al. (1997) demonstrated that at low extracellular Ca^{2+} (0.8mM), there was a partial reduction of LTD while LTP was completely blocked. Studies have suggested that during mGluR receptor-dependent LTD a moderate rise (to ~500nM) was sufficient for the induction of LTD (Otani et al., 1998). Other approaches showed that train of stimuli that normally induce LTP, induce LTD during reduced activation of the NMDA receptor (using AP5) (Cummings et al., 1996). Consequently lowering activity-dependent rises in postsynaptic calcium prevents LTP, but allows LTD induction.

It is also important to consider the temporal parameter, i.e. a very short-lasting Ca^{2+} elevation, if large enough, induces LTP, whereas a long-lasting, moderate Ca^{2+} elevation, is required to induce LTD. Therefore a brief period of Ca^{2+} elevation will not induce LTD but only LTP (Mizuno et al., 2001).

The rise of postsynaptic calcium that results in LTD comes from a variety of sources, such as voltage–gated Ca^{2+} (VGCCs), and mGlu receptor-mediated release of Ca^{2+} from intracellular stores. LTD was found to be blocked by agents that prevent IP_3 mediated release of Ca^{2+} from intracellular stores (Wang et al., 1997). Also, the NMDA receptor-dependent calcium rise was reduced by the use of thapsigargin that depletes intracellular calcium stores (Alford et al., 1992).

1.3.6.6 Intracellular messengers in LTD

Protein phosphatases and CAMKII

As stated previously, Ca^{2+} ions triggered the mechanism of induction of LTD. It is well known that Ca^{2+} ions bind to calmodulin to form a calcium-calmodulin complex that can activate the CAMKII and calcium-calmodulin dependent protein phosphatase (PP2b: calcineurin). A moderate increases in postsynaptic Ca^{2+} with LFS could activate the phospatase, calcineurin PP2b, via the calcium -calmodulin-complex. PP2b may dephosphorylate and inactivate inhibitor 1, activating posphatases (PP1/2) and consequently inducing LTD via dephosphorylation of AMPA receptors or other substrates. Evidence of such mechanism derives from a report showing that LTD was blocked by the use of protein phosphatase 1 and 2A inhibitors, okadaic acid (Mulkey et al., 1993).
In addition, calcineurin inhibitor FK506 blocks or reduces LTD and calcineurin-deficient mice show a lower threshold for LTD induction (Mulkey et al., 1994, Ikegami et al., 1996). Evidence of the involvement of tyrosine kinases is suggested by results where tyrosine kinase inhibitors have been shown to block LTD in the cerebellum and the dentate gyrus (Boxal et al., 1996, Camodeca et al., 1999).

LTD in the CA1 region, which depends on group I mGlu receptor activation, also relies on the activation of PKC. Thus injection of PKC inhibitor peptide, $PKC_{19\text{-}36,}$ blocked LTD in this area (Oliet et al., 1997, Bolshakov et al., 1994). However, the role of PKC in the induction of LTD is still not clear.

Also, PKA seems to be involved in the mechanism of LTD induction as is shown with the use of PKA inhibitors H-89 and KT5720 partially inhibited LTD induction in the medial perforant path of the dentate gyrus in vitro, suggesting that the induction of LTD by LFS involves activation of PKC and PKA following activation of group I and group II metabotropic glutamate receptors (mGluR). Also, the application of PKA blocker KT5720

was found to block LTD in the CA1, suggesting that the activation of PKA is required in LTD (Huang et al., 1999, Brandon et al., 1995, Kameyama et al., 1998).

1.3.7 Metaplasticity

Activity-dependent modulation is an important component in the current understanding of the cellular mechanisms underlying the learning and memory functions of the brain (Abraham and Tate, 1997, Abraham and Bear, 1996). Metaplasticity has been suggested generally to involve some previous synaptic activity that results in a change in the capabilities of synapse to undergo subsequent plasticity (Bear, 1995). Such temporal plasticity of synaptic plasticity can last from minutes to hours and is present in the absence of any discernible effects on baseline levels of synaptic transmission.

Thus, it is becoming increasingly apparent that the induction of synaptic plasticity is sensitive not only to the 'state' imposed by coactive afferents and circulating hormones, but also to the state generated by prior patterns of pre- and postsynaptic activity. This means that this is one way in which synaptic activity can leave a lasting trace.

Let us consider, for instance, the effect of a short burst (30Hz, 150ms) of synaptic stimulation in area CA1 of the hippocampus. By itself, such a burst causes only a transient short-term potentiation (STP) of evoked responses that decays rapidly back to baseline. However, long-lasting effects of this seemingly innocuous activity become apparent during subsequent attempts to induce synaptic plasticity. These effects include both an inhibition of long-term potentiation (Huang et al., 1992), and a facilitation of long-term depression (Holland and Wagner, 1998; Christie and Abraham, 1992; Wagner and Alger, 1995). Similar examples can be found in a variety of neural systems, all indicating that synaptic plasticity can be modulated, sometimes dramatically, by prior synaptic activity. This plasticity has been called 'metaplasticity', which corresponds in meaning to terms such as 'metacognition' (knowledge about one's cognitions) and 'meta-analysis' (a higher order analysis of the results from many other studies or analyses). In the present case, the prefix 'meta-'(Greek for 'beyond' or 'above') is used to indicate a higher level of plasticity, expressed as a change or transformation in the way synaptic plasticity is modified. An

understanding of metaplasticity might yield new insights into how the modifications of synapses are regulated and how information is stored by synapses (Bear, 1995).

It is important to note that metaplasticity is a real phenomenon, as shown by data obtained in the mammalian hippocampus, and that it does not describe a single phenomenon. Rather it is a term, which encompasses a family of effects, which together can set up unique states for individual synapses that regulate the plasticity elicited by subsequent stimuli. While the mechanisms underlying the various effects are not well understood, it is possible to present the evidence as it stands, discuss some likely mechanisms and predict some useful avenues for future research.

Metaplasticity has occurred if prior synaptic or cellular activity (or inactivity) leads to a persistent change in the directions or degree of synaptic plasticity elicited by a given pattern of synaptic activation. Metaplasticity occurs without concurrent changes in synaptic efficacy but, in principle, metaplasticity and synaptic modifications can also be induced simultaneously by the same synaptic activity.

1.3.7.1 Metaplasticity and LTD

Significant examples of metaplasticity are those where prior activity facilities the induction of LTD. When the prior activity induces LTP first, then this facilitated LTD is called depotentiation.

It has been reported that while low-frequency stimulation (1Hz, 100pulses) rarely depressed baseline synaptic transmission in hippocampal area CA1, it could nonetheless depotentiate LTP established 15 min earlier (Barrionuevo et al., 1980). This initial report using anaesthetised rats was subsequently replicated in awake animals, using trains of 1-5Hz stimuli to depotentiate CA1 responses (Staubli and Lynch, 1990).

It has also been found that the induction of NMDAR-dependent LTD is enhanced or inhibited by previous HFS (Holland and Wagner, 1998; Christie and Abraham, 1992; Wagner and Alger, 1995; Wang et al., 1998; Rush et al., 2002, [in press]).

Moreover Bashir and Collengridge (1994) demonstrated that if LTP was first induced in the pathway then LFS generated a depotentiation of LTP. And interestingly, the magnitude of depotentiation was reversibly reduced, by the specific metabotropic glutamate receptor

(mGluR) antagonist MCPG, showing an mGluR-dependent depotentiation. Other studies have described the same effect of an enhancement of LTD by prior LTP (Fujii et al., 1991; O'Dell et al., 1994, Yang et al., 1991, Huang et al., 1999). These results taken together represent a clear demonstration that the modifications in synaptic transmission caused by a certain tetanizing protocol depend upon the history of synaptic efficacy. The history of synaptic efficacy is not necessarily represented by prior LTP induction, because there is evidence showing that the preconditioning stimulation might be represented by a stimulation that is subthreshold for inducing LTP, i.e. short trains of 5-30 Hz can also facilitate LTD (Christie et al., 1992; Wexler et al., 1993). Moreover, a direct action on receptors involved in synaptic plasticity like mGluRs has been shown to modify the degree of LTD. Experiments performed in CA1 of hippocampal slices showed how priming activation of mGluRs can regulate LTD. Activation of Group II mGluRs with DCGIV application, but not group III, and I significantly inhibited the LTD by >50% (Melletin et al., 2001). Li et al (1998) also demonstrated that the application of the group II mGluR antagonist, EGLU prevented the HFS-dependent switch from synaptic facilitation to depression in the *in vitro* amygdala slice. To explain these last results, it has been hypothesised that an alteration of the sensitivity of presynaptic mGluRs by HFS persistently sensitises these autoreceptors, so that there is feedback inhibition, leading to a synaptic depression. In naïve slices generally group II mGluR do not regulate glutamate release. HFS might therefore act as a primer inducing a switch from facilitation to depression (Li et al., 1998). Another report also demonstrates that depotentiation of LTP by prior LFS (the stimulus was delivered 1 or 10 min after LTP induction) involves group II mGluR at the mossy fiber synapses onto CA3 pyramidal neurons. This LFS-induced depotentiation appeared to be mediated by the activation of group II metabotropic glutamate receptors (mGluRs), because it was mimicked by the bath-applied group II agonist (2S,2'R,3'R)-2-(2', 3'-dicarboxycyclopropyl) glycine and was specifically inhibited by the group II antagonists (S)-alpha-methyl-4-carboxyphenylglycine and (alphaS)-alpha-amino-alpha-(1S,2S)-2-carboxycyclopropyl-9H-xanthine-9-propanic acid (Chen et al., 2001).

The involvement of PKC on the priming of LTD has been demonstrated by the work of Wang et al (1998). In this report the effect of PKC activation was investigated by the application of a low concentration of the selective PKC activator (-)-indolactam V (25μM), applied intracellularly via the patch pipette, which enhances the induction of LTD by priming stimulation, and directly inducing LTD. Boxall et al also demonstrated a direct

induction of LTD by stimulation with (-)-indolactam V in the cerebellum (Boxall et al., 1996). In other studies extracellular application of the PKC activator phorbol 12,13-diacetate, which largely enhances synaptic transmission of field EPSPs, showed enhanced homosynaptic LTD in CA1 (Stanton, 1995). This evidence represents a demonstration of the interaction between NMDAR, mGluR, second messengers and therefore the existence of intracellular signalling pathways acting to modulate LTD induction, by previous synaptic activity.

1.3.7.2 Metaplasticity and LTP

There are several other examples of the priming effect on subsequent LTP induction, for examples in the work of Coan et al. (1989) which showed that LTP could not be produced in CA1 region when hippocampal slices were bathed in a nominally Mg^{2+} - free medium. Initially this effect was considerably paradoxical, because by removing Mg^{2+} from the solution they were expecting an LTP increase. Instead, it appeared that the activation of NMDA receptors by baseline test pulses inhibited subsequent LTP. More recent investigations in the CA1 region have confirmed the results discussed above. Also in a series of experiments Huang et al. (1991), induction of LTP by a strong tetanus was inhibited if weak tetani were previously delivered in CA1 of the hippocampus. The effect lasted at least 30 min (less than a hour). This inhibition of LTP was dependent on the activation of NMDA receptor, because normal LTP occurred when AP5 was present during weak tetani. This result could suggest that low-level activation of NMDA receptors in CA1 generates a plastic change that inhibits the subsequent induction of LTP.

O' Dell et al (1994) also find that 5Hz stimulation delivered immediately prior to high-frequency stimulation inhibited the subsequent induction of LTP in the CA1 region of the hippocampus. Moreover, the protein phosphatase inhibitors okadaic acid and calyculin A blocked the subsequent inhibition of LTP by 5Hz stimulation, suggesting that phosphatases play a role in the mechanism of inhibition of LTP.

Furthermore, significantly reduction of the long-term potentiation (depotentiation) was observed by the delivery of a train of low-frequency afferent stimuli (depotentiating stimulation: DPS) before HFS-induced LTP in CA1 neurons of the guinea pig's hippocampal slice. The inhibitory effect on LTP by prior application of DPS was reversed by applying APV during DPS, indicating an important role of NMDAR in the depotentiation of LTP (Fujii et al., 1991).

Interestingly stimulation patterns that facilitate subsequent LTD are also inhibiting LTP (Christie et al., 1992; 1995, Stanton et al., 1995).

Also the use of stimulation patterns above threshold for LTP has been used to inhibit LTP. Frey et al (1995), for instance, demonstrated that prior induction of LTP both in the dentate gyrus in vivo and in the CA1-region in vitro suppress further LTP for the duration of about four hours. Because after this time, the system was still able to produce further potentiation Frey suggested that instead of saturating LTP per se, a mechanism of inhibition of LTP was induced (Frey et al., 1995). Other studies also reported that an excessive number of stimulation trains i.e. 24 or 32 trains of theta-burst stimulation (TBS) produced an "over-stimulation" that inhibited LTP. The effect of this over-stimulation was input specific and dependent on the activation of NMDA and A1 receptor (Abraham et al., 1997).

Several studies also show a facilitation of LTP, by prior activity (Christie et al., 1995, Bortolotto et al., 1994; Cohen et al., 1996; 1999; 1996; Hsu et al., 1999, Huang et al., 2001).

Experimental evidence showed that in the lateral perforant path of the dentate gyrus LTP induction was facilitated by administering five Hz "priming" stimulation (80 pulses, 5Hz) 10 min prior to the conditioning trains. This priming was input specific and was blocked by the muscarinic receptor antagonist atropine sulphate (Christie et al., 1995).

There is direct evidence for mGluRs facilitation of subsequent LTP. Therefore, in order to gain a better understanding of the modulation of synaptic plasticity by priming effect, it is also important to take into consideration the theory formulated by Bortolotto et al. (1994), of the 'molecular switch' (or mGluR 'priming') activated by mGluRs. In this theory mGluRs activation sets a 'molecular switch' that is necessary for persistent LTP occurrence. Such activation could occur prior to HFS-induced LTP and still be effective after tetanisation (Bortolotto et al. 1994). The switch can then stay turned on and facilitate LTP for at least 7 hours, and it can be turned off or reset by LFS (Bortolotto et al.1995). However, these results have been controversial because some reports support the role of mGluRs in LTP (Bliss and Collingridge, 1993), while other studies could not replicate the finding that mGluRs are necessary for the maintenance and induction of LTP (Selig et al., 1995).

Significant findings supporting an important role of mGluRs in metaplasticity are represented by the work of Cohen et al (1996) where a 10-min application of the mGluR

agonist, ACPD, 30 min before the TBS facilitated the induction of LTP in a dose-dependent manner and resulted in an enhanced magnitude and stability of LTP in CA1 (Cohen et al., 1996; 1999). In further studies the receptor specificity and the second messenger pathways involved in the mGluR has also been investigated. It has been demonstrated that phospholipase C coupled to group I mGluRs, are involved in the facilitation of LTP (Cohen et al., 1998).

Taken together the data might raise a question about the mechanisms underlying the process of metaplasticity. However, the mechanisms responsible for metaplasticity remain to be fully characterized, and from the results described above some possibilities are dependent on mGluR and NMDA receptor activation, the increases in intracellular Ca^{2+}, and altered states of protein kinases or phosphatases and other second messengers that interact to increase or decrease synaptic plasticity. Several studies have also demonstrated that protein synthesis inhibitors blocked LTP 2-8 hours after induction. In particular, anysomicin, applied 15 min before HFS, induced a decremented LTP with unchanged peak amplitude immediately after stimulation, but which decayed progressively back to baseline within 2-8 hours (Otani et al., 1992; 1989; Frey et al., 1988).

1.3.7.3 Receptors and the role of Ca^{2+} in metaplasticity

Because of their close proximity, group I mGluRs and NMDARs may be structurally cross-linked via a molecular scaffolding at CA1 synapses in the PDS. Since homer proteins link via Shank to NMDARs and signalling molecules such as PLC and PKC, such scaffolding structure would provide a strategic position for this two receptors (NMDAR and mGluR) to be co-activated in the inhibition of LTP by preconditioning stimulation (Tu et al., 1999, O'Connor et al., 1994). However, it is possible that other signalling pathways, for example cholinergic muscarinic receptors activation, might work together with mGluR activation to regulate the plasticity in the brain (Abraham et al., 1995).

Modulation of NMDA-receptor activation, or biochemical sequelae to Ca^{2+} entry, is likely targets for metaplasticity expression. Thus, it is possible to divide the probable sites of metaplasticity into two broad categories: (1) those processes that regulate the rise in postsynaptic $[Ca^{2+}]$ and (2) the downstream processes that are activated by the rise in

[Ca^{2+}]. As most Ca^{2+} influx is voltage-dependent, one powerful site of regulation might be the regulation of K^+ channels, which could indirectly affect NMDA receptor function by modulating postsynaptic excitability (Ben-Ari et al., 1992).

Other targets of regulation include the NMDAR, and the postsynaptic Ca^{2+} dynamics that results from activating them (Gold et al., 1994). Ca^{2+} diffusion can also be altered, as studies demonstrate, by structural changes in dendrites spines. Preconditioning stimulation might also modify the storage or release of [Ca^{2+}] in response to afferent stimulation by modulation the Ca^{2+} channels or pumps in the endoplasmatic reticulum (Holmes et al., 1990, Ben-Ari et al., 1992; Gold et al., 1994). Furthermore, up-regulation or down-regulation of NMDAR seems to be responsible for the modulation of synaptic plasticity as is shown by previous reports where a depression of NMDAR-mediated responses occurred after LFS-induced LTD (Xie et al., 1992), and up-regulation of NMDAR was observed by tetanisation that produced also LTP (Bashir et al., 1991; Clark et al., 1995). In addition, application of mGluR agonist ACPD can directly cause an enhancement of about 30 min of the isolated NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) and induce a depression of the GABA-mediated EPSPs that should in turn indirectly enhance NMDAR responses (O'Connor et al., 1994; Dudek et al., 1993). NMDAR function should be then a target for a better understanding of the metaplasticity mechanism.

NMDA receptors seem to exert effects in subsequent synaptic plasticity that are in the opposite direction to those exerted by mGluRs. Thus, probably pre-activation of NMDA receptor shifts synaptic plasticity away from LTP (inhibition of LTP) towards LTD, whereas mGluRs seems to predispose synapses towards LTP (LTP facilitation) (Selig et al., 1995, Cummings 1996, O'Leary et al., 1998). Consequently, it seems that separate NMDA receptor and mGluR activation produces different forms of metaplasticity. As an alternative suggestion, there might be a reciprocal feedback interaction between these two receptors that may play a role in synaptic transmission and in the modulation of the direction of synaptic plasticity (Alagarsamy et al., 1999; Luthi et al., 1994). Another factor that might be regulated by these two receptors and also by kinases (PKC, PKA or even MAP kinase) and might then be a target of metaplasticity is the phosphinositide turnover (PI) and also inactivation/desensitisation of mGluRs that might lead to a decrease of synaptic plasticity (Guerineau et al., 1997; Gereau and Heinemann, 1998). There is experimental evidence demonstrating that group I and group II mGluRs present at high concentration in the medial perforant path-granule-cell synapses path (Shigemoto et al., 1997) synergistically interact to stimulate PI hydrolysis in the hippocampus (Schoepp et al., 1996, 1998). To confirm the

important role of mGluRs in metaplasticity, it has previously been reported that the activation of mGluRs prior to HFS enhanced the amplitude of subsequent LTP in CA1 (Cohen and Abraham, 1996) and the activation of group II mGluR inducing a preconditioning HFS altered the response to LFS from the induction of LTP to LTD in the amygdala (Li et al., 1998). However, there are other factors to be taken into consideration, such as hormones, neurotransmitters, enzymes activation, Ca^{2+} accumulation, protein synthesis (Jeffery et al., 1990; Huang and Kandel 1994; Nilsen et al., 2002; Zou et al 2001; Chepkova et al., 2001; Vouimba et al., 2000; Harley et al., 2000), membrane depolarisation, etc., that might contribute to favour in the end, LTD or LTP.

1.3.7.4 Biochemical process that trigger the metaplasticity phenomena

Metaplasticity is also regulated, as is stated above, by biochemical processes. For the regulation of the components downstream following an elevation in $[Ca^{2+}]$ there are numerous possibilities involving the actions of a network of protein kinases and phosphatases (Malenka et al., 1994, Bear et al., 1994). For instance it has been demonstrated that in the hippocampus, transient protein kinase C activation primes LTD and inhibits LTP (Stanton, 1995, Zurner et al., 2000). Another report showed also, that activation and autophosporylation of Ca^{2+}/calmodulin kinase II generates changes (negative charge) in the local electrostatic potential sufficient to hyperpolarize the PDS, and affect the direction of synaptic plasticity. This results in an inhibitory electrostatic effect on subsequent potentiation. However, the target of the synaptic control regulated by CAMKII, seems to be unclear (Tompa et al., 1998). Another candidate that might play an important role in the 'priming event' is the IP_3 receptor. These receptors are calcium- sensitive and the release of Ca^{2+} from ER is a mechanism for amplifying calcium channel-mediated rises of intracellular free calcium. Since IP_3 receptor activity is modulated by phosphorylation sites on the cytoplasmic surface which might recognise PKA and PKC, it means that these activation states may be regulated by prior synaptic activity i.e. they might be a potential site for the regulation of metaplasticity (Obenaus et al., 1989; Behnisch et al., 1995).

Therefore, metaplasticity seems to depend on the same macromolecules as plasticity, while in contrast, plasticity has a feedback-inhibition character that confers stability to synaptic patterns (Tompa et al., 1998).

The understanding of the phenomenology and mechanisms of metaplasticity is still in its infancy. These new insights on how to increase and decrease synaptic strength will refine and further advance our understanding of activity-dependent synaptic modifications and thereby, the mechanisms underlying behavioural learning and memory.

Chapter 2

Materials and methods

2.1 Animals

Male Wistar rats weighing between 40-80g (age 3 and 4 weeks) were used throughout the course of these experiments. The Bioresources Unit, Trinity College Dublin, supplied the animals. During all the experiments the regulations as issued by the Department of Health on the use of animals in scientific research were followed. Particular effort was made to ensure that the conditions, under which the animals were kept, remained stable on a day-to-day basis. The animals were housed in a cage containing a maximum of 10 rats, with food and water available at all times. The animal house in the Bioresources unit was on a twelve hours light / dark cycle, with a constant temperature of 21°C - 23°C.

2.2 Artificial cerebro-spinal fluid (ACSF)

The incubation and experimental solution contained the following unless otherwise stated (in mM): 120 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2.0 $MgSO_4$, 2.0 $CaCl_2$, and 10 D-glucose. The pH was adjusted to 7.35 – 7.45 by gassing with 95 $\%O_2$ / 5$\%CO_2$. In one set of experiments, where the NMDAR mediated component of the synaptic response was isolated, $MgSO_4$ was omitted. Fresh saline solution was prepared each morning before experiments commenced.

All solutions contained 50μM picrotoxin (Sigma, St. Louis, MO) to block $GABA_A$–mediated activity.

2.3 Slice preparation

The brain was rapidly removed after decapitation using a guillotine without anesthesia. Using a pair of dissecting scissors, the excess skin and muscle were cut away from the skull, and an incision was then made at the base of the skull, which facilitated its removal with a pair of forceps. Subsequently a second incision along the midline of the skull was made to allow the removal of the remaining bones with the forceps. Following this the dura was cut using a pair of spring scissors before removing the cerebellum with a scalpel. The brain was removed quickly and submerged in cold (< 5°C) oxygenated (95 $\%O_2$ / 5 $\%CO_2$) saline for the remainder of the dissection. This part of the dissection was accomplished within 1.5-2.0 minutes. The brain was trimmed to size and fixed on a stage with superglue before being placed in the cutting chamber of a Campden Vibroslice (Campden Instruments, Loughborough, UK). Transverse hippocampal slices were cut at a thickness of 350μm setting the Vibroslice at a fast (8) vibration speed and a slow (1) advance speed. On the first advance, the top 4mm of the brain was removed and discarded, exposing the hippocampus. Subsequently, transverse hippocampal slices were removed. The slices were then transferred for recovery to a submersion type incubation chamber (Medical Systems Corporation, New York), and continuously superfused with ACSF at room temperature (20-22°C) and gassed with a mixture of 95 % O_2 / 5 % passing over them.

2.4 Electrophysiological recording

2.4.1 Experimental set-up

The slices were incubated for at least 1 hr in the incubation chamber before an experiment was begun. Slices were then placed in a purpose built

submersion type recording chamber and secured with a nylon mesh. In this type of chamber the slice resulted then completely submerged in the solution inside the chamber. The recording chamber and perfusion ACSF were then warmed to 30-32°C for the duration of the experiment via a stirring heater device (Grant Instruments, Ltd. Cambridge, UK) and the slices were continuously perfused with ACSF saturated with 95 % O_2 / 5 % CO_2 at a rate of ~ 6-7 ml/min for 30 minutes before starting the recording of the electrical activity of the brain. The slices were visualized under illumination, with a microscope with a magnification of 4.5 X, which was set on a steel plate on a table and surrounded by a Faraday cage. The recording and stimulation electrodes were maneuvered and positioned with a Narishige (Japan) and a Prior (England) manipulator respectively.

2.4.2 Electrodes

Excitatory post-synaptic potentials (EPSPs) were recorded extracellularly with standard ACSF filled glass microelectrodes. These recording electrodes were low resistance (~1MΩ) glass microelectrodes, which were pulled using a Flaming/ Brown micropipette puller, P-87 (Sutter Instrument Corp. CA., U.S.A.). The field EPSP population responses were evoked with a bipolar stimulating electrode that was made by twisting two strands of insulated tungsten wire (diameter 0.01mm) together.

2.4.3 EPSP recording

The field EPSPS

The peak amplitude of the field excitatory postsynaptic potential (field EPSP) was used as the measure of the strength of the synaptic transmission in the hippocampus. Both the recording and stimulation electrodes were positioned in the medial perforant path of the dentate gyrus of the hippocampus, at a distance of

1-2mm from each other, and paired pulse depression used as criteria to confirm correct placement. Field EPSPs were evoked by a brief single square wave voltage pulse (0.1ms in duration). The amplitude of f-EPSPs was recorded at a control test frequency of 0.033 Hz. For all the experiments, the amplitude of the test f-EPSP was adjusted to one-third of maximum (which was about 33%), close to 1-1.2 mV. Stimulation was applied through a bipolar, insulated tungsten wire electrode, to the medial perforant path of the dentate gyrus. Before the beginning of each experiment, paired pulse depression was used as criteria to confirm the correct electrodes placement in the medial perforant path of the dentate gyrus. LTP was evoked by high frequency stimulation (HFS) consisting of eight trains, each of eight stimuli at 200 Hz, with an intertrain interval of 2 secs. The stimulation voltage was increased during the HFS so as to elicit an EPSP of double the normal test f-EPSP amplitude. LTD was evoked by LFS consisting of 900 stimuli at 1Hz for 15 min. Priming or 'weak tetani' ('weak' means STP-inducing and not LTP-inducing) was induced using 6 trains of 8 stimuli at 50Hz with an intertrain interval of 2 secs. The stimulation voltage was increased during the weak tetani so as to elicit an EPSP of double the normal test f-EPSP amplitude.

Recording apparatus

A Grass S-88 stimulator (Grass Instruments, Quincy, Mass., U.S.A.) generated the stimulation pulses, and was driven by IBM compatible PC. The stimulation pulses were fed through a stimulus isolation unit before entering the stimulation electrode, to isolate the electrode from ground. The EPSPs were monitored on the screen of a storage oscilloscope (Farnell Instruments ltd., Korea), whilst records of f-EPSPs were recorded by PClamp6 computer software (Axon instruments Inc. Foster city, CA, USA). The EPSPs were amplified by a Grass P16 microelectrode DC amplifier (Grass Instruments, Quincy, Mass., U.S.A.) and converted from analogue to digital form (Axon instruments, Digidata

1200, AD/DA converter) before being stored on a Dell dimension 466 personal computer for subsequent off-line data processing.

2.5 Data analysis and Statistics

Recordings were analyzed using pClamp (Axon Instruments, Foster City, CA). The "normalized" EPSP (%) was calculated by normalizing every value in a particular condition to the 1st EPSP amplitude in that condition.

Summarised results are expressed as normalized EPSP mean ± SEM for *n* slices. The *n*-values refer to the number of times a given results was obtained, which is the same as the number of times the experiment was performed. Each experiment was conducted on a separate slice, each having been obtained from a separate rat (3-4 weeks of age). Examples representative EPSPs (average of 10 single EPSPs) are shown at times indicated by the small case letters. The two–tailed Student's *t* test was used for statistical comparison.

2.6 Compounds

- Bisindolylmaleimide I (Bis-I) from Calbiochem was dissolved in DMSO (the final concentration of DMSO was less than 0.05%).
- (D)-2-amino-5- phosphonopentanoic acid (D-AP5) from Tocris Cookson was dissolved in NaOH.
- (S)-3,5-Dihydroxyphenylglycine ((S)-3,5-DHPG) from Tocris Cookson was dissolved in H_2O.
- (2S)-α-ethylglutamic acid (EGLU) from Tocris Cookson was dissolved in NaOH.
- N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl (H-89, HCl) from Calbiochem was dissolved in H_2O.

- Lavendustin A from Tocris Cookson was dissolved in DMSO (the final concentration of DMSO was less than 0.05%).
- (RS)-a-methyl-4-carboxyphenylglicine (MCPG) from Tocris Cookson was dissolved in NaOH.
- 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP) from Tocris Cookson was dissolved in H_2O.
- 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX) from Tocris Cookson was dissolved in DMSO (the final concentration of DMSO was less than 0.05%).
- ([R]-2-[Methylamino]succinic) acid (NMDA) from Sigma was dissolved in H_2O.
- 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) from Alexis Corporation was dissolved in DMSO (the final concentration of DMSO was less than 0.05%).
- 4-(4-fluoropheny)-2-(4-methylsulfonilphenyl)-5-(4-pyridil) imidazol (SB203580) from Calbiochem, Lucerne Switzerland was dissolved in DMSO (the final concentration of DMSO was less than 0.05%).
- Ro-31-8220 from Calbiochem was dissolved in DMSO (the final concentration of DMSO was less than 0.05%).
- Nifedipine from Tocris Cookson was dissolved in DMSO (the final concentration of DMSO was less than 0.05%).
- Picrotoxin from Sigma was dissolved in DMSO (the final concentration of DMSO was less than 0.05%).

Chapter 3

Preconditioning stimulation inhibits LTP induction in the rat dentate gyrus *in vitro*

3.1 Overview

Initial studies were carried out to determine whether prior weak HFS could inhibit LTP induction in the medial perforant path of the rat dentate gyrus *in vitro*. In control media, high frequency stimulation (HFS) consisting of a series of trains of stimuli at 200Hz induced LTP. The effect of preconditioning was investigated by applying a weak HFS consisting of 6 trains at 2 min interval, each train 8 stimuli at 50 Hz. Such preconditioning stimulation itself only resulted in a short-term change of transmission lasting 1-2 minutes, and did not result in significant LTP. However, applying the preconditioning stimulation 20 minutes prior to the standard HFS resulted in a significant inhibition of LTP induction. In order to determine the duration of the inhibitory influence of the preconditioning stimulation, the preconditioning stimulation was given at different times prior to the standard HFS. The inhibition by preconditioning stimulation occurred in a 'window period'. Thus, the preconditioning stimulation inhibited subsequent HFS-induced LTP, if applied 10 and 20 min, but not 2 or 45 min prior to the HFS. Indeed, LTP induction was slightly enhanced if the interval between the preconditioning stimulation and standard HFS was 2 minutes.

3.2 Introduction

Activity-dependent modulation of synaptic plasticity is an important component in understanding the cellular mechanism underlying memory and learning (Abrahamand Bear, 1996). External signals such as hormones and modulatory neurotransmitters that affect cellular excitability or signalling in biochemical pathways can exert great influence on the direction of synaptic plasticity induced by a particular stimulus protocol. Such changes in the synaptic response can be induced directly, by activation of postsynaptic receptors, or indirectly modulating the activity of afferent principal cells or interneurones (Liu et al., 1993; Wagner et al., 1995; Nilsen et al., 2002; Zou et al 2001; Chepkova et al., 2001; Vouimba et al., 2000; Harley et al., 2000), or finally, as will be demonstrated by work presented in this thesis, by the prior history of synaptic activity. There are several studies demonstrating that prior synaptic activity

can modifying subsequent synaptic plasticity, therefore to inhibit LTP or in other cases facilitate LTP (Huang et al. 1992, Abraham et al., 1997, Bortolotto et al., 1994, Cohen et al., 1996, Stanton, 1995, Zurner et al., 2000). Metaplasticity may serve several functions including: (1) providing a way for synapse to integrate a response across temporally spaced episodes of synaptic activity, or (2) keeping synapses within a dynamic functional range, and thus preventing them from entering states of saturated LTP or LTD (Abraham and Bear, 1997).

3.3 Methods

The animals, ACSF solution and preparation of hippocampal slices were described in Chapter II. LTP was evoked by HFS consisting of 8 trains each of 8 pulses at 200Hz and inter-train interval was 2 seconds. Priming or weak tetani ('weak' means STP-inducing and not LTP-inducing) was induced using 6 trains of 8 stimuli at 50Hz with inter-train interval of 2 sec. The magnitude of LTP was measured at 2, 10, 20, and 45 min post weak tetani.

3.4 Results

3.4.1 Preconditioning stimulation (weak HFS) applied 20 min prior to strong HFS inhibits LTP induction

In control slices, HFS induced a stable LTP measuring 160 ± 3%, n=5, 1h after HFS (Fig. 3.1). The induction of LTP was inhibited if a preconditioning stimulus (6 trains of 8 stimuli at 50Hz, inter-train interval 2 sec) which itself did not induce long-term plasticity, was applied 20 min prior to HFS. Thus, applying the preconditioning stimulation 20 min prior to the standard HFS resulted in a significant inhibition of LTP induction. After 1h LTP was measured at 116 ± 7% (n=5) which was significantly different from the control LTP ($p<0.01$) (Fig. 3.2).

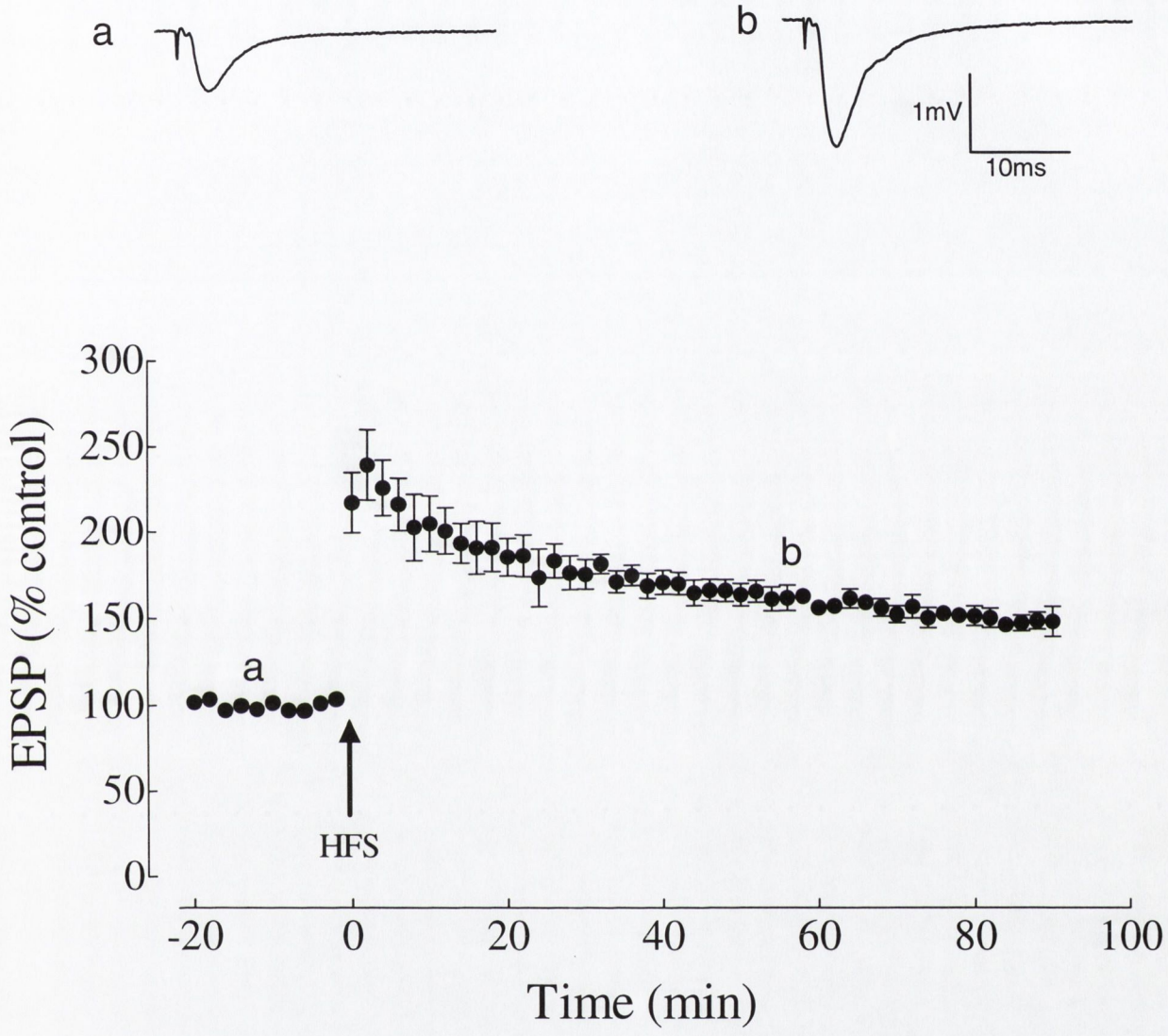


Figure 3.1 LTP in control solution.

When HFS was delivered to slices in control solution (ACSF) LTP was induced that measured 160±3% (n=5) at 1h post HFS. Examples of EPSPs (average of 10 single EPSPS) are shown in **a** (baseline)and **b** (60 min after the induction of LTP).

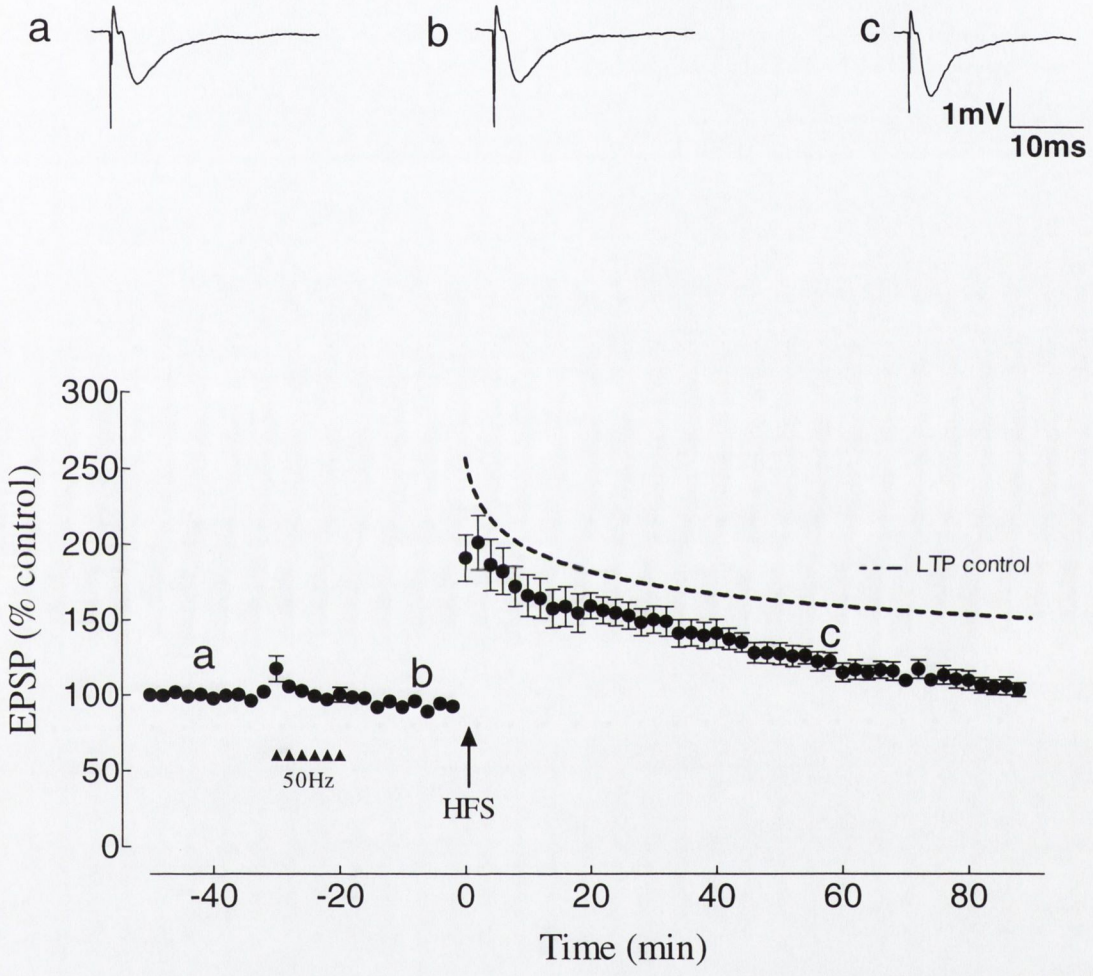


Figure 3.2 Preconditioning stimulation (weak HFS) applied 20 min prior to strong HFS inhibits LTP induction. Applying the preconditioning stimulation 20 min prior to HFS resulted in inhibition of LTP (filled circles), which after 1h measured 116±7% (n=5), a value significantly different ($P<0.01$) from the control LTP(dashed line). Examples of EPSPs are shown in a (baseline), b (after weak tetani) and c (1h after HFS).

3.4.2 'Window period' of the inhibition:

The following experiments indicate that inhibition of LTP by preconditioning stimulation occurred in a 'window period', i.e. 10 and 20 min after weak tetani before HFS, but not after 2 and 45 minutes.

3.4.2.1 Preconditioning stimulation (weak HFS) applied 10 min prior to strong HFS inhibits LTP induction

Applying the preconditioning stimulation (weak tetani) 10 min prior to the standard HFS resulted in a significant inhibition of LTP induction, which after 1h measured 102 ± 4% (n=5) which was significantly different from the control LTP ($p<0.01$) (Fig. 3.3, Fig. 3.1). The inhibition of LTP in this set of experiments was shown to be to be stronger when compared with the set of experiments described previously.

3.4.2.2 Preconditioning stimulation (weak HFS) applied 2 min prior to strong HFS does not inhibits LTP induction

In this set of experiments the time between the application of the preconditioning stimulation and HFS-induced LTP was decrease. Thus, applying weak tetani 2 min prior to HFS resulted in LTP measuring 182 ± 14% (n=5) a slightly enhanced value when compared to the LTP control ($p> 0.1$) (Fig.3.4, Fig.3.1).

3.4.2.3 Preconditioning stimulation (weak HFS) applied 45 min prior to strong HFS does not inhibit LTP induction

These results demonstrated that applying weak tetani 45 min before HFS-induced LTP measuring 156 ± 12%, n=5 a value not significantly different to the control LTP ($p>0.5$) (Fig. 3.5, Fig.3.1).

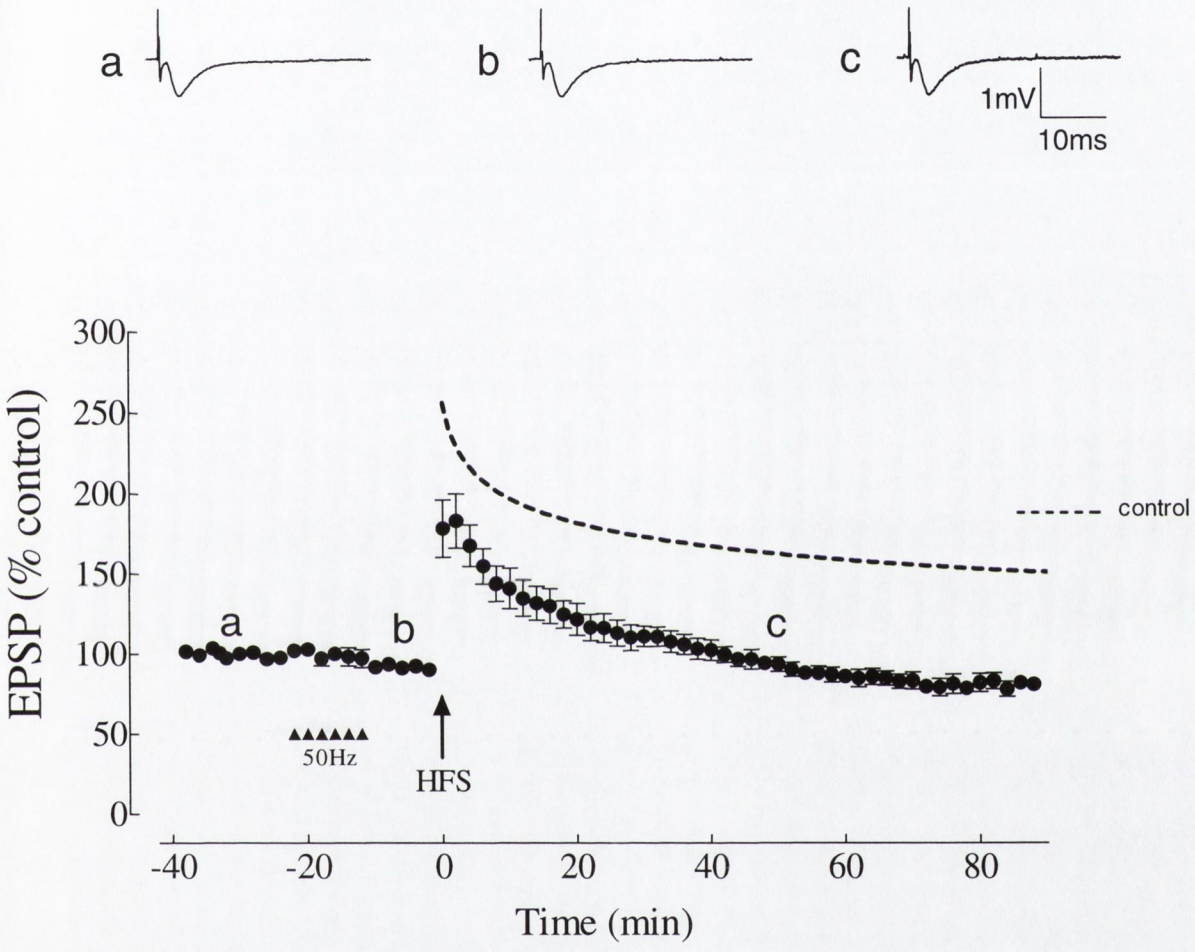


Figure 3.3 Preconditioning stimulation (weak HFS) applied 10 min prior to strong HFS inhibits LTP induction. Applying the preconditioning stimulation 10 min prior to HFS resulted in inhibition of LTP (filled circles), which after 1h measured 102±4% (n=5), a value significantly different ($p<0.01$) from the control LTP(dashed line). Examples of EPSPs are shown in a (baseline), b (after weak tetani) and c (1h after HFS).

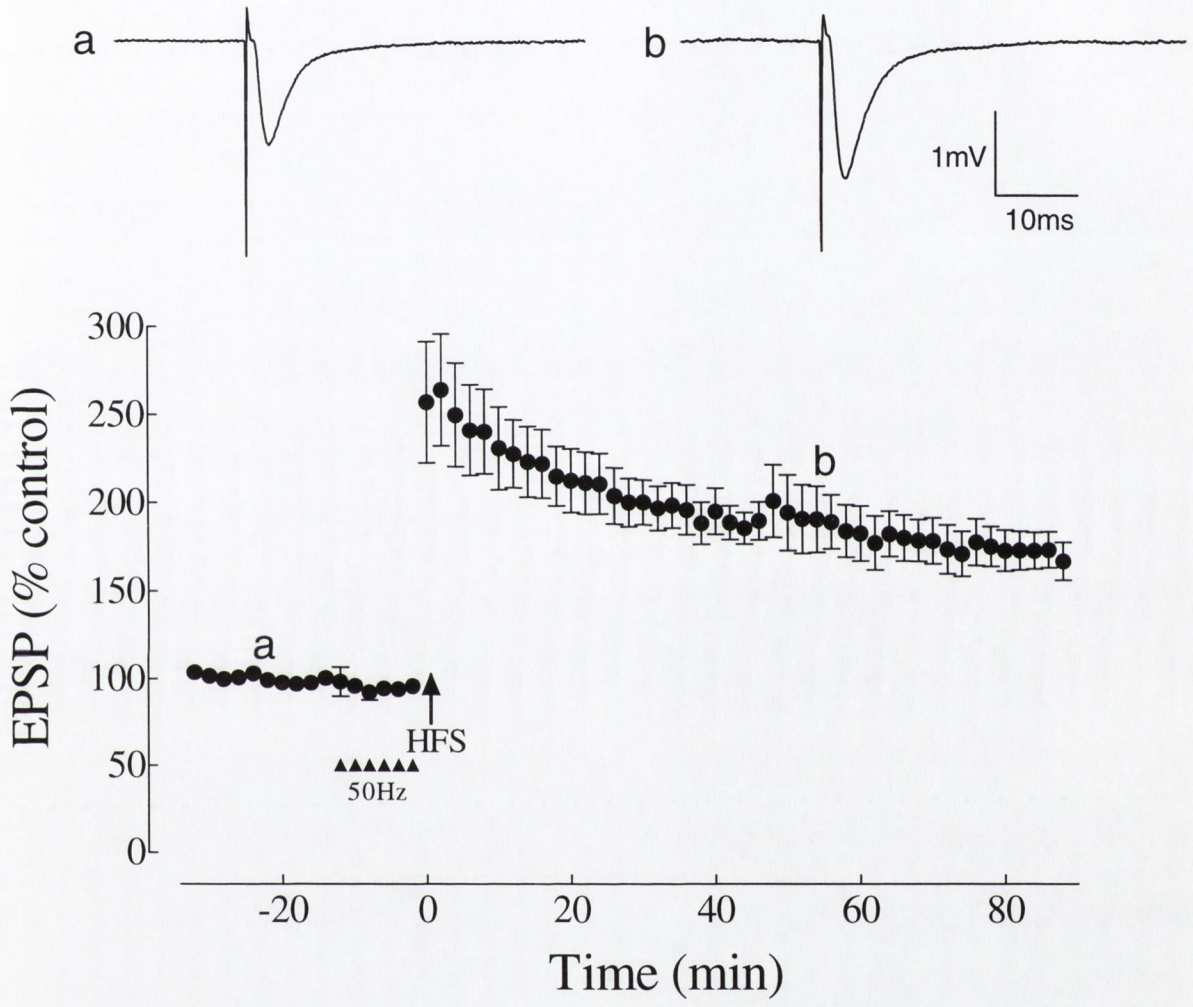


Figure 3.4 Preconditioning stimulation (weak HFS) applied 2 min prior to strong HFS does not inhibit LTP induction. Applying the preconditioning stimulation 2 min prior HFS resulted in LTP, which after 1h measured 182±14% ($P<0.1$, $n=5$). Examples of EPSPs are shown in **a** (baseline) and **b** (1h after HFS).

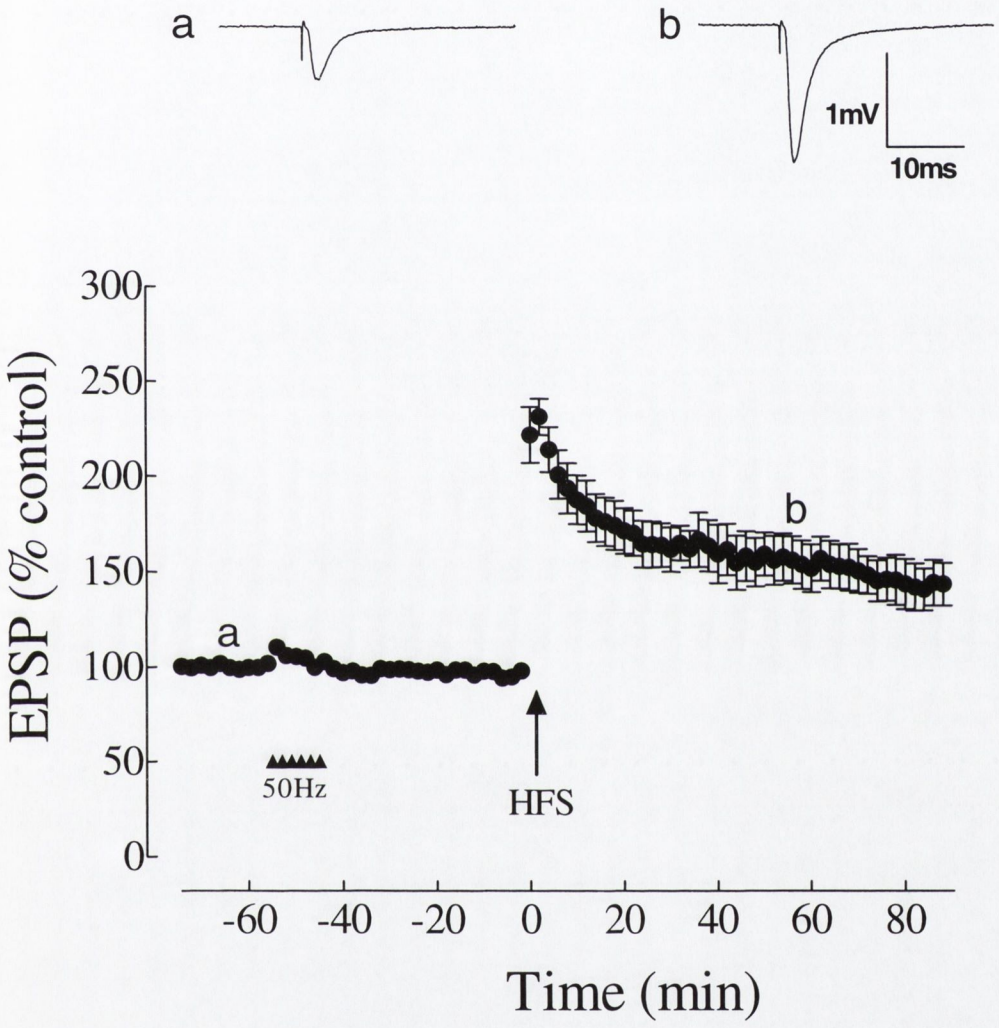


Figure 3.5 Preconditioning stimulation (weak HFS) applied 45 min prior to strong HFS does not inhibit LTP induction. Applying the preconditioning stimulation 45 min prior HFS resulted in LTP, which after 1h measured 156±12% ($p > 0.5$, $n=5$), a value not significantly different from control LTP. Examples of EPSPs are shown in **a** (baseline) and **b** (1h after HFS).

3.5 Discussion

The results described above clearly represent strong evidence of how the previous history of synaptic activity can modulate the direction of synaptic plasticity. The inhibition of LTP by preconditioning stimulation represented by weak tetani has been observed previously in the CA1 region (Huang et al, 1992). The present studies have focused on verifying if similar inhibition could also occur in the medial perforant path of the dentate gyrus. In addition to the work of Huang et al., further experiments described in the following chapters will try to suggest a possible model of the mechanism underlying the inhibition of LTP by previous weak tetani. This model will involve receptors and second messengers that contribute to the inhibitory effect by preconditioning stimulation.

Alteration in the synaptic strengths of synaptic connections has been suggested as being fundamental for the cellular mechanisms that are involved in the acts of learning and memory by the brain (Hebb, 1949). The potential for bi-directional changes in the strengths of synaptic connections seems to give to synapses the capability of change and flexibility to plastic events that occurred in the mechanism of processing information storage in the brain (Bienenstock et al., 1982).

In order to determine the duration of inhibitory influence of the preconditioning stimulation, the preconditioning stimulation was given at different times prior to the standard HFS. The inhibition of LTP was present at 10 and 20 min, but not 2 or 45 min post preconditioning, indicating a time window for the inhibition. The results indicated that the inhibition produced by the preconditioning stimulus took several minutes to develop, and then declined after 30-45 min. Moreover, this temporal window of the inhibition might suggest the evidence of the involvement of second messengers as has already been demonstrated by previous studies (Wang et al., 1998; Boxall et al., 1996; Stanton 1995). This implied by the fact that the inhibition, before it develops, needs a length of time that is sufficient for a second messenger to be produced.

Chapter 4

Receptors involved in the inhibition of LTP induction by preconditioning stimulation in the rat dentate gyrus *in vitro*

4.1 Overview

The involvement of NMDA receptor (NMDAR) and metabotropic glutamate receptors (mGluR) was investigated in the inhibition of LTP by preconditioning stimulation in the medial perforant path of the rat dentate gyrus.

Preconditioning inhibition involved activation of NMDAR and mGluRs, because the presence of NMDAR or mGluR antagonists during weak tetani prevented subsequent LTP inhibition. For this purpose the use of D- AP5, an NMDAR antagonist, prevented LTP inhibition. Similarly, perfusion of the non sub-group selective mGluR antagonist (+)-α-methyl-4-carboxyphenylglycine (MCPG) during the preconditioning stimulation was found to prevent the preconditioning inhibition of LTP induction. In order to determine the particular mGluR group/type involved in the inhibition, a selective inhibitor of group I mGluR was investigated. The selective mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP), applied during the preconditioning stimulation, strongly reversed the preconditioning inhibition of LTP induction. In addition, perfusion of the mGluR group II antagonist, (S)-α-ethylglutamate (EGLU) during weak tetani prevented LTP inhibition, showing the involvement of this group of receptor in the priming inhibition of LTP.

To examine a possible change in the NMDA receptor mediated component of the synaptic response during weak tetani, NBQX was added to block ionic conductances through the AMPA channel and recording were made in Mg^{2+}-free solution to isolate the NMDAR-mediated f-EPSP. These experiments showed a short-term lasting increase in amplitude during weak tetani, but it returned to baseline within 10 min after the preconditioning stimulation.

The use of different types of priming other than weak tetani has also been investigated.

The direct application of NMDA in the medial perforant path of rat dentate gyrus primes LTP. Thus, when applied 20 min before HFS, NMDA facilitated LTP.

In order to determine whether lower frequencies of stimulation than 50 Hz, and especially those inducing LTD, resulted in a subsequent inhibition of LTP, the preconditioning stimulation was given in the form of prolonged LFS (1 Hz, 900 stimuli) terminating 20 min prior to the standard HFS. Subsequent LTP induction was not inhibited by such preconditioning stimulation.

In order to investigate the priming effect on LTP induction through group II mGluRs, hippocampal slices were perfused with DCGIV, a specific group II mGluR agonist. Such priming inhibited LTP.

4.2 Introduction

There is substantial evidence presented in several reports indicating the involvement of NMDAR and mGluRs in the modulation of LTP by previous synaptic activity (Cohen and Abraham, 1996; Huang et al., 1991; Aniksztejn et al., 1992; Harvey et al., 1993; Challiss et al., 1994; Akiva et al., 1996; 1999; Bortolotto et al., 1994).

Since NMDA receptor activation is fundamental for LTP and LTD, considering the possible involvement of this receptor in metaplasticity was an obvious step. In one study in CA1 region, perfusing hippocampal slices with Mg^{2+}-free solution, which enhances activation of NMDAR, resulted in subsequent inhibition of LTP induction (Coan et al., 1989). Other evidence that demonstrates the role of NMDAR in metaplasticity are reports by Huang et al. where inhibition of LTP by weak tetani in CA1 was due to the activation of NMDA receptors because LTP occurred normally when AP5 was present during weak tetani (Huang et al., 1992).

A significant example of the involvement of mGluRs in metaplasticity is given by a study where brief application of the selective antagonist aminocyclopentane- (1S,3R)-dicarboxylate (ACPD) facilitated the stable induction of LTP (Akiva et al., 1996). There is also evidence that group I mGluRs mediate inhibition of synaptic transmission (Cohen et al., 1995; Gereau and Conn 1995).

mGluRs belong to the family of G-protein coupled receptors. There are 8 different subtypes of mGluRs divided into 3 broad groups. There are studies that show that mGluR priming of LTP might result from biochemical cascades triggered by activation of phospholipase C coupled to group I mGluRs, as described in reports where inhibition of phospholipase C by U-73122 completely abolished the priming of LTP by DHPG (Cohen et al., 1998). Activation of this PLC pathway leads to the liberation of inisitol triphosphate (IP_3) and diacylglycerol following hydrolysis of phosphatidydilnositol. Also basal cAMP accumulation is increased by an L-AP3-sensitive mechanism (Winder et al., 1992).

Stimulation of group II mGluR leads to a decrease in forskolin-stimulated cyclic-AMP (cAMP) accumulation. In addition, an increase of cAMP has been demonstrated to be mediated by a synergistic interaction between group I and group II mGluR (Shoepp et al., 1996). It also appears from previous studies that the coactivation of both these last two groups of receptors leads to a priming effect inhibiting LTP induction (O'Leary et al., 1998).

4.3 Methods

The animals, ACSF solution and preparation of hippocampal slices and field potential recordings already were described in Chapter II. The NMDA component was isolated in the set of experiments where the solution was Mg^{2+}-free, and hence $MgSO_4$ free.

LTP was evoked by HFS consisting of 8 trains each of 8 pulses at 200Hz and an inter-train interval of 2 seconds. Preconditioning stimulation or weak tetani was induced using 6 trains of 8 stimuli at 50Hz with an inter-train interval of 2 sec. LTD was evoked by low frequency stimulation (LFS) consisting of 900 stimuli at 1 Hz for 15 min, with a test stimulation voltage remaining at the same amplitude during the LFS.

Drugs used were (D)-2-amino-5- phosphonopentanoic acid (D-AP5), (RS)-a-methyl-4-carboxyphenylglicine (MCPG), 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP), (2S)-α-ethylglutamic acid (EGLU), 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX) (all from Tocris Cookson). D-AP5, MCPG, and EGLU were dissolved in NaOH. MPEP was dissolved in H_2O and NBQX in dimethylsulphoxide (DMSO). The final concentration of DMSO was less than 0.05%. NBQX was perfused for at least 1 hour before start recording.

Other drugs used were ([R]-2-[Methylamino]succinic) acid, NMDA (from Sigma) and (2S,2'R,3'R)-2-(2',3'-Dycarboxycyclopropyl)glycine, DCGIV (from Tocris). Both NMDA and DCGIV were dissolved in H_2O.

4.4 Results

4.4.1 Preconditioning inhibition of LTP is prevented by activation of NMDAR by the application of its antagonist D-AP5

Perfusion of the NMDAR antagonist D-AP5 during the preconditioning stimulation was found to prevent the preconditioning inhibition of LTP induction, with LTP measuring 1h after tetanisation (166 ± 9%, n=5), a value significantly different from the experiments showing that weak tetani applied 20 min prior HFS inhibit LTP ($p<0.05$) (Fig. 4.1, Fig. 3.2). Following a recording period of 20 min at which the test EPSP remained at constant amplitude, D-AP5 (100μM) was perfused for 20 minutes before and during the preconditioning stimulation. The drug was finally washed out 20 min before HFS –induced LTP. This result is therefore evidence of the involvement of NMDAR in the inhibition by preconditioning stimulation.

4.4.2 Preconditioning stimulation does not inhibit the NMDAR component of the EPSP

The purpose of this set of experiments was to determine if the NMDAR component of the EPSP was reduced by the preconditioning stimulation. Hippocampal slices were perfused with Mg^{2+}-free media containing the AMPA receptor antagonist NBQX (20μM) to isolate the pure NMDAR-mediated EPSPs.
After the NMDAR-mediated EPSPs had attained stable amplitude, an identical preconditioning 50 Hz stimulation to those used in previous experiments was applied. A small enhancement of the NMDAR-mediated EPSPs was observed during and immediately following the 50 Hz preconditioning stimulation, but no long-lasting alteration of the NMDAR-mediated EPSPs occurred. Thus at 10 and 20 min after 50 Hz stimulation, the EPSPs measured 100 ± 9% and 95 ± 5% (n=5), a non-significant change from the baseline measuring 95 ± 4% (Fig. 4.2). This result could possibly explain an intrinsic effect of metaplasticity, i.e. that subsequent modification of the direction of synaptic plasticity might occur without concurrent changing of synaptic transmission. It is likely that in the present case NMDARs exert their action by

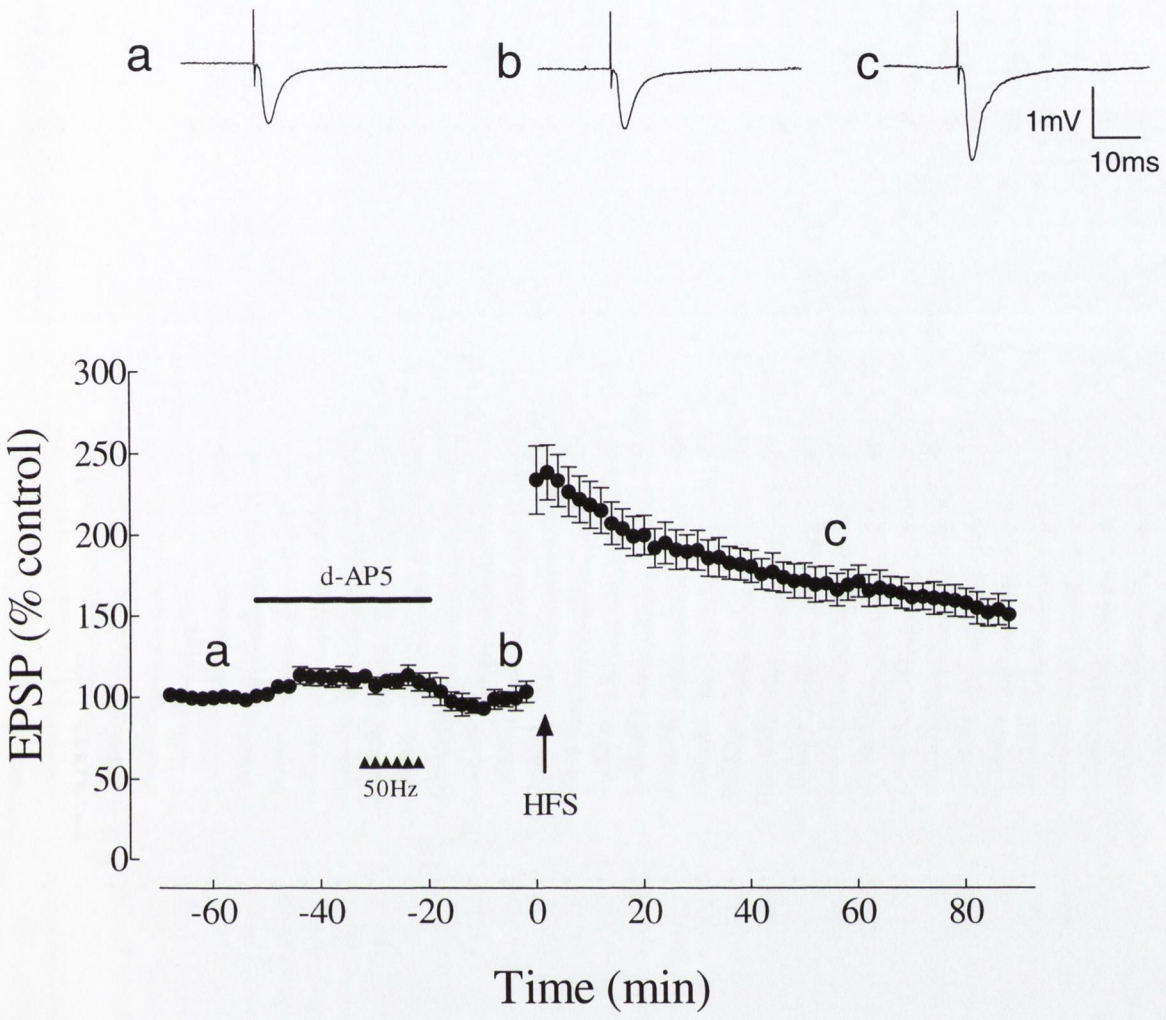


Figure 4.1 Preconditioning inhibition of LTP involves activation of NMDAR. The perfusion of slices with D-AP5 (100μM) for 20 min prior and during the preconditioning stimulation resulted to induce LTP measuring 166±9% (n=5) at 1h after delivery of HFS. This LTP value was significantly different ($p<0.05$) from the experiments showing that preconditioning stimulation inhibit LTP induction. The traces show EPSPs prior to D-AP5 application (a), following washout of D-AP5 (b) and following HFS-induced LTP (c).

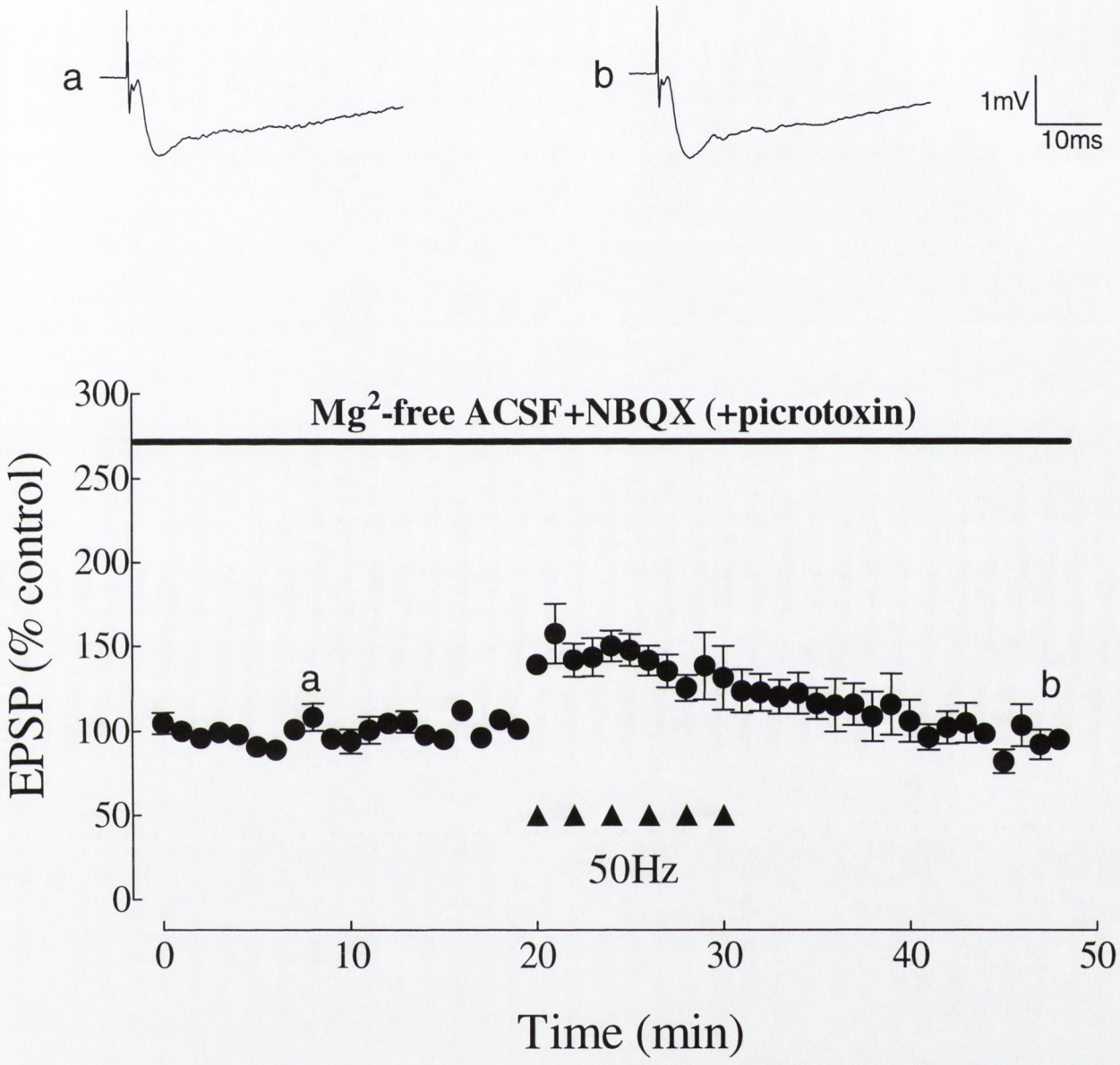


Figure 4.2 Preconditioning stimulation did not inhibit the NMDAR component of the EPSP. The application of preconditioning stimulation in the presence of Mg^{2+}-free media containing NBQX (20μM) did alter the NMDAR-mediated EPSPs, which at 20 min post 50 Hz stimulation measured 95±5% (n=5),a non-significant change from the baseline measuring 95±4%. The traces show the amplitude of the EPSPs prior (a) and after (b) preconditioning stimulation.

interacting with second messengers or receptors like mGluRs or even by inducing protein synthesis that inhibit subsequent LTP.

4.4.3 Priming of LTP by prior activation of NMDA receptor by its agonist NMDA facilitates LTP in rat dentate gyrus in vitro

The treatment of hippocampal slices with 10μM of an NMDAR agonist, NMDA, for 20 min and in the further 20 min washing out of NMDA before the application of HFS lead to an increase of LTP measuring 188 ± 4%, n=5 a value significantly greater than control ($P<0.01$). The application of NMDA induced a depression of field EPSPs. After NMDA application field EPSPs measured 60 ± 7% (n=5; $p<0.01$) (Fig. 4.3, Fig.3.1).

4.4.4 Preconditioning inhibition of LTP is prevented by activation of mGluR by the application of its antagonist MCPG

The involvement of mGluRs in the preconditioning stimulation was investigated applying the non-sub-group selective mGluR antagonist MCPG during the preconditioning stimulation. MCPG prevented the preconditioning inhibition of LTP induction, with LTP measuring 145 ± 9%, n=5, a value significantly different from the experiments showing that weak tetani applied 20 min prior HFS inhibit LTP ($p<0.05$) (Fig. 4.4, Fig. 3.2). Thus, after 20 min of baseline MCPG (500μM) was perfused in the hippocampal slices for further 20 min before and during weak tetani. MCPG was then washed out 20 min before HFS- induced LTP.

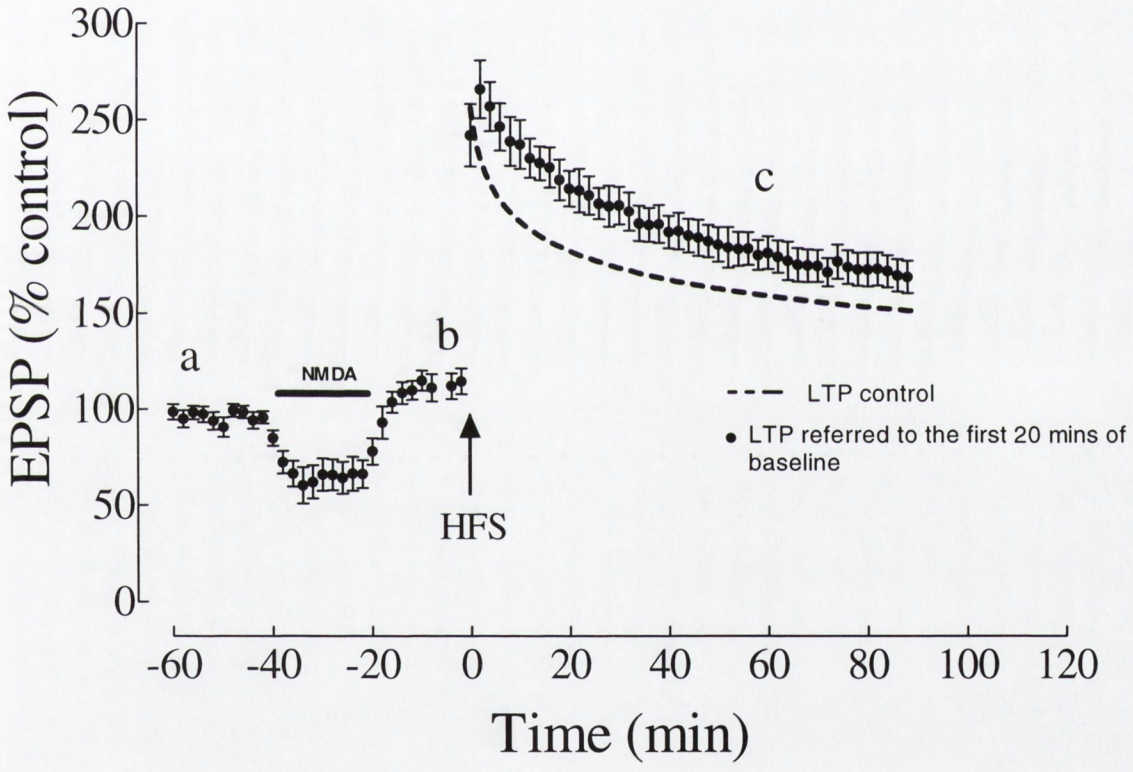


Figure 4.3 The NMDAR antagonist NMDA facilitates the induction of LTP .
Perfusion of NMDA (10μM) for 20 min prior HFS induced a depression of field EPSPs to 60 ±7% ($p<0.01$; n=5) and increased LTP (filled circles) measuring 188±4%, n=5 a value significantly different ($p< 0.01$) from control (dashed line).
The traces show EPSPs prior to application (a), washout of NMDA (b) and following HFS-induced LTP (c).

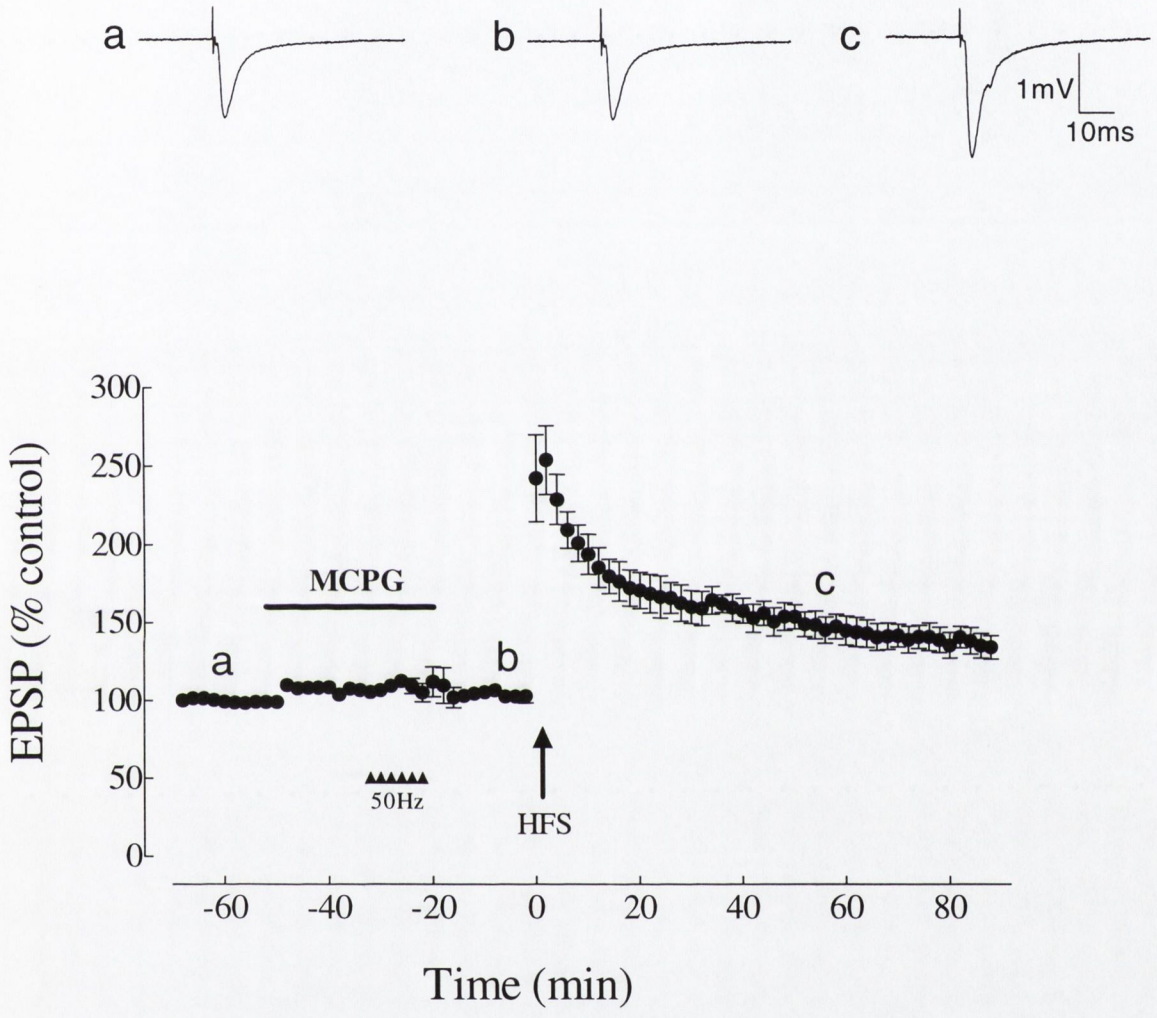


Figure 4.4 Preconditioning inhibition of LTP involves the activation of mGluR. The perfusion of slices with MCPG (500μM) for 20 min prior and during the preconditioning stimulation resulted to induce LTP measuring 145±9% (n=5) at 1h after delivery of HFS. This LTP value was significantly different ($p<0.05$) from the experiments showing that preconditioning stimulation inhibit LTP induction. The traces show EPSPs prior to MCPG application (a), following washout of MCPG (b) and following HFS-induced LTP (c).

4.4.5 Preconditioning inhibition of LTP is prevented by activation of mGluR group I by the application of its antagonist MPEP

A set of experiments was carried out in order to determine the particular mGluR group/type involved in the inhibition. Therefore a selective inhibitor of group I mGluR was investigated, i.e. 2-methyl-6-(phenylethynyl)pyridine (MPEP) which is a potent and highly selective non-competitive antagonist at the $mGlu_5$ receptor subtype (IC_{50} = 36 nM). MPEP (3μM) applied 20 min before and during the preconditioning stimulation, strongly reversed the preconditioning inhibition of LTP induction, which measured 146 ± 12%, n=5, a value significantly greater then the experiments showing that weak tetani applied 20 min prior HFS inhibit LTP ($p<0.05$) (Fig. 4.5, Fig. 3.2).

4.4.6 Priming of LTP by prior activation of group I mGluRs by its agonist DHPG inhibits LTP in rat dentate gyrus in vitro

Bath administration of the group I mGluR agonist DHPG (100μM) for 20 min and then washing out the drug for a further 5 min inhibited LTP induction when applied prior to HFS-induced LTP. DHPG caused a transient mild depression of the fEPSP, which largely washed out quickly upon return to the control solution. Thus, DHPG applied prior to HFS led to inhibition of LTP, measuring only 124 ± 2%, n=5 (1h after HFS) a value significantly different from control ($p<0.01$) (Fig. 4.6, Fig.3.1).

4.4.7 Preconditioning inhibition of LTP is prevented by blocking activation of group II mGluRs by the application of EGLU

In order to investigate the role of group II mGluRs in the inhibition by preconditioning stimulation, a presumed group II mGlu receptor antagonist (2S)-α-ethylglutamic acid (EGLU) was used to see if this compounds was able to block the

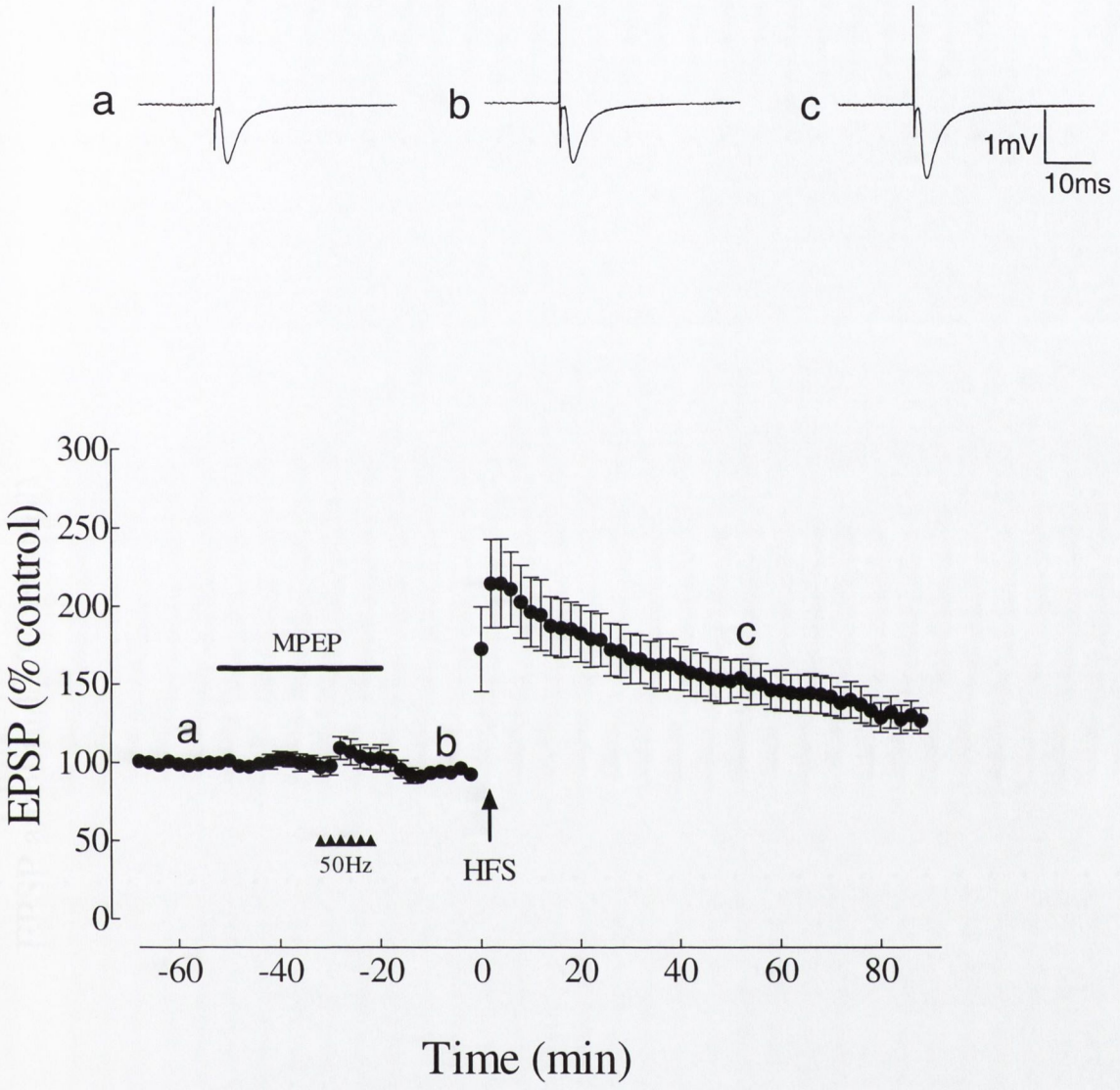


Figure 4.5 Preconditioning inhibition of LTPinvolves activation of $mGluR_5$ receptor subtype. The perfusion of slices with MPEP (3μM) for 20 min prior and during the preconditioning stimulation resulted to induce LTP measuring 146±12% (n=5) at 1h after delivery of HFS. This LTP value was significantly different ($p<0.05$) from the experiments showing that preconditioning stimulation inhibit LTP induction. The traces show EPSPs prior to MPEP application (a), following washout of MPEP (b) and following HFS-induced LTP (c).

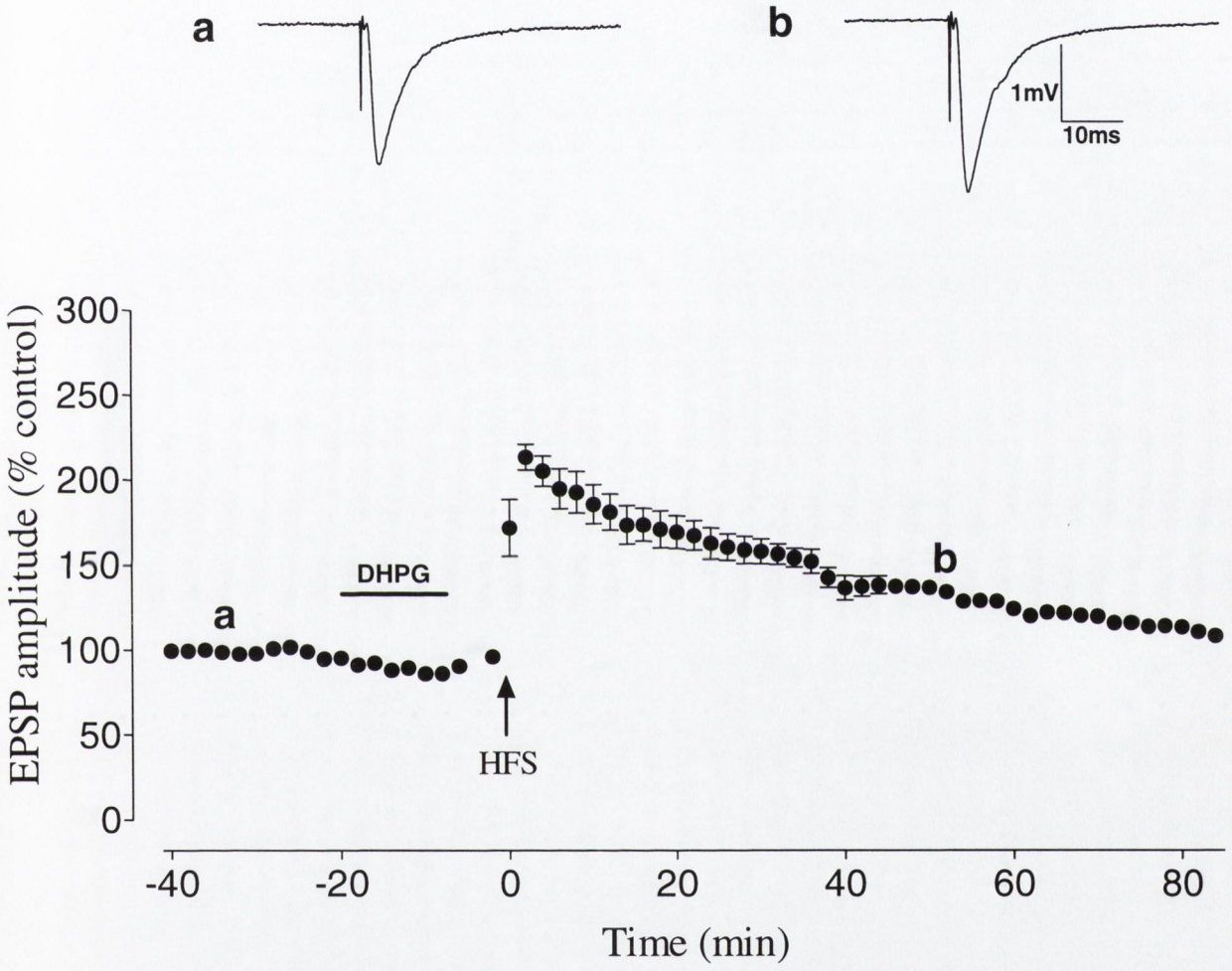


Figure 4.6 mGluR group I agonist DHPG inhibits the induction of LTP. Perfusion of DHPG (100μM) for 20 min and washout 5 min prior to HFS inhibited LTP measuring $124 \pm 2\%$, n=5 a value significantly different from control ($p<0.01$). Examples of EPSPs are shown in a (baseline) and b (1h after the induction of LTP).

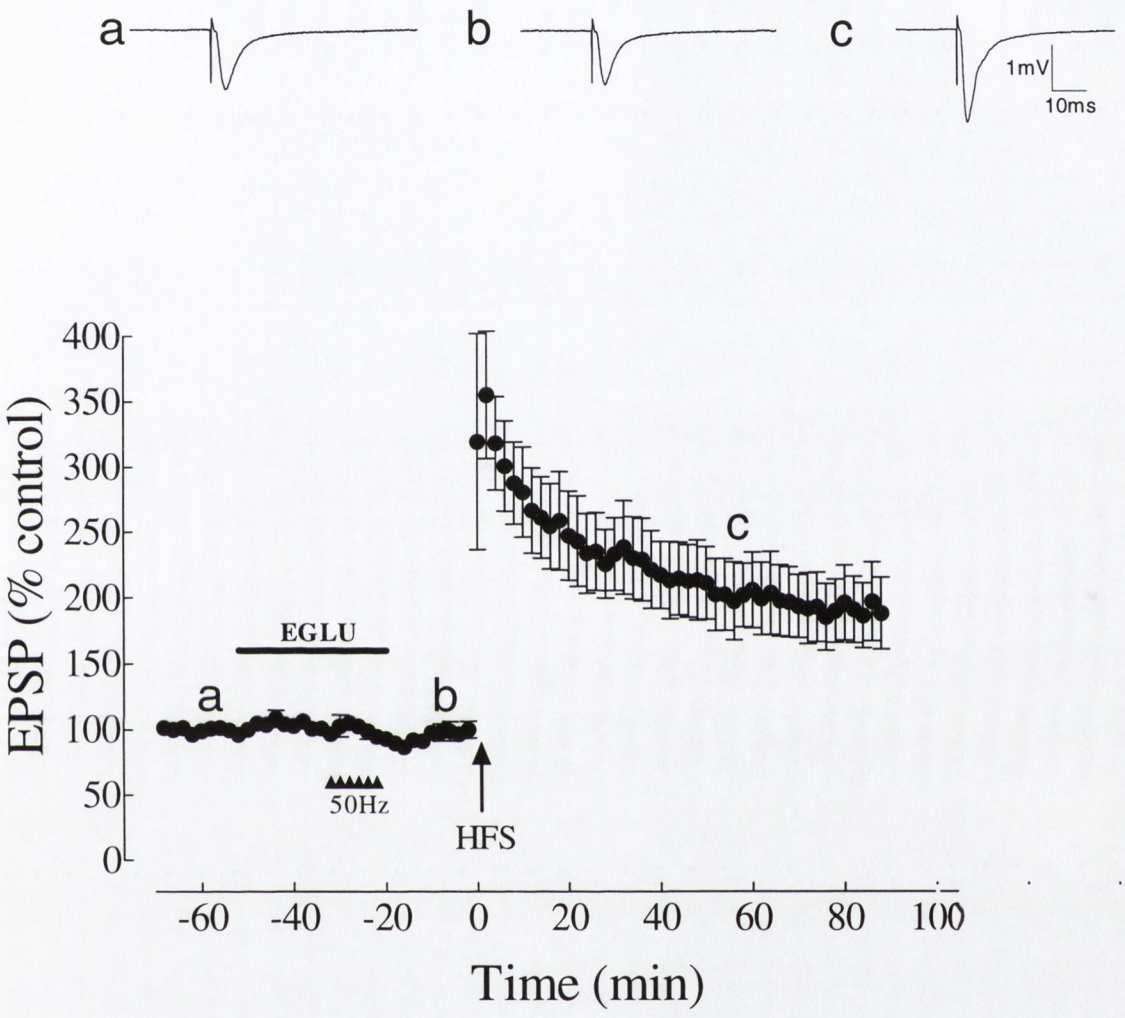


Figure 4.7 Preconditioning inhibition of LTP involves activation of group II mGluRs. The perfusion of slices with EGLU (250μM) for 20 min prior and during the preconditioning stimulation induced LTP measuring 195± 12%, n=5 at 1h after delivery of HFS. This LTP value was significantly different ($p<0.05$) from the experiments showing that preconditioning stimulation inhibited LTP induction. The traces show EPSPs prior to EGLU application (a), following washout of EGLU (b) and following HFS-induced LTP (c).

inhibition. EGLU (250μM) was applied 20 min before and during weak tetani and finally washed out for 20 minutes before HFS- induce LTP. The application of EGLU blocked the inhibition by preconditioning stimulation inducing an LTP, which measured 195±5%, n=5, a value significantly greater than the experiments showing that weak tetani applied 20 min prior HFS inhibit LTP ($p<0.05$) (Fig. 4.7, Fig.3.2).

4.4.8 Priming of LTP by prior activation of group II mGluR by its agonist DCG-IV inhibits LTP in rat dentate gyrus in vitro

Brief application (5min) of the group II mGluR agonist, DCGIV (2μM) in rat hippocampal slices prior to HFS lead to inhibition of LTP, measuring only 117 ± 5%, n=5, value significantly different from control ($p<0.01$) (Fig. 4.8, Fig.3.1). DCGIV was washed out 20 min before the application of HFS-induced LTP.

4.4.9 Application of LFS-induced LTD 20 mins prior HFS does not inhibit LTP induction

In order to determine whether lower frequencies of stimulation than 50 Hz, and especially those generating LTD, resulted in subsequent inhibition of LTP, the preconditioning stimulation was given in the form of prolonged LFS (1 Hz, 900 stimuli, 15min) terminating 20 min prior to the standard HFS. The LFS induced enhanced LTD with EPSPs measuring 62 ± 2% (n=5; $p<0.01$). Subsequent LTP induction was not inhibited by such preconditioning stimulation, measuring 168 ± 7% a value that is not significantly different from control ($p>0.5$) (Fig. 4.9, Fig.3.1).

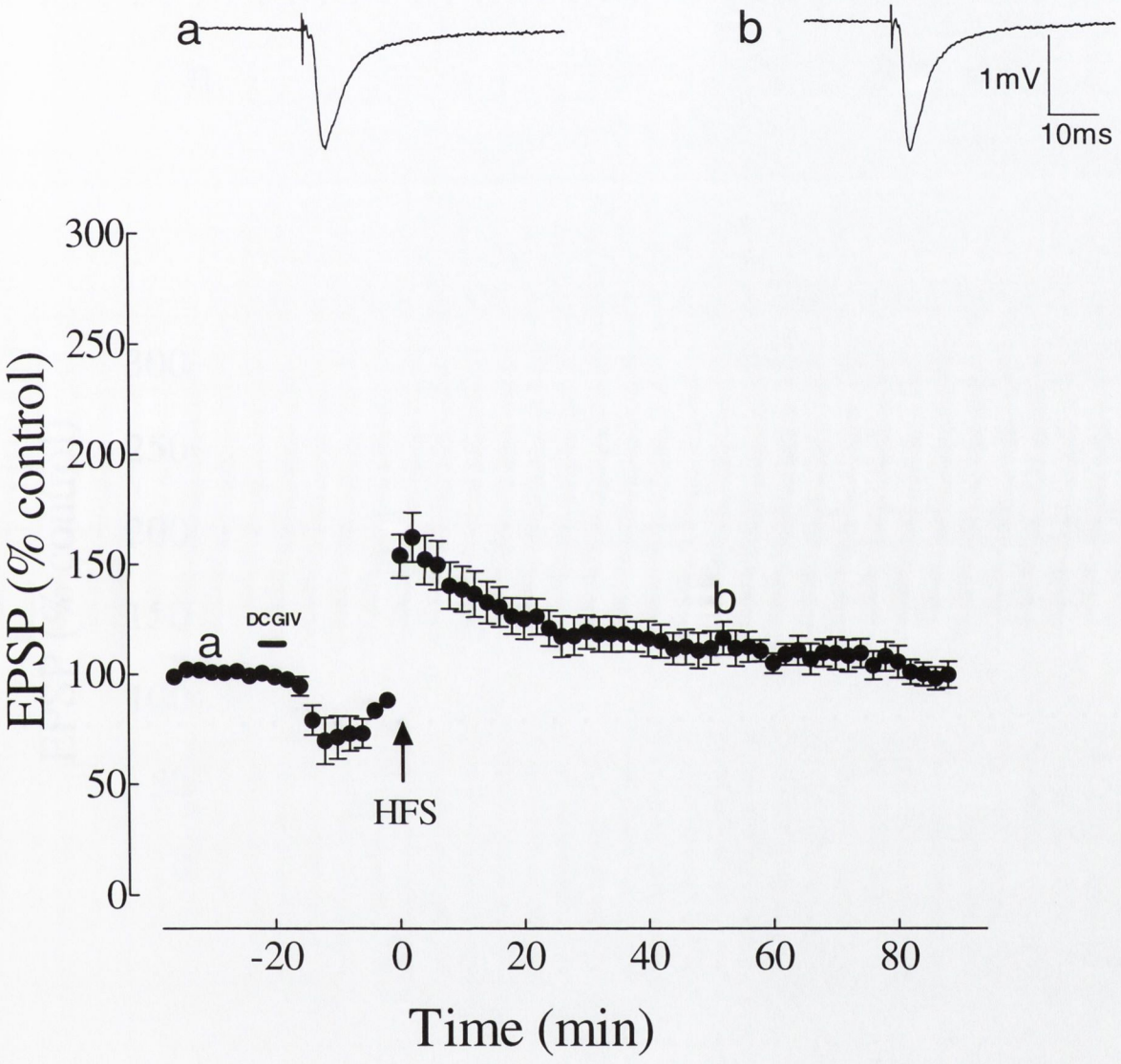


Figure 4.8 The mGluR group II agonist DCGIV inhibits the induction of LTP. Perfusion of DCGIV (2μM) for 5 min caused a depression of the EPSPs measuring 20± 3% (n=5; $p<0.01$). DCGIV applied 20 min prior to HFS inhibited LTP measuring 117 ± 5%, n=5 a value significantly different from control ($p<0.01$). Examples of EPSPs are shown in **a** (baseline) and **b** (1h after the induction of LTP).

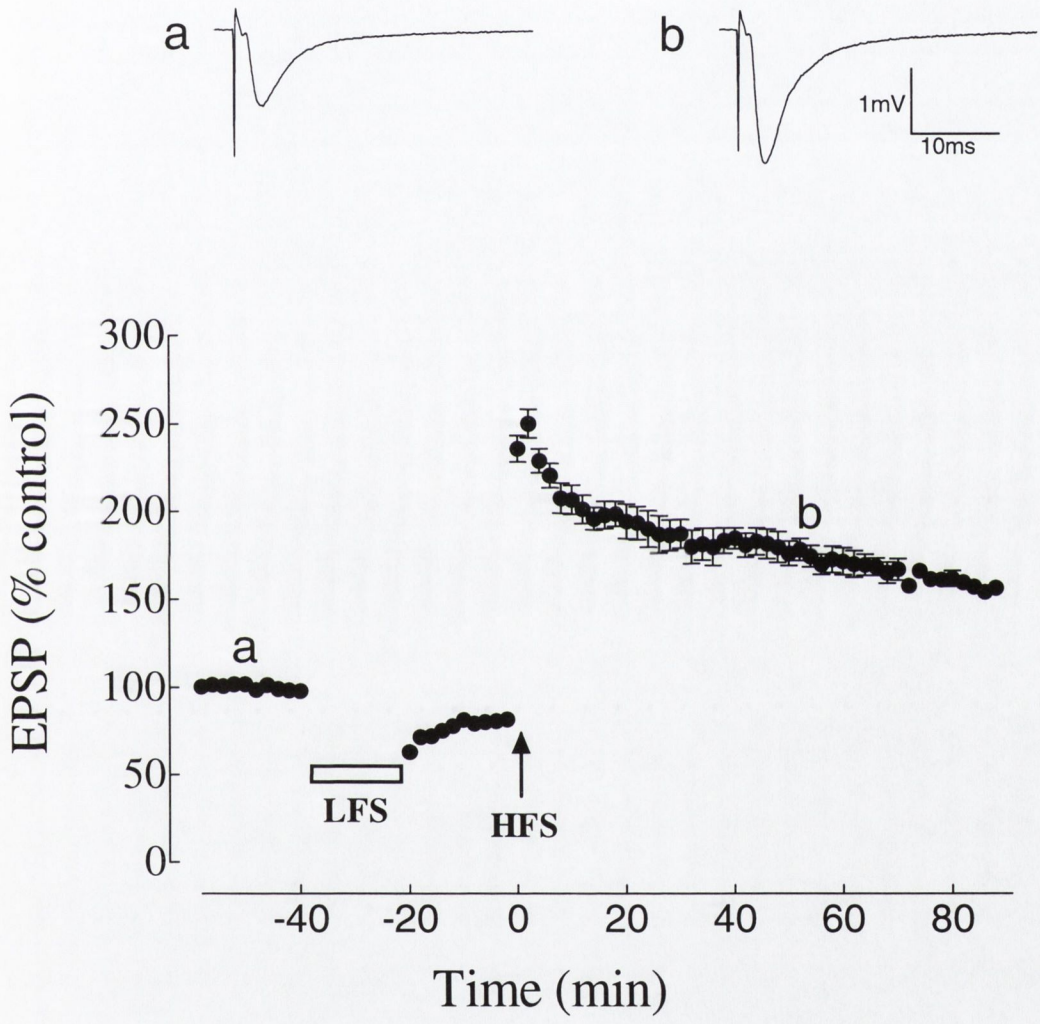


Figure 4.9 Low-frequency stimulation (LFS) does not result in subsequent inhibition of LTP. LFS (900 stimuli at 1Hz for 15 min) induced a significantly enhanced LTD with EPSPs measuring 62±2% ($p<0.01$; $n=5$). Subsequent LTP induction measured 168±7% a value not significantly different from control ($p>0.5$) was not inhibited by LFS.

4.5 Discussion

The present studies have presented evidence that both NMDA receptors and mGluR receptors are both involved in the inhibition of LTP by preconditioning stimulation.

These findings support previous studies describing the essential role of these receptors in the process of metaplasticity (Cohen and Abraham, 1996; Akiva et al., 1996; 1999 Huang et al., 1991; Aniksztejn et al., 1992; Harvey et al., 1993; Challiss et al., 1994; Bortolotto et al., 1994; Kato et al., 1999; Attucci et al., 2001; Tompa et al., 1998; Selig et al., 1995; Cummings 1996; O'Leary et al., 1998).

To investigate the involvement of mGluRs and NMDARs on the inhibition of LTP by preconditioning stimulation two different types of priming have been used. In the first type weak tetani (with the receptor antagonist) and in the second one a pharmacological means like the agonist activating the receptor under investigation.

The application of the group I mGluR agonist, DHPG, and of the group II mGluR agonist DCGIV, for 5 min and 20 min respectively before HFS, both inhibited subsequent LTP induction. This is consistent with other results where weak tetani in the presence of mGluR antagonists, inhibits subsequent LTP induction. In contrast, the application of NMDA prior to HFS facilitated LTP, but the application of NMDA during weak tetani seems to have the opposite effect because it is involved in the subsequent LTP inhibition.

An increase or decrease of synaptic plasticity seems therefore strictly dependent on the nature of the stimulation, and particularly on the timing and pattern of activation. This means also that only certain patterns of activation can have a modulatory effect on subsequent synaptic plasticity. In fact, application of LFS-induced LTD 20 mins prior to HFS did not inhibit LTP induction and the LTP value was then not different from the value of the LTP –control (Fig. 4.9, Fig.3.1) (Fujii et al., 1991; Huang et al., 1992; Christie et al., 1992; 1995; Frey et al., 1995; Abraham et al., 2001; 1997; 1996; Mockett et al., 2002; Cohen et al., 1996). Therefore different types of priming might have different effects dependent on the nature of the pattern of stimulation. Because different receptors have different functions in the complex machinery of synaptic plasticity, the consequent metaplastic effect on synapses will change on the basis of the different action expressed by these different receptors in conjunction with other macromolecules interacting with th

Chapter 5

Second messengers involved in the inhibition of LTP induction by preconditioning stimulation in the rat dentate gyrus *in vitro*

5.1 Overview

The involvement of second messengers such as protein kinase C (PKC), protein kinase A (PKA), MAP kinases and tyrosine kinase in the inhibition of LTP by preconditioning stimulation, was investigated in the medial perforant pathway of the dentate gyrus in *vitro*.

Evidence of the involvement of PKC in the inhibition of LTP by preconditioning stimulation was demonstrated by experiments in which the presence of the PKC inhibitor Bis-1 and Ro-31-8220 during preconditioning stimulation was able to prevent the inhibition of LTP. Also a selective known PKA inhibitor, H-89, was applied during weak tetani. H-89 reversed the inhibition of LTP by preconditioning stimulation. Two inhibitors of MAP kinase pathways were investigated on the preconditioning inhibition of LTP induction, SB 203560, which inhibits the p38 MAP kinase and PD 98059 which blocks MEK upstream to p42/44 MAP kinase. SB 203560 blocked the inhibition of LTP induction by preconditioning stimulation, but PD 98059 did not block the inhibition of LTP by priming stimulation.

The involvement of tyrosine kinase in the inhibition of LTP induction by preconditioning stimulation was investigated using the selective tyrosine kinase inhibitor Lavendustin A. The presence of lavendustin A during the preconditioning stimulation did not block the preconditioning stimulation inhibition of LTP induction.

The possible involvement of Ca^{2+} channels was investigated. For this purpose nifidipine, a specific L-type calcium channel blocker was applied throughout the experiments, but it did not prevent LTP inhibition by the priming stimulation.

5.2 Introduction

The involvement of second messengers in metaplasticity has been shown in previous studies (Akiva et al., 1996; Stanton et al., 1995; Bortolotto et al., 2000; Coogan et al., 1999; Malenka et al., 1994; Bear et al., 1994; Zurner et al., 2000), but second messenger involvement seems to be extremely complex and the impact on the targets of each second messengers and the interaction between them in the priming modulation of LTP induction is not completely clear.

Since activation of PKC seems to be an event that accompanies, and may contribute to, the induction of LTP, it has been hypothesised that activation of PKC might also alter the sensitivity of synapses for subsequent induction of LTP or LTD (Stanton et al., 1995, Bortolotto and Collingridgeet, 2000). To support this hypothesis there are studies that show how PKC activation could prime synapses of CA1 region enhancing LTD and inhibiting LTP induction. In these studies PKC can elicit a metaplastic effect through phorbol esters, which transiently activate PKC, reducing LTP but facilitating LTD induction (Stanton et al., 1995). Also PKA represents a candidate for mediating priming of LTP, although its role as a primer is not still well known. PKA is a cAMP dependent kinase that has been implicated in NMDA receptor-dependent LTP. It has been suggested that PKA might be important for the late phase of LTP (Frey et al., 1993), but opposite conclusions has also been reported by other studies (Otmakhov et al., 2002).

Activation of mGluRs can also modify the activity of protein kinases (PKA and PKC). More specifically, mGluRs has been classified in into three general groups of receptors by sequence homology and agonist affinity methods. Group I mGluRs increase phospholipid metabolism and PKC activity and group II/III decrease cAMP levels and PKA activity. It has also been suggested that PKA levels are not increased directly as an effect of mGluR activation, but rather as result of activation of PKC (Ramakers et al., 1997, Conn et al., 1997, Pin et al., 1995). Interestingly, PKA in turn inhibits mGluR2 coupling to G-proteins by direct receptor phosphorylation. This last result demonstrates a reciprocal interaction between PKA and group II mGluRs, probably in response to the nature of the stimulus interacting with them (Shaffhauser et al., 2000). The understanding of the interaction between these receptors and protein kinases might give new insight in to the comprehension of metaplastic changes at synapses. In the present study the role of two types of MAP kinases in the inhibition of LTP by preconditioning stimulation has been investigated, i.e. the p38 MAP kinase and the mitogen-activated protein kinase (MAPK). Three subgroups of the MAPK superfamily have been identified: the extracellular responsive kinases ($p42/44^{MAPK}$); the c-Jun N-terminal kinases ($p46/54^{JNK}$), also known as stress-activated protein kinases; and the $p38^{MAPK}$.

It has been suggested that MAPK and $p38^{MAPK}$, might regulate opposing forms of synaptic plasticity (Bolshakov et al., 2000). However, this theory has not been tested yet (Bolshakov et al., 2000). MAPK belongs to the group of serine/threonine dual

specificity kinases that seems to be activated by growth factors and in cellular proliferation and differentiation (Seger and Krebs, 1995). There are several reports indicating that the activation of the MAPK cascade is a requirement for the maintenance of LTP, since the activity-dependent activation of MAPK underlie some cellular processes, especially gene transcription and CREB transcriptional pathways (Adam et al., 2000; Robertson et al., 1999; English et al., 1996; 1997, Pereira et al., 2002; Impey et al., 1999). In contrast to the effect of MAPK, much experimental evidence indicates an inhibitory role of $p38^{MAPK}$ on LTP in the rat hippocampus. In one study for instance the use of the $p38^{MAPK}$ inhibitor SB203580 blocked the LTP inhibition (Coogan et al., 1999). Similar results confirm an inhibitory effect of $p38^{MAPK}$ on LTP (O'Donnell et al., 2000, Saleshando et al., 2000).

This last finding of the inhibitory role of $p38^{MAPK}$ on LTP is in agreement with the present work, where $p38^{MAPK}$ is involved in the inhibition of LTP by preconditioning stimulation. Moreover, P38 MAPK seems to be present in the pyramidal neurones in both CA1, CA3 regions and in the dentate gyrus, a strategic position for regulation of synaptic plasticity (Bolshakov et al., 2000).

5.3 Material and methods

The animals, ACSF solution and preparation of hippocampal slices and field potential were described in Chapter II.

Drugs used were N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl (H-89, HCl), Ro-31-8220 and Bisindolylmaleimide I (Bis-I) (all from Calbiochem). 4-(4-fluoropheny)-2-(4-methylsulfonilphenyl)-5-(4-pyridil) imidazol (SB203580) (Calbiochem, Lucerne Switzerland) and 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) (Alexis Corporation) were dissolved in DMSO. H-89, HCl and Bisindolylmaleimide I were dissolved in H_2O and Ro-31-8220 in DMSO. All these kinases inhibitors were perfused for at least 1 hour before the application of weak tetani, during which neither inhibitor altered baseline EPSPs.

Lavendustin A and Nifedipine were dissolved in DMSO (Tocris). The final concentration of DMSO was less than 0.05%.

5.4 Results

5.4.1 The PKC inhibitor Bisindolylmaleimide I does not block LTP induced by HFS at synapses receiving only test stimulation

Bisindolylmaleimide I (BIS I) is a highly selective protein kinase C (PKC) inhibitor with a K_i of 10 nM (Nixon et al., 1992). BIS I (1μM) was preperfused for at least 1 hr before the application of HFS, during which the test EPSPs was not altered in amplitude. Bis I was then perfused throughout the experiment and HFS induced LTP measuring 174 ± 6%, n=4, a value not significantly different ($p>0.05$) from that in the absence of Bis I (Fig. 5.1, Fig. 3.1). Therefore in these control studies, BIS I did not alter the amplitude of LTP induced by HFS.

5.4.2 Inhibition of PKC by Bisindolylmaleimide I prevents the block of LTP by preconditioning stimulation

The involvement of PKC in the preconditioning stimulation was investigated by applying Bis I (1μM), 1 hour before weak tetani and throughout the experiment. BIS I reversed the preconditioning stimulation inhibition of LTP induction, LTP measuring 174 ± 9%, n=5, a value significantly different from the experiments showing that weak tetani applied 20 min prior HFS inhibit LTP ($p<0.01$) (Fig. 5.2, Fig. 3.2).

5.4.3 Inhibition of PKC by Ro-31-8220 prevents the block of LTP by pre - conditioning stimulation

To confirm the results of the previous set of experiments another PKC inhibitor, Ro-31-8220, which also has a high affinity for PKC (K_i = 10 nM) (Nixon et al., 1992) was applied during weak tetani. Ro-31-8220 also reversed the preconditioning inhibition of PKC. Thus LTP measured 159 ± 6%, n=4 following preconditioning stimulation in the presence of Ro-31-8220 (2.5 μM), perfused

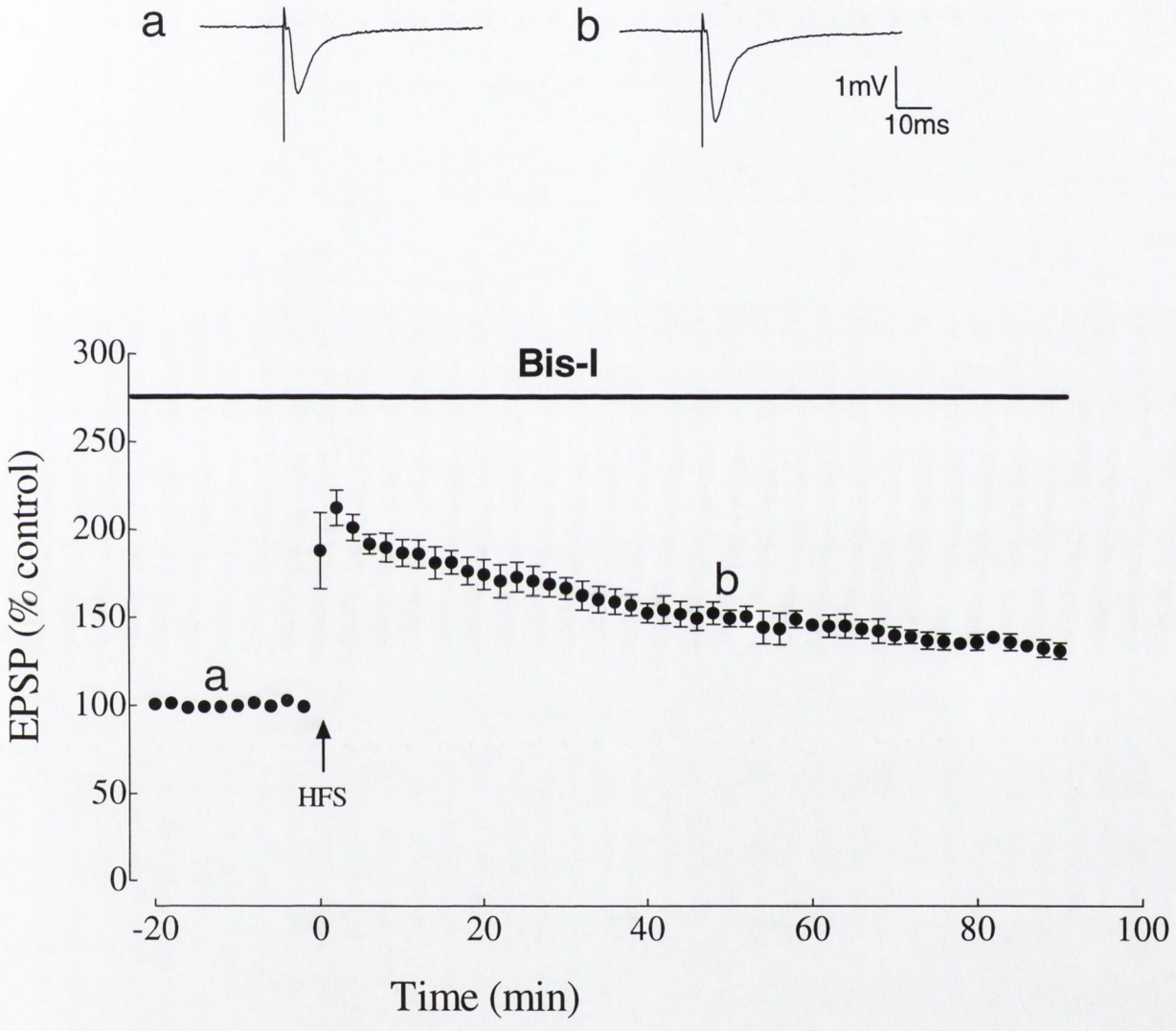


Figure 5.1 LTP is not inhibited by PKC inhibitor. In the presence of the PKC inhibitor Bis-I at a concentration of 1μM, HFS induced an LTP measuring 174±6% (n=4) at 1h after the delivery of HFS. This LTP was not significantly different from the LTP induced in control solution ($p>0.05$) Examples of EPSPs are shown in **a** (baseline) and **b** (after the induction of LTP). Calibration bar is 1mV, 10 ms.

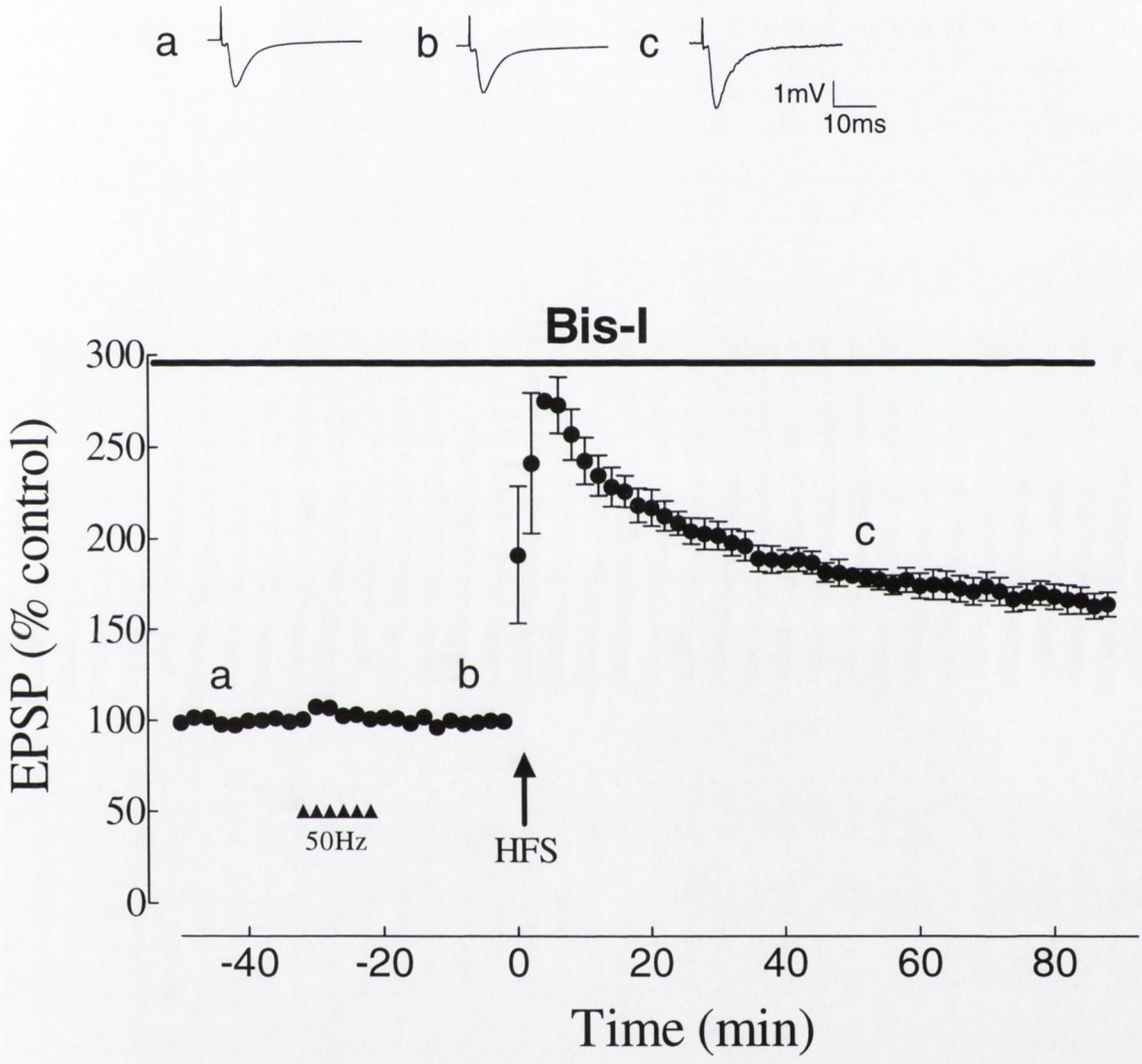


Figure 5.2 Inhibition of PKC prevents the block of LTP by preconditioning stimulation. In the presence of the PKC inhibitor Bis-I (1μM), preconditioning stimulation did not inhibit LTP measuring 174±9% (n=5) at 1h after the delivery of HFS. This LTP value was significantly different ($p<0.01$) from the experiments showing that preconditioning stimulation inhibit LTP. The traces show field EPSPs prior to (a), following (b) preconditioning stimulation and (c) after application of HFS (1h after the induction of LTP).

throughout the experiment and for at least 1 hour before weak tetani without any change in baseline EPSPs ($P<0.01$) (Fig. 5.3, Fig. 3.2)

5.4.4 Inhibition of PKA by H-89 Dihydrochloride prevents the block of LTP by preconditioning stimulation

The involvement of PKA in the inhibition of LTP induction by preconditioning stimulation was investigated using the selective PKA inhibitor H89. This agent inhibits the catalytic site of PKA with a K_i of 48 nM (Nixon et al., 1992). H89 (10μM) was preperfused for 1 hr, during which baseline EPSPs were not altered in amplitude. The presence of H89 during the preconditioning stimulation was found to reverse the preconditioning inhibition of LTP, LTP measuring 181 ± 9%, n=5, a value significantly different from the experiments showing that weak tetani applied 20 min prior to HFS inhibit LTP ($p<0.01$) (Fig. 5.4, Fig. 3.2).

5.4.5 Tyrosine kinase inhibitor Lavendustin A does not block LTP induced by HFS at synapses receiving only test stimulation

The involvement of tyrosine kinase in the inhibition of LTP induction by preconditioning stimulation was investigated using the selective tyrosine kinase inhibitor Lavendustin A (IC_{50} for pp60 c-src = 500 nM). Lavendustin A (5 μM) was preperfused for 1 hr, during which baseline EPSPs were not altered in amplitude and HFS induced LTP measuring (161 ± 5%, n=5), a value not significantly different ($p>0.5$) from that in the absence of Lavendustin A (Fig. 5.5, Fig. 3.1). Therefore in this control study, Lavendustin A did not alter the amplitude of LTP induced by HFS.

5.4.6 Tyrosine kinase inhibitor Lavendustin A did not reverse the block of LTP by preconditioning stimulation

The presence of the selective tyrosine kinase inhibitor lavendustin A (5μM) during the preconditioning stimulation did not reverse the preconditioning stimulation inhibition of LTP induction, LTP measuring 126 ± 6%, n=5, a value not significantly

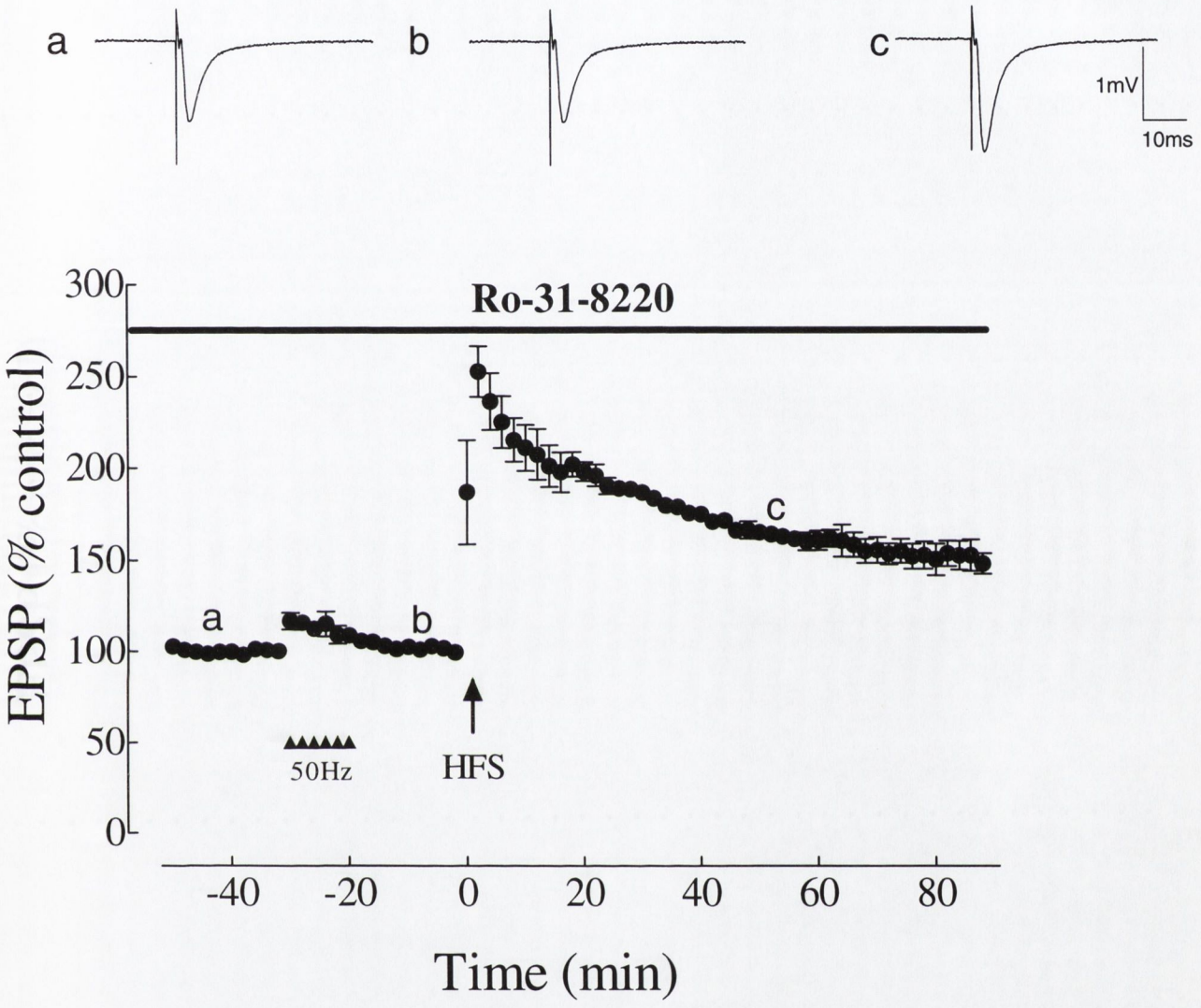


Figure 5.3 Inhibition of PKC by Ro-31-8220 prevents the block of LTP by preconditioning stimulation. In the presence of the PKC inhibitor Ro-31-8220 at a concentration of 2.5μM, preconditioning stimulation did not inhibit LTP measuring 159 ±6% (n=4) at 1h after delivery of HFS. This LTP value was significantly different ($p<0.05$) from the experiments showing that preconditioning stimulation inhibit LTP induction. The traces show EPSPs prior to (a), following preconditioning stimulation (b) and after 1h from the application of HFS (c).

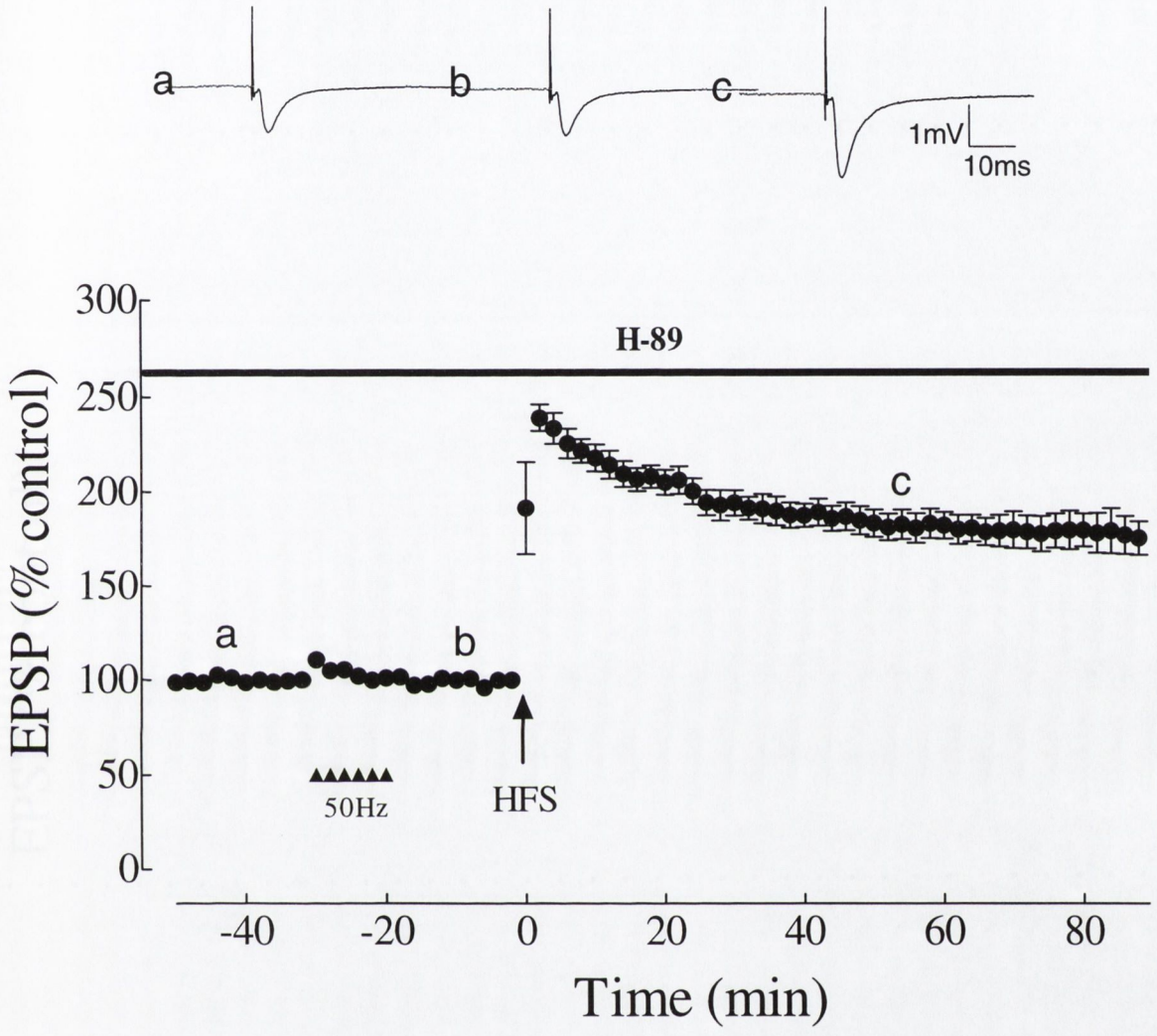


Figure 5.4 Inhibition of PKA by H-89 prevents the block of LTP by preconditioning stimulation. In the presence of the PK A inhibitor H-89 at a concentration of 10μM, preconditioning stimulation did not inhibit LTP measuring 181±9% (n=5) at 1h after delivery of HFS. This LTP value was significantly different ($p<0.01$) from the experiments showing that preconditioning stimulation inhibit LTP induction. The traces show EPSPs prior to (a), following preconditioning stimulation (b) and after 1h from the application of HFS (c).

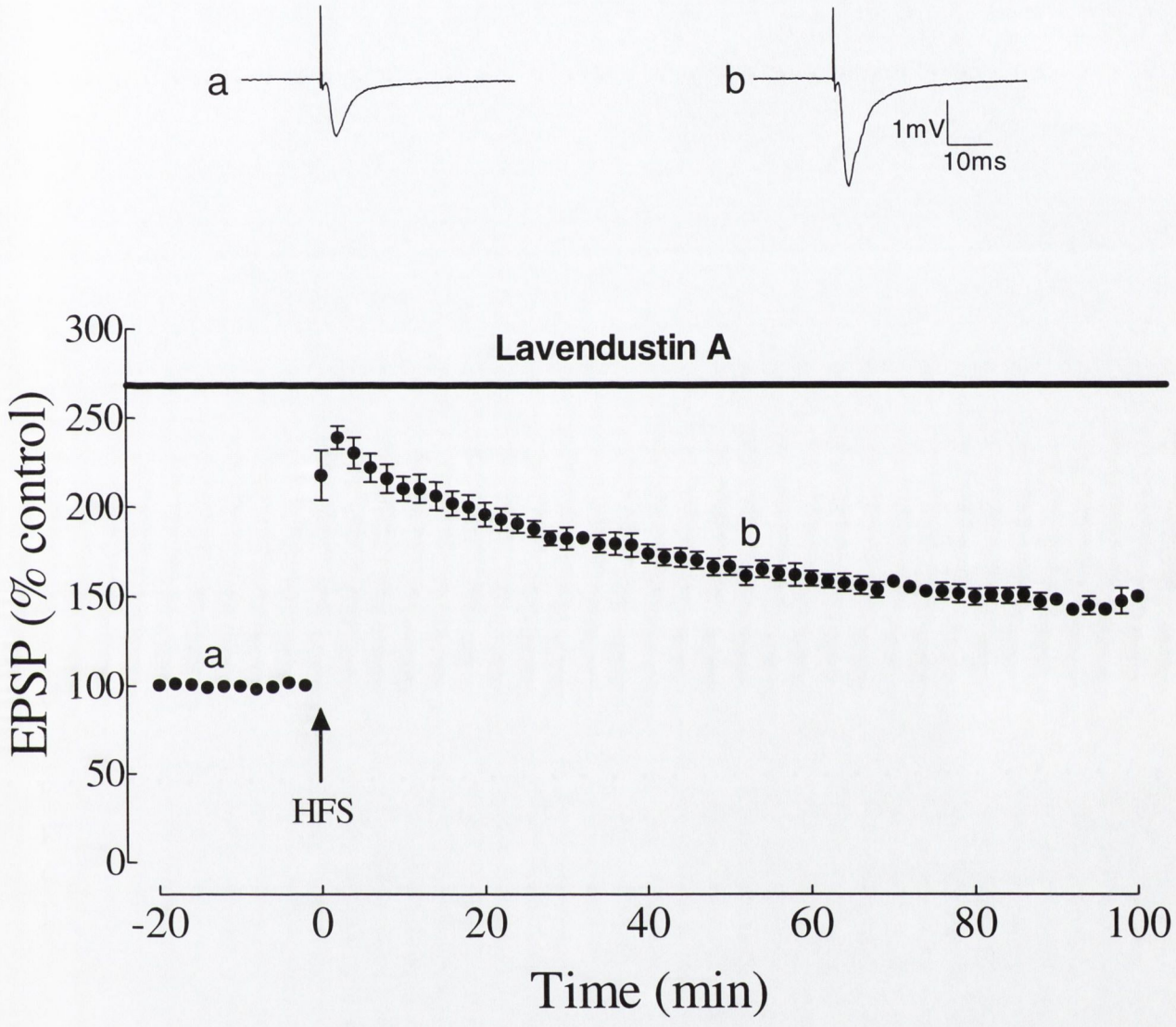


Figure 5.5 LTP is not inhibited by tyrosine kinase in the presence of the tyrosine kinase inhibitor Lavendustin A at a concentration of 5μM, HFS induced an LTP measuring 161±6% (n=5) at 1h after the delivery of HFS. This LTP was not significantly different from the LTP induced in control solution ($p>0.5$)
Examples of EPSPs are shown in **a** (baseline) and **b** (1h after the induction of LTP). Calibration bar is 1mV, 10 ms.

different from the experiments showing that weak tetani applied 20 min prior HFS inhibit LTP ($p>0.5$) (Fig.5.6, Fig. 3.2).

5.4.7 The MAPKK/MEK inhibitor PD-98059 does not block LTP induced by HFS at synapses receiving only test stimulation

The MAPKK inhibitor PD-98059 (50μM) that blocks MEK upstream to p42/44 MAP kinase (English et al., 1996) was preperfused 1 hour prior to HFS-induced LTP during which baseline EPSPs were not altered in amplitude and HFS induced LTP measuring (161 ± 11%, n=5), a value not significantly different ($p>0.5$) from that in the absence of PD-98050 (Fig. 5.7, Fig. 3.1). Therefore in this control studies, PD-98050 did not alter the amplitude of LTP induced by HFS.

5.4.8 The MAPKK/MEK inhibitor PD-98059 does not prevent the block of LTP by preconditioning stimulation

The effect of the MAPKK inhibitor PD-98059 inhibitor was investigated on the preconditioning inhibition of LTP induction. For this purpose PD-98059 (50μM) was preperfused for 1 hr prior to the preconditioning stimulation, and throughout the experiment. PD-98059 did not reverse the inhibition of LTP induction by preconditioning stimulation, LTP measuring 109 ± 10%, n=5, a value not significantly different from the experiments showing that weak tetani applied 20 min prior to HFS inhibit LTP ($p>0.5$) (Fig.5.8, Fig. 3.2).

5.4.9 The p38 MAP kinase ininhibitor SB-203580 reverses the inhibition of LTP by preconditioning stimulation

A second type of MAP kinase, P38 MAP kinase was investigated in the preconditioning mechanism of LTP. SB-203580 is a highly selective inhibitor of the P38 MAP kinase with an IC_{50} value of 34 nM (Lee et al., 1994) that has been proven to have no effect in controlling LTP (Coogan et al., 1999). Application of SB-203580 (1μM) in hippocampal slices during preconditioning stimulation, in contrast to the effect of the previous MAP kinase inhibitor, reversed the subsequent LTP inhibition

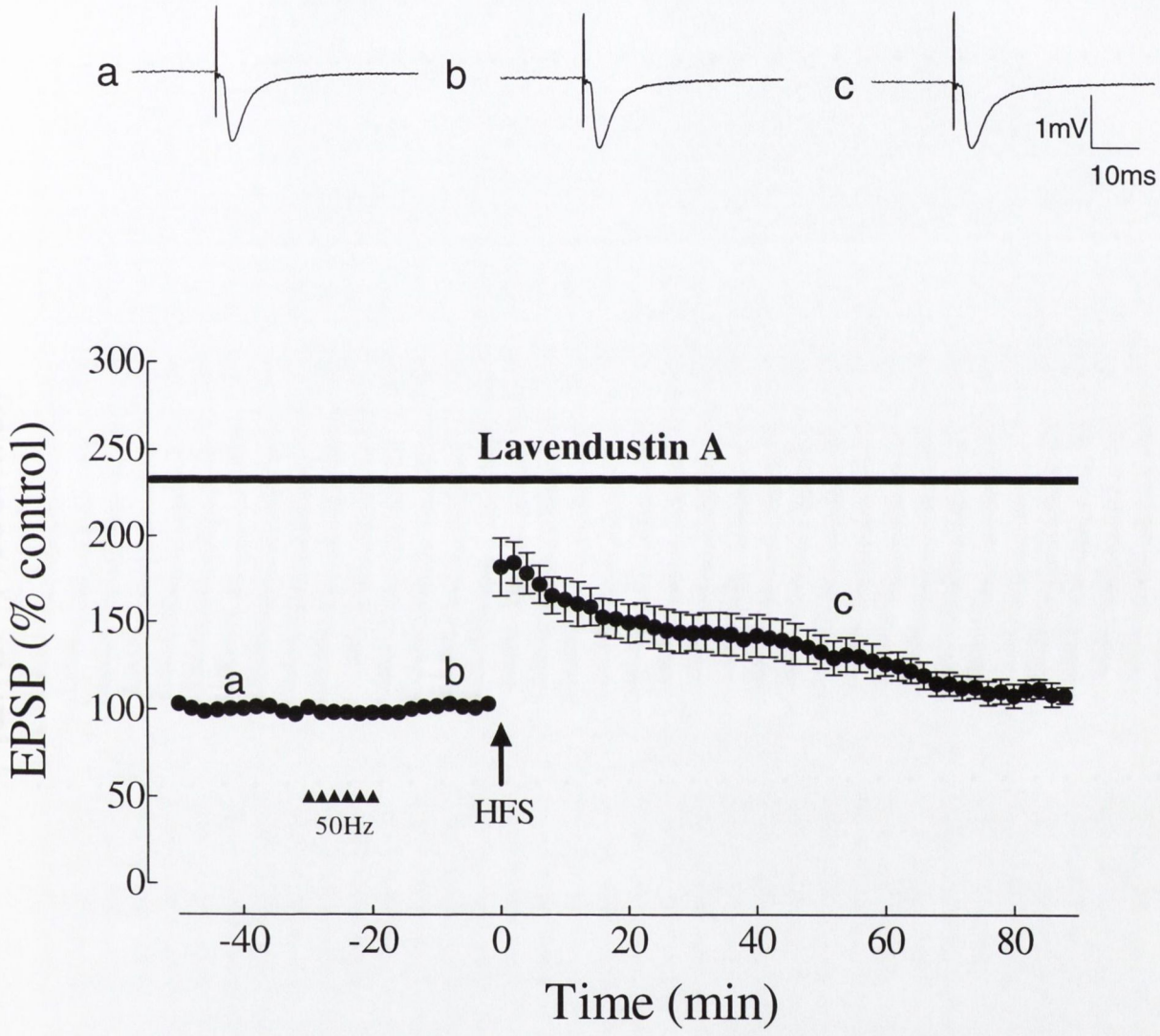


Figure 5.6 Preconditioning inhibition of LTP does not involve the activation of tyrosine kinase. In the presence of the tyrosine kinase inhibitor Lavendustin A (5μM), preconditioning stimulation inhibited LTP measuring 126±6% (n=5) at 1h after the delivery of HFS. This LTP was not significantly different from the experiments showing that preconditioning inhibit LTP ($p>0.5$). The traces show field EPSPs prior to (a), following preconditioning stimulation (b) and (c) after application of HFS (1h after the induction of LTP).

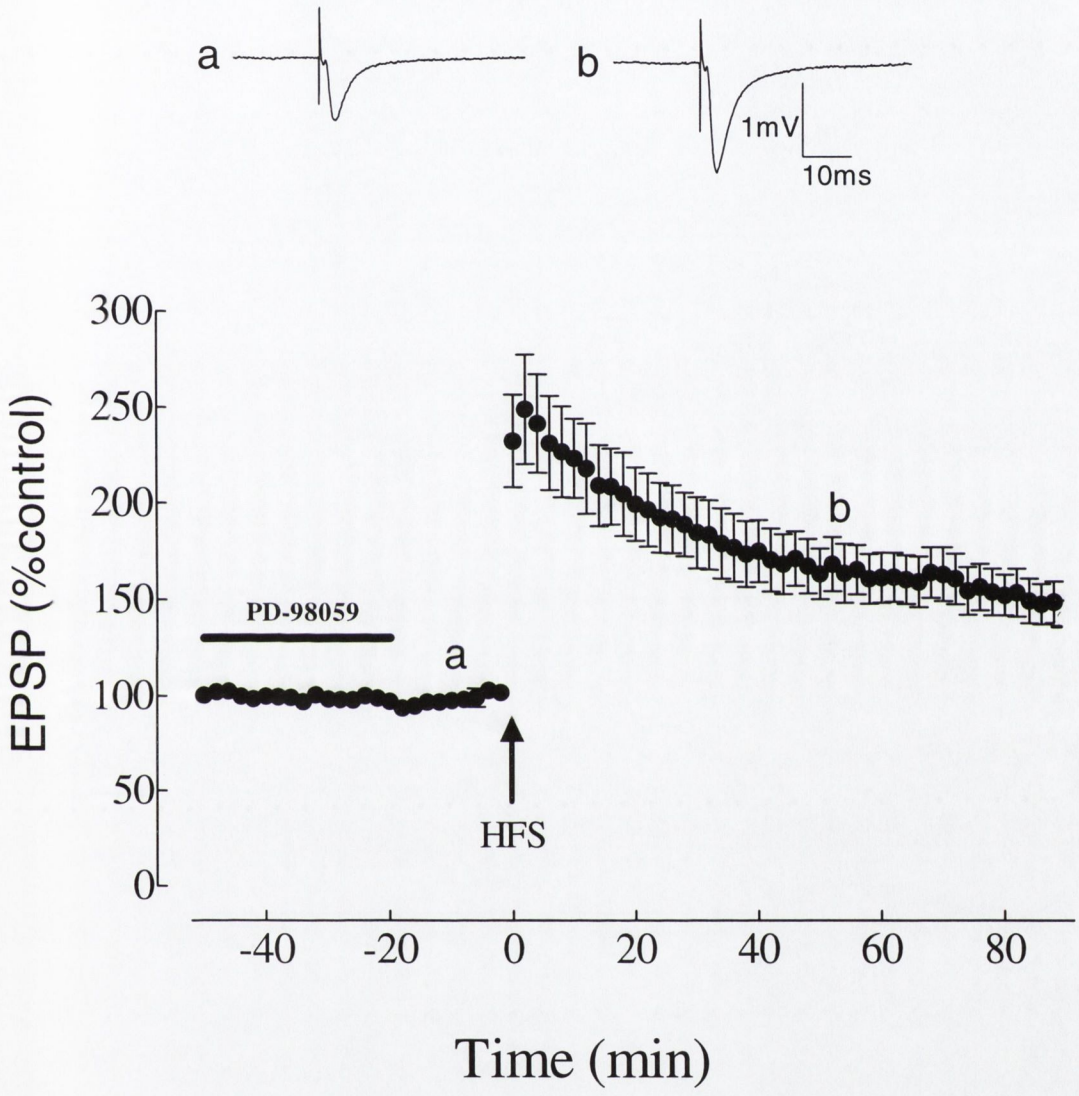


Figure 5.7 LTP is not inhibited by MAPKK/MEK inhibitor or at least not when perfused -50 to -20 min pre-HFS. The MAPKK/MEK inhibitor PD-98059 (50μM) preperfused 1h and washout 20 min prior HFS induced an LTP measuring 161±11% (n=5) at 1h after the delivery of HFS. This LTP was not significantly different from the LTP (Fig.3.1) induced in control solution ($p>0.5$) Examples of EPSPs are shown in **a** (baseline) and **b** (after the induction of LTP). Calibration bar is 1mV, 10 ms.

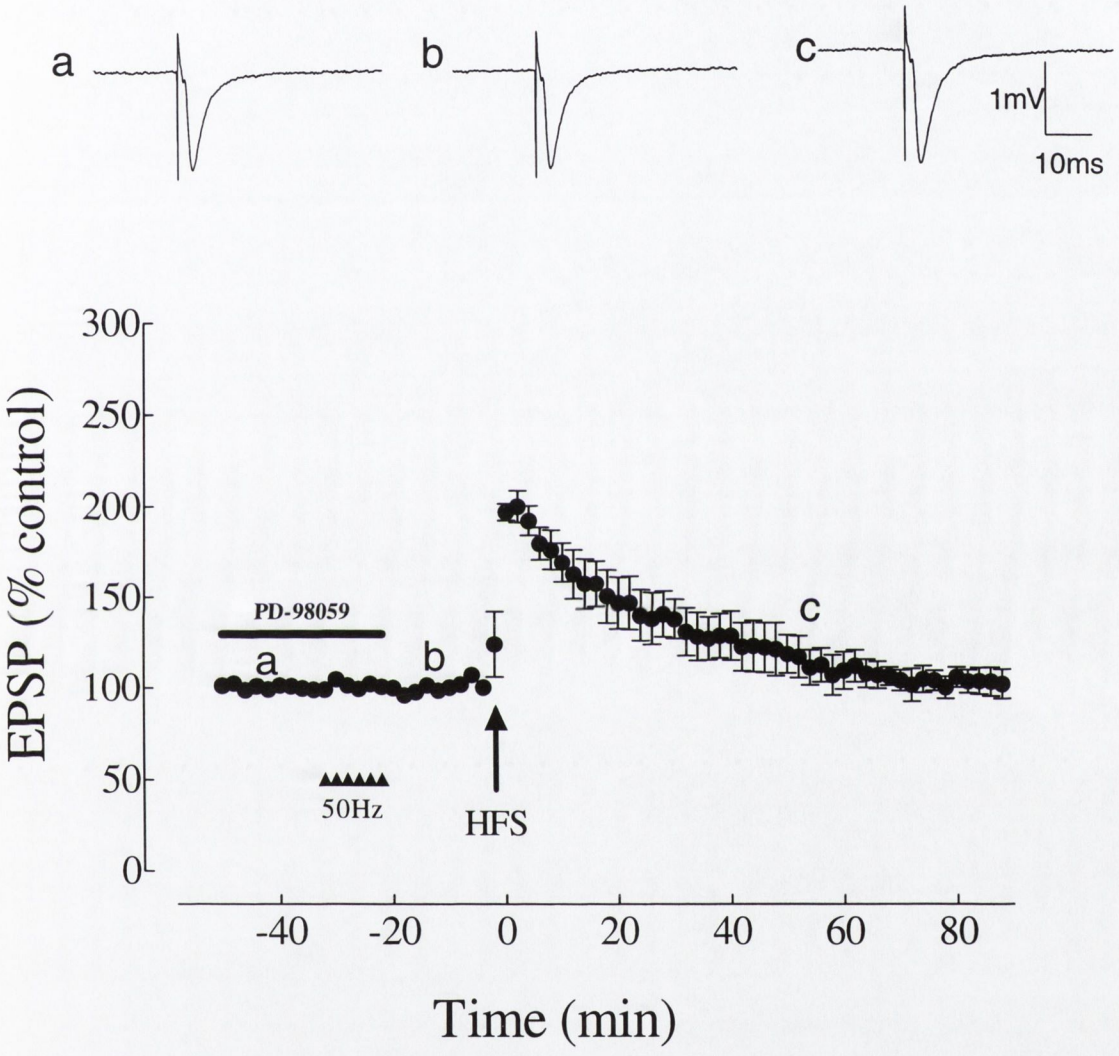


Figure 5. 8 Preconditioning stimulation (weak HFS) does not involve MAPKK/MEK. In the presence of the MAPKK/MEK inhibitor PD-98059 (50μM), preconditioning stimulation inhibited LTP measuring 109±10% (n=5) at 1h after the delivery of HFS. This LTP was not significantly different from the experiments (Fig. 3.2) showing that preconditioning inhibit LTP ($p>0.5$). The traces show field EPSPs prior to (a), following preconditioning stimulation (b) and (c) after application of HFS (1h after the induction of LTP).

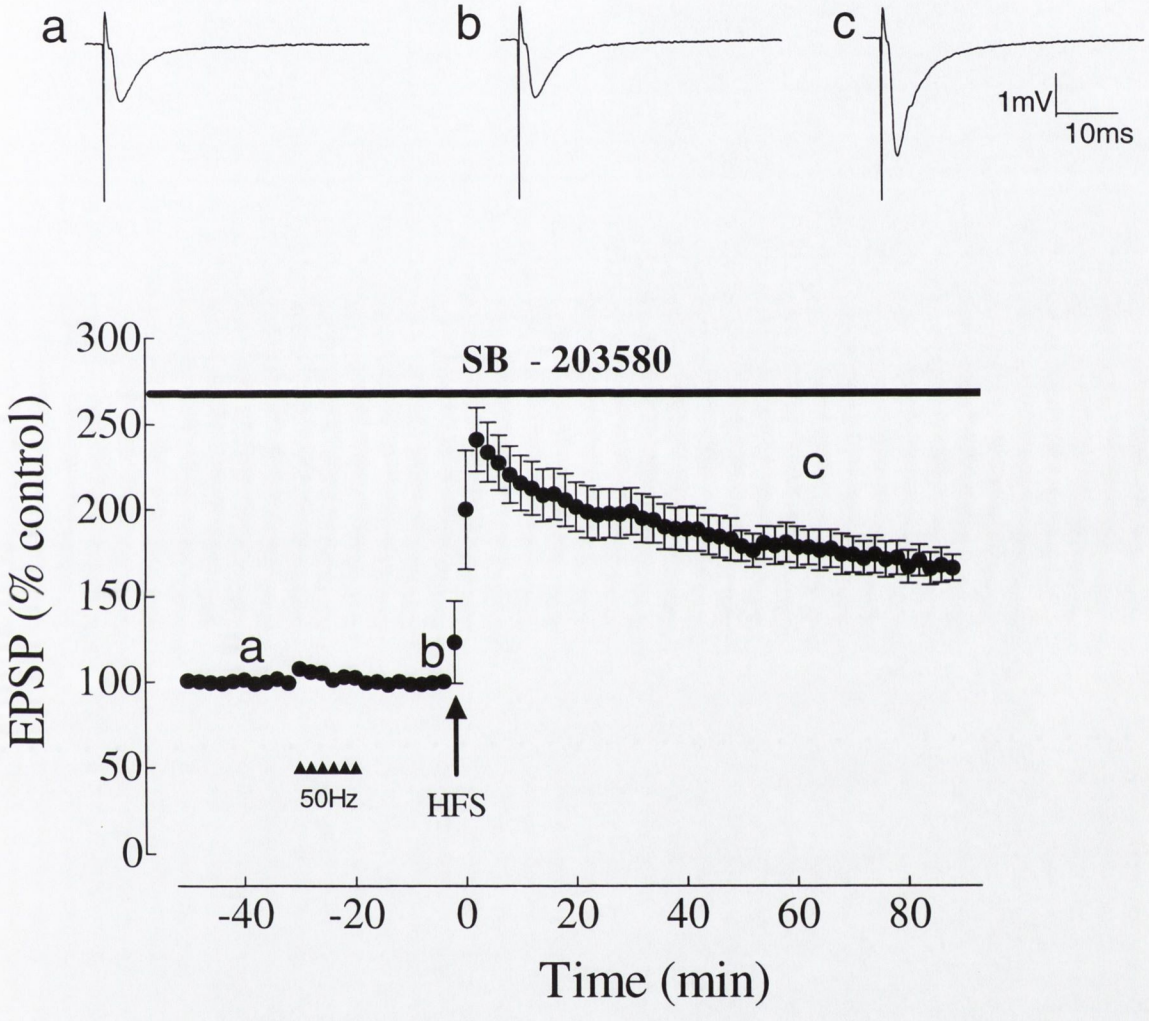


Figure 5.9 The p38 MAP kinase inhibitor SB-203580 reverses the inhibition of LTP by preconditioning stimulation. In the presence of the p38 MAP kinase inhibitor sb-203580 (1 μM), preconditioning stimulation did not inhibit LTP measuring 178±9% (n=5) at 1h after the delivery of HFS. This LTP value was significantly different ($p<0.5$) from the experiments showing that preconditioning stimulation inhibit LTP. The traces show field EPSPs prior to (a), following (b) preconditioning stimulation and (c) after application of HFS (1h after the induction of LTP).

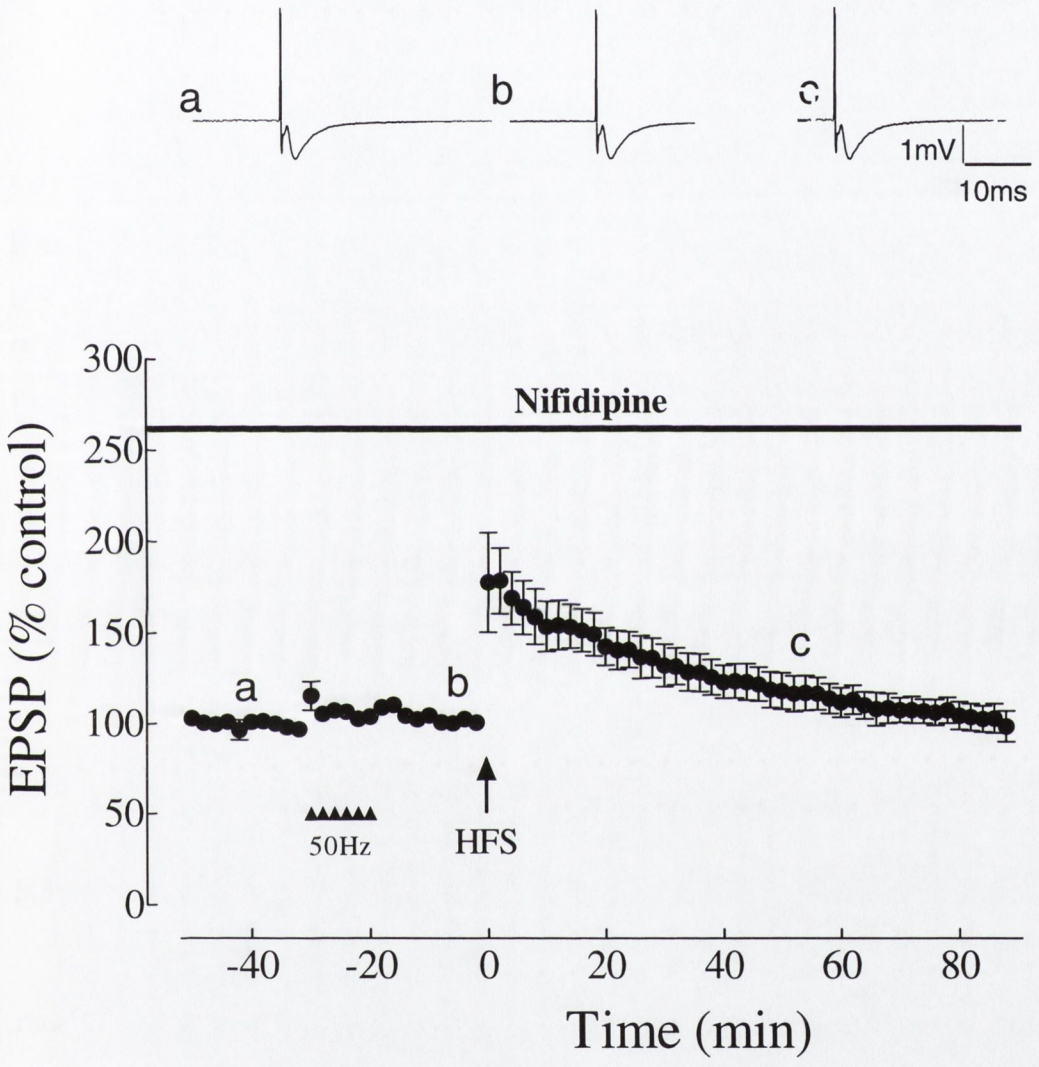


Figure 5.10 Preconditioning stimulation (weak HFS) does not involve activation of L-type Ca^{2+} channel. In the presence of the L-type Ca^{2+} channel inhibitor Nifidipine (10μM), preconditioning stimulation inhibited LTP measuring 111± 6% (n=4) at 1h after the delivery of HFS. This LTP was not significantly different from the experiments showing that preconditioning inhibit LTP ($p>0.5$). The traces show field EPSPs prior to (a), following preconditioning stimulation (b) and (c) after application of HFS (1h after the induction of LTP).

with LTP measuring 178 ± 9%, n=5, a value significantly different from the experiments showing that weak tetani applied 20 min prior HFS inhibit LTP ($p<0.5$) (Fig.5.9, Fig. 3.2).

5.4.10 The L-type Ca^{2+} inhibitor Nifidipine does not prevent the block of LTP by preconditioning stimulation

A possible involvement of Ca^{2+} channel has been investigated using an L-type calcium channel blocker, Nifidipine. Nifedipine (10 μM) was preperfused for 1 hr prior to preconditioning stimulation. The presence of nifedipine during the preconditioning stimulation did not reverse the preconditioning stimulation inhibition of LTP induction, LTP measuring 111 ± 6%, n=4, a value not significantly different from the experiments showing that weak tetani applied 20 min prior HFS inhibit LTP ($p>0.5$) (Fig. 5.10, Fig. 3.2).

5.5 Discussion

The present study has presented evidence that activation of a second messengers chain involving protein kinases (PKA, PKC) and the p38 -MAP kinase is responsible for the inhibition of LTP by preconditioning stimulation. In this complex event it seems that most of the macromolecules involved in the 'synaptic plasticity machinery' are the same as those involved in metaplasticity.

Since it seems that Ca^{2+} might be an important component in triggering the priming effect, the role of L-type Ca^{2+} channel has also been tested in the inhibition of LTP, which showed that Nifidipine, an L-type Ca^{2+} channel inhibitor did not prevent LTP inhibition, by weak tetani. This is not surprising since the priming effect might also involve other Ca^{2+} sources, for instance intracellular Ca^{2+} stores.

Previous experimental evidence relating to the involvement of protein kinases in the priming mechanism supports the findings of the present work (Stanton et al., 1995; Bortolotto et al., 2000). Taken together, the results presented in the present and in the previous chapters represent the most likely mechanism of inhibition of LTP being through the PKC-mediated inactivation of group I mGluRs via a feedback loop, with the activation of mGluRs by the preconditioning stimulation resulting in activation of PKC and the subsequent inactivation of the group I mGluRs. PKC might then desensitise mGluR via a mechanism that has already been described in previous studies (Alagarsamy et al., 1999; 2001, De Blasi et al., 2001; Schoepp et al., 1988; Guerineau et al., 1997; Gereau et al., 1997; 1998). There might be also a crosstalk between PKC and PKA as has been reported in previous studies (Borner et al., 1989; Wooten et al., 1996) and a synergistic effect between group I and II mGluRs, in which group II mGluR agonists were found to potentiate PI hydrolysis mediated via activation of group I mGluRs (Nicoletti et al., 1993; Genazzani et al., 1994). mGluRs might in turn downregulate NMDARs to finally inhibit LTP by preconditioning stimulation (Nicoletti et al., 1993; De Blasi et al., 2001). P38 MAP kinase might contribute to this chain of effects stimulating DAG and consequently activate PKC as has been shown in previous reports (Shimizu et al., 1999).

Chapter 6

General discussion

6. General discussion

The results described in the preceding chapters demonstrated clearly how synaptic plasticity like LTP might be modified by the previous history of synaptic activity. The inhibition of LTP by preconditioning stimulation represented by weak tetani has been observed previously in CA1 (Huang et al, 1992). The results reported in this thesis have verified that similar inhibition does occur in the medial perforant path of the dentate gyrus. In addition this work complements the initial finding of Huang et al., with results that might suggest a possible model (Fig. 5.11) of the mechanism underlying the inhibition of LTP by previous weak tetani. This model represents a novelty of the interaction between receptors and second messengers that contribute to the inhibitory effect by preconditioning stimulation.

Substantial evidence for the modulation of LTP by previous modification of synaptic activity can be found in the literature (Cohen and Abraham, 1996; Huang et al., 1991;Anikstejn et al., 1992; Harvey et al., 1993; Challiss et al., 1994; Akiva et al., 1996; 1999; Bortolotto et al., 1994). The 'priming event' represented by weak tetani that inhibits the subsequent induction of synaptic plasticity may provide examples of shifting the sliding modification threshold, θ_M, proposed by Bienenstock, Cooper and Munro (1982). The concept of a sliding modification threshold is important for the preservation of a dynamic response range of the neuron to synaptic stimulation, thus preventing them from entering states of saturated LTP or LTD, and also providing a way for synapse to integrate a response across temporally spaced episodes of synaptic activity (Abraham et al., 1997). θ_M might then correspond to the LTD/LTP crossover point in conditioning frequency –response experiments (Dudek et al., 1992). In this theoretical model, activity mildly above the spontaneous level results in LTD while that above the modification threshold (θ_M) leads to LTP. The non-linear function θ is not fixed but shifts according to the history of prior activity (Bienenstock, 1982).

This concept is crucial in the understanding of how metaplasticity occurs in keeping synaptic strengths within a dynamic range that is optimal for the learning process. Therefore, a synapse that has been long-term potentiated through the application of HFS should have a mechanism by which it can reduce the enhanced response back

towards its original baseline (Tsumoto, 1993). This is particularly true if LTP is to be used as a cellular correlate in the modelling of information storage in the brain (Bliss and Collingridge, 1993). If this mechanism did not exist, sooner or later all synapses would become fully potentiated and no new information could be acquired. Therefore, a reversal process, in the form of a metaplastic event must take place to allow synapses to produce more synaptic plasticity. In light of this general explanation of the purpose of metaplasticity, the inhibition of LTP by weak tetani as 'priming event' might have such functional meaning.

The fact that the inhibition of LTP was present at 10 and 20 min, but not 2 or 45 min post preconditioning, indicates a temporal window for the inhibition. The fact that the inhibition occurred at 10-20 min indicates that it took several minutes to develop, suggesting evidence of the involvement of second messengers as has already been demonstrated by previous studies (Wang et al., 1998, Boxall et al., 1996; Stanton 1995). In fact, as is demonstrated in chapter V, preconditioning inhibition of LTP involves the activation of a second messengers chain constituted by protein kinases (PKA, PKC) and the p38 -MAP kinase.

This study also emphasises the important role played by the activation of mGluRs and NMDARs in the decreased level of synaptic plasticity after the 'priming event'.

There is substantial evidence presented in previous studies indicating the involvement of NMDAR and mGluRs in the modulation of LTP by previous modification of synaptic activity (Cohen and Abraham, 1996; Huang et al., 1991; Aniksztejn et al., 1992; Harvey et al., 1993; Challiss et al., 1994; Akiva et al., 1996; 1999; Bortolotto et al., 1994; Abraham et al., 2001). Experiments (see chapter IV) were carried out either by synaptic stimulation (weak tetani) or by pharmacological means using pharmacological agents to prime subsequent LTP induction.

Since NMDA receptor activation is fundamental for LTP and LTD it was obvious to consider the possible involvement of this receptor in metaplasticity. The application of D-AP5 during weak tetani restored the subsequent LTP induction, but in contrast the direct activation of this receptor with bath-application of NMDA prior to HFS-induced LTP induced an enhancement of LTP induction. Unfortunately it has been difficult to give a satisfactory explanation of this discrepancy of effect on LTP by weak tetani (with D-AP5) and NMDA application prior to HFS-induced LTP. A possible, general explanation might refer to the fact that different types of priming might have different effects on the modulation of synaptic activity. When discussing

different types of priming, I referred to the nature of the pattern of stimulation. For instance, that different frequencies of stimulation and also differences in the time between each frequency might modulate differently synaptic plasticity as has already been shown from previous studies (Fujii et al., 1991; Huang et al., 1992; Christie et al., 1992; 1995; Frey et al., 1995; Abraham et al., 2001; 1997; 1996; Mockett et al., 2002). The different application times and the drug concentration might also affect differently synaptic plasticity in a dose-dependent manner. In fact as Cohen et al (1996) demonstrated in CA1 and CA3 slices, the application of ACPD 30 min before the TBS facilitated the induction of LTP in a dose-dependent manner and resulted in an enhanced magnitude and stability of LTP.

A number of other authors support the finding that the activation of NMDAR might lead to the inhibition of LTP induction. In fact Coan et al (1989) find that perfusing hippocampal slices with Mg^{2+}-free solution, which enhances activation of NMDAR resulted in subsequent inhibition of LTP induction. Other experimental evidence in Huang et al (1992) showed that inhibition of LTP by weak tetani in CA1 was due to the activation of NMDA receptors because LTP occurred normally when AP5 was present during weak tetani. Also, the use of a low concentration of NMDA in hippocampal slices HFS inhibits LTP (Izumi et al., 1992; Kato et al., 1999). However the work of Coan et al, Izumi et al, and Kato et al, does not technically fall within the general definition of metaplasticity, because pharmacological manipulation was made right up to the time that tetanisation was given.

Moreover, a set of experiments also shows that there in no change in the NMDA component of field EPSP during weak tetani (Fig.4.2). These results might lead at first to the thought that NMDAR might not contribute to the inhibition of LTP. This apparent contradiction might find explanation in similar results in Kato et al. (1999) where in the CA1 region, inhibition of LTP by priming activation of NMDAR showed no effect on isolated NMDAR EPSCs (NMDAR –mediated excitatory postsynaptic currents). NMDARs might therefore contribute during the application of weak tetani to inhibit LTP through the activation of Ca^{2+}-dependent processes, induction of second messengers included phosphatases or protein kinases (see chapter V) or protein synthesis that might have an inhibitory effect on the maintenance of LTP (Wexler et al., 1993, Stanton 1995, Cohen et al., 1995). In fact the individual subunits of the NMDAR can be modified by dynamic post-translational phosphorylation and by their interaction with other specific proteins. For instance calmodulin might

interact with the NR1 subunit in a Ca^{2+}-dependent manner, reducing the probability of the open state of the channel significantly, operating as a feedback signal to control the response of the receptor to incoming signals (Ehlers et al., 1996). Furthermore there are postsynaptic density proteins, like the PSD-95 (or SAP-90) that belong to the family of the guanylyl kinases, which interact directly with the NMDA subunits NR2A and 2B, providing a binding surface for concentrating other protein involved in plasticity and regulating the activity of the receptors. NMDARs are linked to intracellular cytoskeletal and signalling molecules via the PSD-95 protein complex (Naisbitt, S. et al., 1999; Rossum, et al., 1999) and the activity of NMDAR itself can be modulated by its phosphorylation (see chapter V). Such mechanisms controlling the state of the receptors might be critical in determining the metaplastic state of synapses.

As stated above, the present study presents strong evidence of the activation of mGluRs in the mechanism of inhibition of LTP by preconditioning stimulation. Both, the pharmacological activation of mGluR with its agonist and weak tetani (with mGluR antagonist) confirm the inhibitory role of these receptors in the inhibition of LTP by a 'priming event'.

In some reports it seems that the priming effect does not require co-activation of both NMDAR and mGluR (Cohen et al., 1996; Akiva et al., 1996; 1999). However since a number of researchers now believe that NMDARs and mGluRs may work synergistically to generate LTP (Behnish et al., 1993; Otani et al., 1993) it seems reasonable to think that in dependence of the nature of the priming stimulation and also in view of the results described in the present work, metaplasticity might occur in a complex cross-talk between different receptors, second messengers (see chapter V) and protein synthesis. Metaplasticity is a dynamic and articulate event that might require the interaction of synaptic elements, i.e. the PSD complex, acting to produce a well-orchestrated response to changes in activity. Consequently, metaplasticity does not follow a single mechanism because the plasticity upon which it operates has diverse mechanisms and loci of expression (Artola et al., 1993).

Since mGluR5 has also been involved in the inhibition and there are several studies proving the interaction between NMDAR and mGluR5, consequently there might also be a reciprocal positive feedback interaction between these two receptors leading to implications in the regulation of synaptic plasticity. It has therefore been

demonstrated that NMDAR activation potentiates mGluR5-mediated responses in slices hippocampal and also that activation of mGluR5 can potentiate NMDAR current via a phospholipase C (PLC)- initiated cascades (Alagarsamy et al., 1999; Luthi et al., 1994). There might be also activation of Ca^{2+}-dependent phosphatase (calcineurin?) and desphosphorylation of PKC phosphoryaltion sites that participate in mGluR5 desensitisation (Alagarsamy et al., 1999). Moreover, as group I mGluRs are localised in the perisynaptic region in juxtaposition to NMDARs at glutamatergic synapses, the reciprocal feedback interaction between these two receptors may play a role in synaptic transmission and in the modulation of the direction of synaptic plasticity. In addition to their close proximity, group I mGluRs and NMDARs may be structurally cross-linked via a molecular scaffolding at CA1 synapses in the PDS. Since homer proteins link via Shank to NMDARs and signalling molecules such as PLC and PKC, such scaffolding structure would provide a strategic position for these two receptors to be co-activated in the inhibition of LTP by preconditioning stimulation (Tu et al., 1999). It has been shown that the process activated by mGluR5 agonists may lead to phosphorylation of protein belonging to the NMDA receptor complex that in turn might regulate the phosphorylation state and functional responses of mGluR5. Such interaction between these two receptors seems to suggest that they might act synergistically in a coordinate manner in order to finely tune the synaptic responses (Alagarsamy et al., 1999; Skeberdis et al., 2001). This final observation could be relevant to designing a new strategy, through selective stimulation of mGluR5, for the treatment of learning pathologies. In fact experimental evidence has demonstrated that mice lacking in mGluR5 with gene targeting approaches had significant learning deficits (Lu et al., 1997).

The activation of group I mGluR seems to be transduced by diverse second messenger pathways beginning from the activation of PLC, that in turn induces the liberation of IP_3 and activation of PKC by DAG and elevation of cAMP concentration (Akiva et al., 1996).

The present study shows that there is another receptor mediating priming of LTP, i.e. group II mGluRs. Synergistic effects between mGluR group I and II has also been reported, in reports where selective mGluR group II agonists were found to potentiate PI hydrolysis mediated via activation of group I. In other studies the use of (1S-3R)-ACPD, which activates both mGluR, group II and I was found to increase cAMP formation by synergistic interaction between mGluRs group II and I (Gennazzani et

al., 1994; Nicoletti et al., 1993; Shoepp et al., 1996). In addition, since that immunohistochemical studies (Shigemoto et al., 1997) have also showed that mGluR5 are distributed throughout the hippocampus, and group II mGuRs seems to be particularly prominent in the medial perforant path of the dentate gyrus, it is not surprising that they would both be good candidates for involvement in the regulation of the mechanism of inhibition of LTP by preconditioning stimulation.

As I stated above further studies were carried out to verify a possible interaction between NMDAR and mGluRs with second messengers such as protein kinases (PKA, PKC) or MAP kinases in the inhibition of LTP by preconditioning stimulation. The present study clearly demonstrates that there is a chain of second messengers involved in such 'priming event'.

The involvement of protein kinases activity in the priming mechanism is consistent with previous findings (Stanton et al., 1995; Bortolotto et al., 2000). A strong interaction between PKC and mGluR5 in the modulation of synaptic plasticity has also been reported. There might be a kind of inter-relationship between phosphorylation and dephosphorylation of mGluRs that seems to be an important determinant in regulating mGluR function and the subsequent neuromodulatory event elicited by activation of mGluRs. In particular, these studies are characterised by the PKC-mediated inactivation of group I mGluRs via a feedback loop. This has been hypothesised an inhibitory mechanism through a PKC-mediated desensitisation of group I mGluRs (Alagarsamy et al., 1999; 2001; De Blasi et al., 2001; Schoepp et al., 1988; Guerineau et al., 1997; Gereau et al., 1997; 1998).

In the present work the priming inhibition of LTP, as described in the previous chapter, involves mGluR5 and group II mGluR. These finding might suggest a possible interaction between PKC and PKA with these receptors in the inhibition of LTP. There might be an interaction, or parallel action through two different pathways of these two protein kinases (Borner et al., 1989; Wooten et al., 1996) converging to inhibit LTP. Consequently parallel mechanisms by which selective down-regulation of a specific-dependent signalling pathway can be achieved by a cross-talk between different second messenger cascades; which might in turn contribute to modifying the chain of events normally associated with synaptic stimulation and as a result, that prime the inhibition of LTP.

With these findings taken together; what kind of model might be suggested as explaining the interaction between NMDA receptors, mGluRs, PKC, PKA and MAP kinase pathway in the priming mechanism of LTP?

It has been demonstrated that the p38 MAPK activated in the preconditioning stimulation is physically associated with delta1 isoform of phospholipase C (PLC-delta1), which hydrolyzed phosphatidylinositol bisphosphate into diacylglycerol, an activator of PKC. SB203580 also blocked the activation of PKC-alpha (Shimizu et al., 1999). This finding can lead to the hypothesis that after priming stimulation there might be an increase of extracellular Ca^{2+} concentration inducing the activation of the p38 MAPK that seems to be sensitive to the extracellular Ca^{2+} oscillation. p38 MAPK in turn might increase DAG concentration and activate PKC.

PKC seems to play a widespread role in the regulation of group I, group II and III mGluRs and NMDARs in the hippocampus (Alagarsamy et al., 1999; 2001; De Blasi et al., 2001; Schoepp et al., 1988; Guerineau et al., 1997; Gereau et al., 1997; 1998). Since mGluR5 and group II are involved in the priming inhibition of LTP, they might be probable target for PKC and PKA. PKC might then induce a desensitisation of mGluR5 by direct phosphorylation of the receptor. In fact there are several studies, which describe the role of PKC in the desensitisation of mGluR5 (Alagarsamy et al., 1999; 2001, De Blasi et al., 2001; Schoepp et al., 1988; Guerineau et al., 1997; Gereau et al., 1997; 1998). mGluR5 in turn could probably down-regulate NMDAR activity (De Blasi et al., 2001). LTP could then be inhibited as result of all this cascade of events, triggered by interaction between second messengers and receptors. PKC might also have a dual action on the presynaptic group II mGluR, together with PKA to regulate presynaptic transmitter release. In fact one of the primary physiological roles of group II mGluRs is to presynaptically reduce synaptic transmission at glutamatergic synapses (Shoepp, 2001).

Experiments carried out in the future might examine the possibility of varying the frequency of conditioning stimulation in the dentate gyrus of rat hippocampal slices. The design of a frequency-response function might then be compared with the modification function of the BCM theory. These new insights might give a contribution in the understanding of the brain activity and how information is stored in the learning processes.

6.1 Conclusion

Initial hypothesis:

A prominent example of metaplasticity is the inhibition of LTP by prior preconditioning stimulation (weak HFS) in CA1 (Huang et al., 1999). The mechanisms of LTP priming via receptors and second messengers was not investigated. The aim of the present research was:

1. To examine priming in a different hippocampal area (DG)
2. To elucidate the mechanisms involved (via receptors and second messengers)
3. To examine (inhibition or facilitation of LTP induction?) different types of priming from weak HFS (pharmacological agents acting at the same receptors involved in the inhibition of LTP by weak HFS) in the rat dentate gyrus

The above hypothesis has been tested and it has been found that:

- Preconditioning stimulation was found to inhibit subsequent LTP induction

- The inhibition of LTP was present at 10 & 20 min, but not 2 or 45 mins post preconditioning, indicating a time window for the inhibition. These results indicate that the preconditioning stimulation took several minutes to develop, and then declined after 30-45 mins

- Activation of both NMDAR and group I/II mGluR during the preconditioning stimulation was required for inhibition of LTP induction

- Stimulation of PKC, PKA and p38 MAP kinase, but not tyrosine kinase or p42/44 MAP kinase during the preconditioning stimulation was required for inhibition of LTP induction

- The preconditioning stimulation did not involve L-type calcium channels

- The preconditioning stimulation did not inhibit NMDA receptor-mediated transmission

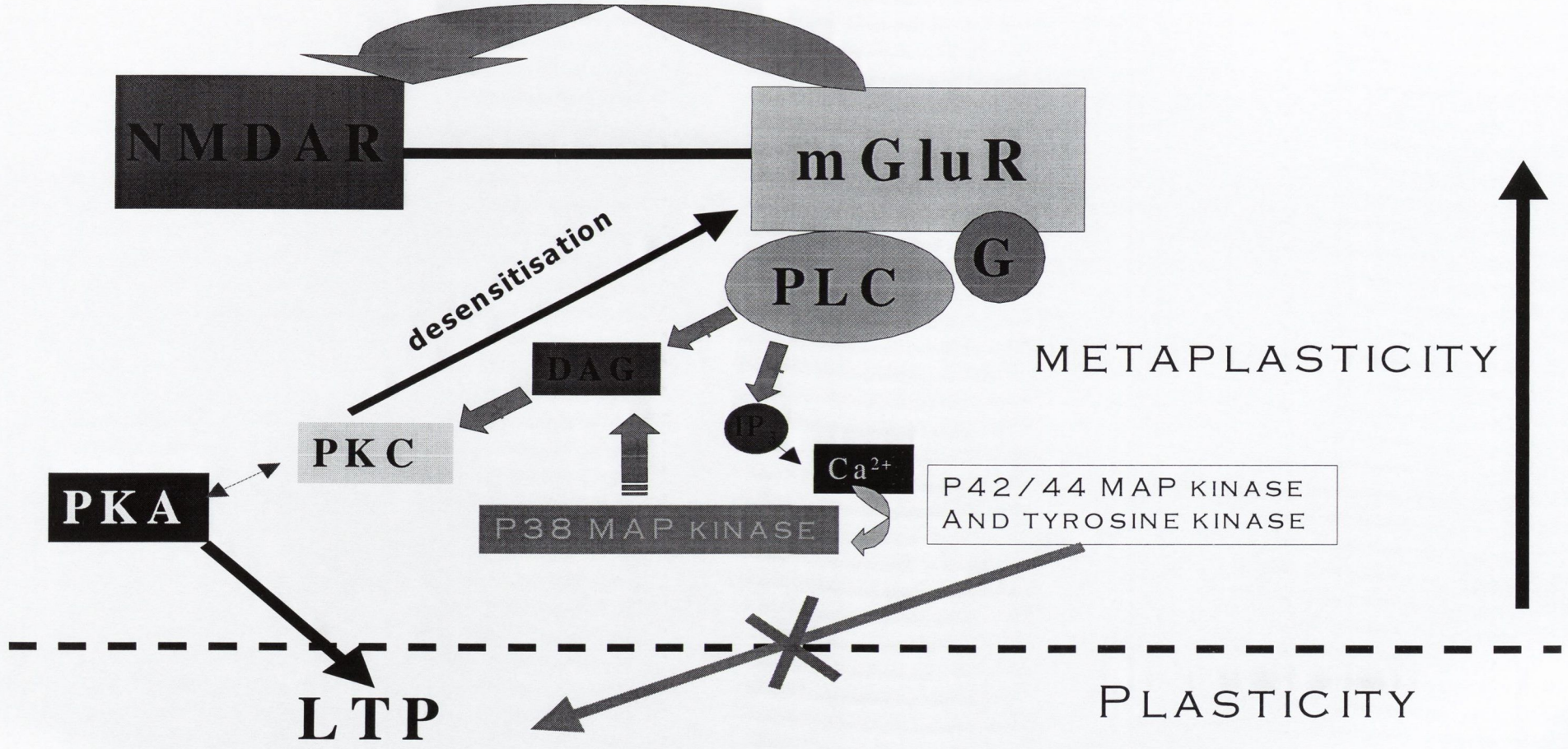
- LTP was not inhibited by prior low frequency stimulation induced LTD

- Priming of LTP by prior activation of group I mGluR by its agonist DHPG inhibits LTP in rat dentate gyrus in *vitro*

- Priming of LTP by prior activation of group II mGluR by its agonist DCG-IV inhibits LTP in rat dentate gyrus in *vitro*

- Priming of LTP by prior activation of NMDAR by its agonist NMDA facilitates LTP in rat dentate gyrus in *vitro*

Fig.5.11 Schematic of the inhibition of LTP by weak HFS.



Chapter 7

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