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**The role of Ca²⁺ and metabotropic glutamate receptors
(mGluRs) in the induction of synaptic plasticity in the rat
dentate gyrus *in vitro*.**

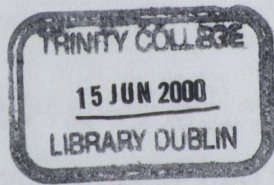
By

Nicoletta Camodeca, Laurea

**A dissertation submitted for the degree of Doctor of Philosophy of the
University of Dublin, Trinity College,
Dublin 2, Ireland**

**This research was conducted in the Department of Physiology of the
Faculty of Health Science.**

April 1999



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Declaration

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Summary

This thesis is about the investigation of some properties of synaptic plasticity in the rat dentate gyrus *in vitro*. For the experiments brain slices were cut from unanaesthetized rats aged between 3 and 5 weeks. Extracellular field potentials were evoked in order to investigate the synaptic plasticity in the slices.

In chapter 3 the involvement of mGluR group III in the induction of long-term potentiation (LTP) and long-term depression (LTD) was investigated.

The effects of L-AP4, a potent mGluR group III agonist, and of MAP4, a selective mGluR group III antagonist were investigated in the induction of LTP by high-frequency stimulation (HFS) and LTD by low-frequency stimulation (LFS) in the medial perforant pathway of the dentate gyrus. It was found that activation of group III mGluRs by L-AP4 affected neither LTP nor LTD, while the antagonizing of group III mGluRs with MAP4 had no effects on LTP but significantly reduced LTD.

In chapter 4 the induction of LTD by first lowering and then increasing the Ca^{2+} concentration in the extracellular media was examined, named Ca^{2+} -induced LTD. This form of LTD shared common intracellular induction/maintenance pathways with the LFS-induced LTD and like the latter was not accompanied by a change in the probability of transmitter release. Influx of Ca^{2+} via activation of NMDA receptors and opening of low voltage activated Ca^{2+} channels as well as release of Ca^{2+} from the intracellular Ca^{2+} stores was revealed to be essential for the generation of the Ca^{2+} induced LTD.

In chapter 5 the properties of an LTD induced by activation of mGluR group I were analysed. Activation of mGluR group I by application of the selective agonist DHPG induced an LTD, that was named DHPG-induced LTD. This LTD shared common intracellular induction/maintenance pathway with LFS-induced LTD and, like the latter, was not accompanied by a change in the probability of transmitter release. DHPG-induced LTD was not affected by increased excitability in the preparation and was not dependent upon activation of NMDA receptors. However the DHPG-induced LTD was blocked by the specific group I mGluR antagonist AIDA, which significantly reduced also the LFS induced LTD. Two subtypes, mGluR1 and mGluR5, constitute group I mGluRs and they are both present in the dentate gyrus (Shigemoto *et al.*, 1997). Using specific agonists for mGluR5 (UPF

596 and CHPG) and knockout mice lacking the gene for mGluR5 it was demonstrated that mGluR5 is a possible candidate responsible for the generation of the DHPG-induced LTD. Because the activation of group I mGluRs leads to activation of PLC with consequent activation of PKC and IP₃ production, the intracellular pathways underlying the induction of the DHPG-induced LTD were investigated. It was demonstrated that inhibition of PKC by bisindolylmaleimide I and inhibition of non-receptor protein tyrosine kinases (PTKs) with lavendustin A strongly inhibited the DHPG-induced LTD and affected the LFS-induced LTD. However the inhibition of PKA with H89 affected neither the DHPG-induced LTD nor the subsequent LFS-induced LTD.

Publications

Papers

1. Camodeca, N., Rowan, M.J. and Anwyl, R. (1998) Induction of LTD by increasing extracellular Ca^{2+} from a low level in the dentate gyrus *in vitro*. *Neurosci.Lett.* **225**, 53-56.
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Abstract

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List of abbreviations

°C	Celsius degree
1S,3R-ACPD	(1S, 3R)-1-aminocyclopentane-1,3-dicarboxylic acid
ACSF	artificial cerebral spinal fluid
AIDA	(RS)-1-aminoidan-1,5-dicarboxylic acid
AMPA	α -amino-3-hydroxy-5-methyl-4-isonxazole propionic acid
AMPAR	AMPA receptor
BAPTA	1,2-bis(2aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
cAMP	cyclic AMP
CHPG	(RS)-2-chloro-5-hydroxyphenylglycine
CNS	central nervous system
D-AP5	(-)-2-aminophosphonopentanoic acid
DAG	diacylglycerol
DMSO	dimethylsulfoxide
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EPSP	excitatory post-synaptic potential
g	gram(s)
GABA	γ -aminobutyric acid
GABA _A	GABA A receptor
h	hour(s)
HFS	high frequency stimulation
Hz	Herz
IP ₃	inositol triphosphate
L-AP4	L-(+)-2-aminophosphonobutyric acid
LFS	low frequency stimulation
LTD	long-term depression
LTP	long-term potentiation

M	molar
MAP kinase	mytogen-activated protein kinase
MAP4	(S)-2-amino-2-methyl-4-phosphonobutanoic acid
MCPG	(+) α -methyl-4-carboxyphenylglycine
mGluR	metabotropic glutamate receptor
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
μ m	micrometer
μ M	micromolar
msec	millisecond
MSOP	(RS)- α -methylserine-O-phosphate
mV	millivolt
M Ω	megaohm
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTK	protein tyrosine kinase
RS-DHPG	(RS)-3,5-dihydroxyphenylglycine
S-DHPG	(S)-3,5-dihydroxyphenylglycine
SE	standard error
sec	second(s)
STP	short-term potentiation

1 Introduction

1.1 The aim of this thesis.

The aim of this thesis is the investigation of the role played by calcium and the activation of metabotropic glutamate receptors in the induction of synaptic plasticity in the rat dentate gyrus. The role of activation of group III metabotropic glutamate receptors in the induction of long-term potentiation (LTP) was briefly analysed in chapter 3, but the main objective of this work was on the effects of alteration of the extracellular calcium concentration and the effects of activation of metabotropic glutamate receptors in the induction of long-term depression (LTD). These investigations were focused on elucidating the role played by various second messengers consequent to the activation of certain receptors and/or channels in the induction of some forms of long term synaptic plasticity in the dentate gyrus. The goal was to establish the molecular basis of some properties of long-term depression (LTD) and long-term potentiation (LTP) in this region of the hippocampus.

1.2 The hippocampus.

The hippocampal formation or archicortex is a component of the allocortex of the mammalian telencephalon (Gastaut and Lammers, 1961) and more recently it has been associated with the limbic system (Swanson, 1983).

The hippocampal formation includes the dentate gyrus (or fascia dentata), the Ammon's horn (cornu Ammonis or proper hippocampus) and the subiculum (Gastaut and Lammers, 1961). The Ammon's horn can be further subdivided into 4 areas: CA1, CA2, CA3 and CA4 going from the subiculum towards the dentate gyrus (Lorente de Nó, 1934). In the rat, as in all the mammals, the hippocampus

consist of two C-shaped interlocking cell layers: the granular cell layer of the dentate gyrus and the pyramidal cell layer of the Ammon's horn (Lopes da Silva *et al.*, 1990). The basic architecture of the hippocampal subfields is very similar, in that they all consist of a single lamina of neurones whose apical dendrites extend into a cell-poor zone, the stratum moleculare in the dentate gyrus and the subiculum and the stratum lacunosum/moleculare and stratum radiatum in Ammon's horn (Witter 1989).

1.2.1 Hippocampal neurones.

The different types of hippocampal neurones were described after the introduction of the silver impregnation technique by Golgi (Golgi 1886, Sala 1891, Schaffer 1892, Lugaro 1893, Koelliker 1896, Cajal 1911 and Lorente de Nó 1934). Using these studies and more recent investigations (Braak 1974, Amaral and Woodward, 1977, Amaral 1978, Tömböl *et al.*, 1978, Ribak and Seress, 1983, Seress and Ribak, 1985, Schlander and Frotscher, 1986, Misgeld and Frotscher, 1986, Somogyi *et al.*, 1983) four main types of neurones can be distinguished in the hippocampus on the basis of their morphological characteristics:

- a) CA1 pyramidal neurones.
- b) CA3 pyramidal neurones.
- c) Dentate granule neurones.
- d) Nonpyramidal neurones.

- a) CA1 pyramidal neurones.

In the CA1 region the somata of pyramidal neurones are loosely packed in the two or three cell wide stratum radiatum. The thick apical dendrites from cell bodies pass through the stratum radiatum to the stratum lacunosum-moleculare, where they form contacts with afferent fibres. The space between the somata is filled with horizontal dendritic branches.

b) CA3 pyramidal neurones.

The pyramidal neurones in CA3 are distinguished by being much larger than the cells in the CA1 region. The large branched spines (called excrescences) appear on proximal dendritic segments of CA3 pyramidal cells, which form the contacts with mossy fibres.

c) Dentate granule neurones.

The granule cells are the main cell type in the dentate gyrus. Their small somata (approximately 7 μm in diameter) are densely packed in the granular layer. Only apical dendrites arise from the perikaryon. These branch in the molecular layer and are densely covered with spines. The mossy fibre axon originating from the basal pole of the granule cell perikaryon runs downwards to the hilar region.

d) Nonpyramidal neurones.

The majority of the nonpyramidal neurones in the hippocampus are interneurones, most of which are GABAergic inhibitory neurones. GABAergic interneurones in the hippocampus are found throughout all the hippocampal regions (dentate gyrus, CA3 and CA1), both adjacent to the cell body layers and in dendritic regions. They are primarily intrinsic interneurones although some cells send axons to the contralateral hippocampus or septum. One particularly important class of interneurone common to both the dentate gyrus and the hippocampus proper is the basket cell. These neurones have fairly large somata, located close to stratum pyramidale and stratum granulosum, and widely branching aspiny dendrites which may or may not cross the cell body layer, allowing them to receive excitatory synapses from many sources. Most importantly their axons ramify extensively and make prominent inhibitory synapses with the somata of pyramidal and granule cells.

1.2.2 Connectivity in the hippocampus.

The lamellar organisation of the hippocampus has been established by anatomical (Blackstad *et al.*, 1970, Hjorth-Simonsen and Jeune, 1972) and electrophysiological (Andersen *et al.*, 1971) investigations.

Each lamella contains a sequence of unidirectional connections from the dentate gyrus to the subiculum via CA3 and CA1 (Witter, 1989). The dentate gyrus represents the major input structure of the hippocampus with major cortical afferents arising from the entorhinal cortex (Witter, 1989). The afferent fibre projection that originates from the entorhinal cortex is the perforant pathway (McNaughton, 1980). It can be subdivided into two components that originate from different parts of the entorhinal cortex and differ in respect to their distribution in the hippocampus (Witter, 1989) and electrophysiological properties (McNaughton, 1980). Another main input into the hippocampus is constituted by the commissural fibres originating in the contralateral hippocampus (Blackstad, 1956).

Within the hippocampus, the dentate granule cells distribute the mossy fibres to the CA3 area and the pyramidal cells in the CA3 field originate the Schaffer collaterals that distribute to the CA1 region (Lopes da Silva *et al.*, 1990).

1.3 Glutamate receptors.

In 1959 Curtis *et al.* demonstrated that L-glutamate, among other naturally occurring acidic amino acids excited single neurones in the mammalian brain. More recently it has been commonly accepted that L-glutamate may be the main mediator of fast excitatory neurotransmission in vertebrate neurones (Collingridge and Lester, 1989).

Glutamate receptors play a major role in the induction and maintenance of synaptic plasticity (for review see Collingridge and Lester, 1989, Pin and Duvoisin, 1995, Bliss and Collingridge, 1993, Bear and Abraham, 1996).

They can be divided into two categories (Nakanishi *et al.*, 1994):

- a) Ionotropic glutamate receptors.
- b) Metabotropic glutamate receptors.

1.3.1 Ionotropic glutamate receptors.

Most fast glutamatergic synapses use ionotropic glutamate receptors, which possess an integral ion permeable pore, to generate a dual component excitatory post-synaptic potential). The two components are mediated by different receptor subclasses: a fast component is mediated by receptors that are activated by α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and a slow component mediated by receptors activated by N-methyl D-aspartate (NMDA) (Collingridge *et al.*, 1988, Forsythe and Westbrook, 1988).

The AMPA receptors are also activated by quisqualate/kainate and they are linked to non-selective cation channels (Keinänen *et al.*, 1990). Initially they were considered to be only slightly permeable to Ca^{2+} . However it has been recently demonstrated that there are AMPA receptors in the hippocampus that exhibit high Ca^{2+} permeability (Isa *et al.*, 1996).

NMDA receptors are ionotropic glutamate receptors potently activated by NMDA (Curtis and Watkins, 1963). The channel linked to the NMDA receptors is a cationic channel permeable to Na^+ , K^+ and Ca^{2+} (Ascher *et al.*, 1988, Ascher *et al.*, 1986). Another property of the NMDA receptor is that the channel linked to the receptor is blocked by the Mg^{2+} present in the extracellular medium in a voltage dependent manner (Nowak *et al.*, 1984).

1.3.2 Metabotropic glutamate receptors.

Metabotropic glutamate receptors are a class of receptors present in the CNS activated by glutamate. They are G-protein coupled receptors that regulate electrical signalling by influencing intracellular metabolic processes (Knöpfel *et al.*,

1995). The existence of mGluRs was first postulated in 1985 based on biochemical studies demonstrating that L-glutamate stimulates inositol triphosphate (IP₃) accumulation in the CNS (Sladeczek *et al.*, 1985, Nicoletti *et al.*, 1986).

Eight different mGluRs have been identified and they have been classified into three different groups on the basis of their genetic sequence homology, their pharmacological properties and the second messenger pathway that they employ (for review see Pin and Duvoisin, 1995, Conn and Pin, 1997).

Group I mGluR comprises mGluR1 and mGluR5. These receptors are coupled to phospholipase C (PLC) (Masu *et al.*, 1991, Abe *et al.*, 1992). In the hippocampus they are present only on the post-synaptic elements (Shigemoto *et al.*, 1997). Both mGluR1 and mGluR5 are present on pyramidal cells and on granule cells throughout the CA3 region the dentate gyrus. In the CA1 area, mGluR5 is present in the dendritic fields, while mGluR1 is restricted to cell bodies and dendrites of interneurons in the stratum oriens/alveus border (Shigemoto *et al.*, 1997).

Group II mGluR comprises mGluR2 and mGluR3. These receptors are linked to inhibition of forskolin stimulated cyclic AMP (cAMP) formation (Tanabe *et al.*, 1992, Tanabe *et al.*, 1993). In the hippocampus mGluR2 and mGluR3 are expressed predominantly on the pre-synaptic membrane in the dentate gyrus and the CA3 area, with higher density in the middle than in the outer one-third of the stratum moleculare. In the CA1 area staining for mGluR2/3 is found only in the neuropil (Shigemoto *et al.*, 1997)

Group III mGluR comprises mGluR4, mGluR6, mGluR7 and mGluR8. These receptors are also linked to inhibition of forskolin stimulated cAMP. However, they differ from group II mGluRs because of their pharmacological properties, being selectively activated by L-(+)-2-amino-phosphonoutyric acid (L-AP4) (Tanabe *et al.*, 1993, Nakajima *et al.*, 1993, Saugstad *et al.*, 1994, Duvoisin *et al.*, 1995) and distribution (Shigemoto *et al.*, 1997). In the hippocampus only mGluR4a, mGluR7a, mGluR7b and mGluR8 are expressed and they are located on the pre-synaptic membrane. More specifically, in the dentate gyrus mGluR4a is present in

the terminal zone of the associational/commissural path, mGluR7a predominantly in the medial perforant path terminal zone and mGluR8 in the terminal zone of the lateral perforant path. In the CA3 area mGluR 7a is present in the inner layer of the stratum lacunosum moleculare, mGluR7b is restricted to the mossy fibre terminal zone and mGluR8 is expressed in the outer layer of the stratum lacunosum moleculare (Shigemoto *et al.*, 1997).

1.4 Synaptic plasticity in the hippocampus.

Hebb (1949) was one of the first scientists to formulate a theory for synaptic plasticity that could form the basis for learning and memory formation.

Several years later, Bliss and Lomo (1973) were the first to observe the phenomenon of long-lasting potentiation of synaptic transmission following repeated electrical stimulation. This constituted the first empirical evidence that there was a physical mechanism underlying the theory proposed by Hebb.

These first experiments (Bliss and Lomo, 1973) were carried out in the dentate gyrus region of the hippocampus of adult anaesthetised rabbits. It was discovered that the stimulation of the perforant path for several seconds (10-15 sec at 10-20 Hz or 3-4 sec at 100 Hz) produced a potentiation of the synaptic response (long-term potentiation or LTP) that lasted for several hours. The authors discovered also that the effect of this stimulation in the animals tested was confined to the stimulated pathway.

Other studies were carried out (Bliss and Gardner-Medwin, 1973) on unanaesthetized rabbits. Following administration of single periods of stimulation identical to those used in the experiments on the anaesthetised rabbits, a long-term potentiation of the responses lasting from 1 to 72 hours was observed, indicating that long-term synaptic changes could be produced in animals in which the physiological conditions were kept closest to normal.

Later, Alger and Teyler (1976) showed for the first time that in rat hippocampal slices LTP was present in the CA1 and CA3 regions and in the dentate gyrus,

following 4-6 trains of stimulation at 15 Hz, each with a duration of 15 sec. In the same study the response of hippocampal slices to low- frequency stimulation (10 sec at 1 Hz or 10 sec at frequencies varying between 0.1 and 15 Hz) was also analysed. While pyramidal cells in the CA1 and CA3 regions showed a potentiation during the stimulation at all the frequencies tested, in the dentate gyrus a depression was detected. However the responses in all three regions showed a rapid recovery within 35 seconds from the termination of the low frequency stimulation.

Another form of long-term synaptic plasticity strictly related to LTP was reported by Lynch *et al.* in 1977. In these experiments when a train was delivered to the slice preparation in the CA1 area of the hippocampus, an LTP of the stimulated path was obtained while the non stimulated pathway exhibited a persistent depression, termed heterosynaptic long-term depression (heterosynaptic LTD).

It was only a decade later when Bramham and Srebro (1987) showed that when low frequency stimulation (15 Hz) was applied to the dentate gyrus in anaesthetised rats a homosynaptic long-term depression (homosynaptic LTD) was induced and it lasted up to 4 hours.

However Dudek and Bear in 1992 described for the first time a stimulation paradigm that reliably induced homosynaptic LTD in slices. In their paper they reported that a stimulation train of 900 pulses delivered at 1-3 Hz consistently produced homosynaptic LTD in the CA1 area of the hippocampus.

Since their discovery, there have been extensive studies on LTP and the various forms of LTD in order to understand the biochemical processes and the electrophysiological modifications underlying these forms of synaptic plasticity. Because the experiments for this thesis concerned LTP and homosynaptic LTD, only these two forms of synaptic plasticity will be described.

1.4.1 Long-term potentiation (LTP).

Long-term potentiation (LTP) can be defined as a long-lasting enhancement of the synaptic response subsequent to a strong stimulation (Bliss and Lomo, 1973).

LTP in rat hippocampus develops within milliseconds and lasts for several hours in the *in vitro* preparation (Alger and Teyler, 1975) and up to days in freely moving animals (Bliss and Gardner-Medwin, 1973).

Generally there is a distinction based on the duration and the time of occurrence following the stimulation between short-term potentiation (STP) of the synaptic response which decreases back to baseline after some minutes (Bliss and Collingridge, 1993) and LTP, which is by definition a long-term change of response.

Furthermore, hippocampal LTP can be classified on the basis of whether or not the activation of the *N*-methyl-D-aspartate (NMDA) glutamate receptors is required for its induction. LTP is dependent upon activation of NMDA receptors in the area CA1 (Collingridge *et al.*, 1983, Harris *et al.*, 1984) and the dentate gyrus (Burgard *et al.*, 1989). However LTP observed in the area CA3 at the mossy-fibre synapses in the area CA3 does not require the activation of NMDA receptors (Zalutsky and Nicoll, 1990).

Hippocampal LTP is characterised by three basic properties: input specificity, cooperativity and associativity.

Bliss and Lomo in 1973 reported for the first time input specificity of LTP. During their investigations they discovered that the effect of the train to induce LTP was restricted to the experimental pathway while the control pathway lacked a similar effect.

Cooperativity was described by McNaughton *et al.* in 1978. The authors reported that in order to induce any potentiation, a threshold stimulus intensity during the train given in order to induce LTP was required and that this threshold was remarkably above the stimulus threshold for observing a minimal synaptic response.

In the same study by McNaughton *et al.* in 1978, and in 1979 by Levy and Steward the property of associativity of LTP was described. In their studies it was shown that concurrent stimulation of two separate but convergent inputs produced enhancement at intensities where identical but independent stimulation of the same failed or was less effective.

Since this thesis is about synaptic plasticity in the dentate gyrus and LTP in this area of the hippocampus is NMDA-dependent, only the induction and maintenance of this type of LTP will be discussed and it will be referred to as LTP.

1.4.1.1 The induction of LTP: The role of Ca^{2+} .

Ca^{2+} influx is essential for the induction of LTP. If the Ca^{2+} in the extracellular medium is reduced to less than half of its original concentration LTP cannot be induced (Dunwiddie and Lynch, 1979).

In the pre-synaptic terminal, Ca^{2+} enters the neurone through voltage activated Ca^{2+} channels that are opened by a depolarisation (Reichardt and Kelly, 1983). Here the Ca^{2+} triggers fusion of synaptic vesicles with the plasma membrane and the consequent release of neurotransmitter (Kennedy, 1989).

In the post-synaptic cell Ca^{2+} influx is essential for the induction of LTP. When EGTA, a Ca^{2+} chelator, is injected in the post-synaptic cell, LTP induction is prevented (Lynch *et al.*, 1983). In 1988 Malenka *et al.* showed that an increase in post-synaptic Ca^{2+} is necessary to induce LTP and sufficient to potentiate synaptic transmission.

It has been hypothesised that the influx of Ca^{2+} ions in the post-synaptic cell starts a series of processes that result in LTP (Eccles, 1983).

One candidate, which could mediate the postsynaptic Ca^{2+} influx was activation of the NMDA receptor/channel. It had already been shown that activation of NMDA receptors in the CA1 area were essential for the induction of LTP (Harris *et al.*, 1984). However, direct evidence that NMDA are permeable to Ca^{2+} receptors was given by MacDermott *et al.* in 1986. They showed that NMDA elevated

intracellular free Ca^{2+} in voltage clamped cultured spinal cord neurones. In 1988 Connor *et al.* demonstrated that selective activation of NMDA receptors by local application of either glutamate or NMDA induced extremely long-lasting Ca^{2+} gradients in apical dendrites of CA1 hippocampal neurones.

NMDA receptors do not constitute the sole source of Ca^{2+} responsible for the increase in the intracellular Ca^{2+} concentration necessary for the induction of LTP. Additional sources of Ca^{2+} may originate from influx via AMPA receptors (Isa *et al.*, 1996), opening of voltage-dependent Ca^{2+} channels (Grover and Teyler, 1990, Kullmann *et al.*, 1992) and activation of metabotropic glutamate receptors (mGluRs) with consequent release of Ca^{2+} from intracellular organelles (Bortolotto and Collingridge, 1993).

Activation of mGluRs in the CA1 area induces LTP (Bortolotto and Collingridge, 1993) and in the presence of the mGluR antagonist (RS)- α -methyl-4-carboxyphenylglycine (MCPG) tetanus-induced LTP is blocked (Bashir *et al.*, 1993). In the dentate gyrus, activation of mGluRs induces a potentiation of the NMDA component of the synaptic response which is mutually exclusive with long-term potentiation of the same NMDA currents (O'Connor *et al.*, 1994). Furthermore, selective activation of mGluR group I with specific agonists has been shown to facilitate LTP induced by a weak tetanus in the dentate gyrus in freely moving animals (Manhan-Vaughan and Reymann, 1996). Similar results were obtained in the CA1 area *in vitro* (Cohen and Abraham, 1996).

Activation of group III mGluRs has been shown to block LTP induction both in the CA1 area and in the dentate gyrus of freely moving animals (Manhan-Vaughan and Reymann, 1995).

Additionally the activation of group II mGluRs in the CA1 area blocked the induction of LTP in the anaesthetised rats (Hölscher *et al.*, 1997). Similarly, in the dentate gyrus *in vitro* the activation of mGluR group II blocked LTP induction (Huang *et al.*, 1997).

1.4.1.2 The expression of LTP.

There is no evidence for a distinct locus of expression of LTP, however several hypotheses have been postulated and demonstrated by various groups (for a review see Bliss and Collingridge, 1993).

The increase in the post-synaptic response generated at the potentiated synapses could be due to:

- Presynaptic modifications that result in an increase in the amount of transmitter released per impulse;
- Post-synaptic changes, such as an increase in the number of receptors or a change in their functional characteristics;
- An extra synaptic modification, such as a reduction in uptake of neurotransmitter by glial cells leading to increased glutamate availability at the receptors;
- Morphological modifications.

A possible scenario is that a combination of these changes, with different time courses occurs.

Several Ca^{2+} -sensitive proteins or Ca^{2+} -dependent transcription factors, as well as other proteins activated by different intracellular second messengers which may be produced during LTP induction may serve as targets for converting the induction signal to a permanent change.

One of the proteins that serve this function is protein kinase C (PKC). This enzyme is activated by a rise in cytosolic Ca^{2+} and by diacylglycerol (DAG) (Nishizuka, 1995). Among the effectors of PKC are protein tyrosine kinase, Ras proteins, Jun protein activated by MAP kinase, channels, pumps, receptors (including NMDA) and nuclear protein factors essential for gene transcription (Whittemore *et al.*, 1993, Nishizuka, 1995). Early studies in the dentate gyrus of anaesthetised animals revealed that stimulation of PKC resulted in a prolonged enhancement by acting specifically at tetanized synapses (Lovinger and

Routtenberg, 1988). A variety of protein kinase inhibitors have been shown to block the expression of LTP when applied during the induction of LTP (Malinow *et al.*, 1988, Denny *et al.*, 1990). Because these inhibitors affected other enzymes, additional evidence for the involvement of PKC in the expression of LTP has come from studies using selective inhibitor peptides injected in the post-synaptic cell (Malinow *et al.*, 1989). However, it has also been demonstrated that activation of PKC with phorbol esters does not result in LTP since synaptic responses return to baseline following washout of the drug (Muller *et al.*, 1988).

Protein kinase A (PKA) is another enzyme considered to play a role in the induction of LTP (for review see Roberson *et al.*, 1996). PKA is activated by cAMP, one of the most widely used second messengers. The specific role played by PKA in LTP is unknown, but it is hypothesised that this enzyme causes inhibition of phosphatase activity, promoting in this way the action of PKC, CaMKII and other kinases (Blitzer *et al.*, 1995, Roberson *et al.*, 1996).

Ca²⁺-Calmodulin Kinase II (CaMKII) is a serine-threonine kinase that is activated by the complex formed by Ca²⁺ with the protein calmodulin. Among the effectors of CaMKII are AMPA receptors (Barria *et al.*, 1997) and NMDA receptors (Gardoni *et al.*, 1999). CaMKII can function as a 'memory device' switching to an active state when exposed to Ca²⁺/calmodulin and then remaining active even after the Ca²⁺ is withdrawn (Lisman, 1985). This is because CaMKII is able to autophosphorylate as well as phosphorylate other proteins, so that even in the absence of Ca²⁺, when in the autophosphorylated state, this enzyme remains active beyond the duration of the initial stimulating Ca²⁺ signal (Miller and Kennedy, 1986). The CaMKII inhibitor KN62 has been shown to block LTP in the CA1 area *in vitro* (Ito *et al.*, 1991). Additional evidence for the involvement of CaMKII in LTP has come from studies in which the use of inhibitor peptides injected in the postsynaptic neurone blocked LTP (Malinow *et al.*, 1989).

Protein tyrosine kinases (PTK) are widely expressed in the hippocampus (for review see Boxall and Lancaster, 1998). These enzymes can be divided into two categories; those which are integral component of receptors, such as growth

factor receptors, and those which are not linked to specific receptors. Non-receptor PTKs are localised on the inner surface of the cells. Among the factors that can activate the non-receptor PTKs are PKC (Liebenhof *et al.*, 1993) and elevated intracellular free Ca^{2+} (Siciliano *et al.*, 1994, Lev *et al.*, 1995). It has been shown that non-receptor PTKs modulate NMDA receptors (Wang and Salter, 1994), GABA_A receptors (Moss *et al.*, 1995) and AMPA receptors (Grant *et al.*, 1992). Non-receptor PTKs are essential for LTP induction since in the presence of specific inhibitors LTP in the CA1 area *in vitro* was blocked (O'Dell *et al.*, 1991). Similar results were observed in the dentate gyrus of anaesthetised animals (Abe and Saito, 1993).

1.4.2 Homosynaptic long-term depression (LTD).

Homosynaptic long-term depression can be described as an activity dependent decrease in the strength of synaptic transmission (Mulkey and Malenka, 1992). LTD in the CA1 area in the *in vitro* preparation can be obtained by repetitive stimulation at frequencies below the threshold for inducing LTP, typically 900 pulses at 1-3 Hz (Dudek and Bear, 1992). Homosynaptic LTD in the *in vitro* preparation has been demonstrated to occur also in the CA3 area (Kobayashi *et al.*, 1996) and in the dentate gyrus (O'Mara *et al.*, 1995a).

Although Bramham and Srebro (1987) reported that they could induce LTD in the dentate gyrus of anaesthetised animals, other groups could not reproduce the same experiments (Doyère *et al.*, 1996). In the same study the authors showed that in the dentate gyrus LTD could not be induced with a protocol that is effective to induce LTD in CA1 *in vivo* called burst-pair stimulation (Thiels *et al.*, 1994). However Thiels *et al.* (1996) showed that in their preparation the burst-pair protocol was effective in inducing LTD in the dentate gyrus of anaesthetised animals.

In the slice preparation, homosynaptic LTD in the CA1 area has been shown to be specific for the stimulated input and dependent upon activation of NMDA receptors (Mulkey and Malenka, 1992).

A rise in the intracellular Ca^{2+} concentration in the postsynaptic cell is essential for the induction of this LTD since injections of BAPTA, a Ca^{2+} chelator, in the postsynaptic cells prevented the induction of LTD (Mulkey and Malenka, 1992).

However in a younger group of animals Bolshakov and Siegelbaum (1994) showed that the induction of LTD in CA1 pyramidal neurones depends on postsynaptic Ca^{2+} entry through L-type voltage gated Ca^{2+} channels, activation of mGluRs and is not dependent upon activation of NMDA receptors.

These different results are explained by the existence of two distinct forms of LTD in CA1, one dependent on the activation of mGluRs and the other on the activation of NMDA receptors (Oliet *et al.*, 1997).

One common feature to all these different forms of LTD, NMDA-dependent and mGluR dependent, is a raise in the intracellular Ca^{2+} concentration (Mulkey and Malenka, 1992, Cummings *et al.*, 1996, Otani and Connor, 1996, Neveu and Zucker, 1996a, b and Otani and Connor, 1998).

This rise can occur through activation of NMDA receptors (Mulkey and Malenka, 1992, Oliet *et al.*, 1997), activation of mGluR receptors (Oliet *et al.*, 1997, Otani and Connor, 1998), rapid influx through voltage sensitive Ca^{2+} channels (Bolshakov and Siegelbaum, 1994, Christie *et al.*, 1996, Oliet *et al.*, 1997, Christie *et al.*, 1997 and Wang *et al.*, 1997) and release of Ca^{2+} from the intracellular stores (O'Mara *et al.*, 1995b, Reyes and Stanton, 1996).

Metabotropic glutamate receptors have been shown to play an important role in the induction of LTD. Activation of mGluRs with 1S,3R-ACPD was shown to induce LTP in the dentate gyrus in vitro (O'Mara *et al.*, 1995a and Overstreet *et al.*, 1997). These preliminary studies did not discriminate between the different mGluR subtypes. Later studies demonstrated that the activation of mGluR group II is necessary for the induction of LTD in the dentate gyrus (Huang *et al.*, 1997) and

that activation of mGluR group I in CA1 is able to induce LTD (Palmer *et al.*, 1997).

The increase in the intracellular Ca^{2+} concentration in conjunction with the activation of mGluRs constitutes the first step in the biochemical cascade underlying LTD.

Depletion of intracellular Ca^{2+} stores prevents the induction of LTD in CA1 (Reyes and Stanton, 1996) and in the dentate gyrus (Wang *et al.*, 1997). The connection between the activation of mGluRs in the induction of LTD and the release of Ca^{2+} from the intracellular stores was provided by direct evidence that inhibition of PLC prevents the induction of LTP (Reyes-Harde and Stanton, 1998).

Activation of protein phosphatases plays an essential role in the induction of LTD in the CA1 area (Mulkey *et al.*, 1993). In addition to this it has been demonstrated that a cascade involving calcineurin, inhibitor-1 phosphatase and protein phosphatase 1 is required for the induction of LTD (Mulkey *et al.*, 1994).

Based on the hypothesis that synaptic plasticity may be under the bidirectional control of phosphatases and kinases, based on the fact that kinase activation inhibits phosphatase activity, Coussens and Teyler (1996) demonstrated that enhanced phosphatase activity, by reducing kinase activity, allowed the induction of LTD using a stimulation frequency that in normal conditions did not elicit any synaptic plasticity. Furthermore, a reduction in protein kinase activity has been shown to induce a long-term depression that mimics and occludes homosynaptic LTD (Hrabetova and Sacktor, 1996).

However more recently Stanton and Gage (1996) demonstrated that activation of CaMKII is essential for the induction of LTD.

2 Materials and Methods

2.1 Animals

Male Wistar rats (inbred strain, BioResources Unit, Trinity College, Dublin) were used for all the experiments. The rats were between 40g and 70g in weight and 4-6 weeks of age. The animals were kept in the BioResources Unit in a room with a 12 hours light/dark cycle at a temperature between 20 °C and 23 °C.

One group of experiments was carried out with knockout mice lacking the gene for mGluR5. The mice were obtained from Dr Conquet from Glaxo Wellcome in Lausanne, Switzerland. After shipment the animals were kept in the facilities of the BioResources Unit in Trinity College and were left 10 days to recover before experiments.

Approximately 300 rats were used for the experiments reported in this thesis and only one slice from each animal was used.

2.2 Solutions

2.2.1 Standard solution

The solution used for all the experiments was Artificial Cerebral Spinal Fluid (ACSF).

All the experiments were carried out in the presence of 50 mM picrotoxin (Sigma) in order to block the GABA_A –mediated activity in the slices unless otherwise stated. A stock solution 4 times concentrated was prepared weekly. This stock solution did not contain glucose, to minimise the bacterial growth, nor Ca²⁺ to minimise the formation of precipitates. An aliquot of the stock solution was diluted

daily in order to obtain the standard solution and glucose and Ca^{2+} , from a 1M stock solution, were added at the time of dilution.

Table 2.1 shows the concentrations of the substances present in the standard solution.

All the chemicals in the solution were purchased from Sigma-Aldrich, Ireland.

Table 1.1 Chemical composition of standard solution (ACSF)

Substance	Concentration (mM)
NaCl	120.00
NaHCO ₃	26.00
Na ₂ HPO ₄	1.25
KCl	2.50
MgSO ₄ (6H ₂ O)	2.00
CaCl ₂ (2H ₂ O)	2.00
Glucose	10.00

2.2.2 Solutions with altered Ca^{2+} concentration

For some experiments a solution with altered Ca^{2+} concentration was used. This was obtained by changing the concentration of Ca^{2+} while making the solutions. In this solution the Mg^{2+} concentration was not altered. None of the changes greatly affected the osmolarity or the pH of the solution.

2.3 Slice preparation

This technique was previously described by O'Connor *et al.* (1994). The rat was decapitated and the brain was rapidly removed and placed in oxygenated (95% O₂/5% CO₂) ACSF at a temperature between 4°C and 8°C. Hippocampal slices

were cut at a thickness of 350 μm using a 752M vibroslice (Campden Instruments) with the solution in the cutting chamber kept at 4-8 $^{\circ}\text{C}$. Immediately after cutting, the slices were put into a holding chamber with oxygenated (95% O_2 /5% CO_2) ACSF at room temperature (20-23 $^{\circ}\text{C}$) and left there to recover for one hour.

2.4 Compounds

The following compounds were used for some experiments of this thesis. They were all dissolved in different solvents, which are individually specified for each of them, to form a stock solution. In order to reach the final concentration of the compound for each experiment, aliquots of the stock solution were diluted in the ACSF used for the recordings. Before using a compound for the first time a control experiment was carried out using only the solvent. This was done in order to rule out any possible effects that the solvent could have on the preparation.

AIDA ((R,S)-1-Aminoidan-1,5-dicarboxylic acid)

AIDA was purchased from Tocris, UK. It was dissolved in 0.1 M NaOH to form a 100 mM stock solution.

L-AP4 (L-(+)-2-Aminophosphonobutyric acid)

L-AP4 was purchased from Tocris, UK. It was dissolved in 0.1 M NaOH to form a 100 mM stock solution.

D-AP5 (D(-)-2-Amino-5-phosphonopentanoic acid)

D-AP5 was purchased from Tocris, UK. It was dissolved in H_2O to form a 200 mM stock solution.

Bisindolylmaleimide I (GF 109203X) (3-[1-(3-Dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)-maleimide)

Bisindolylmaleimide I was purchased from Calbiochem, CA, USA. It was dissolved in H_2O to form a 5 mM stock solution.

CHPG ((R,S)-2-Chloro-5-hydroxyphenylglycine)

CHPG was purchased from Tocris, UK. It was dissolved in 0.1 M NaOH to form a 100 mM stock solution.

(R,S)-DHPG ((R,S)-3,5-Dihydroxyphenylglycine)

(R,S)-DHPG was purchased from Tocris, UK. It was dissolved in H₂O to form a 50 mM stock solution.

(S)-DHPG ((S)-3,5-Dihydroxyphenylglycine)

(S)-DHPG was purchased from Tocris, UK. It was dissolved in H₂O to form a 50 mM stock solution.

Econazole (1-(2-[(4-Chlorophenyl)methoxy]-2-[2,4-dichlorophenyl]ethyl)-1H-imidazole)

Econazole was purchased from Sigma-Aldrich, Ireland. It was dissolved in DMSO to form a 200 mM stock solution. Unlike any other compound used, econazole was added to ACSF containing 0 Ca²⁺. This solution had to be constantly bubbled with 95% O₂/5% CO₂ to prevent the formation of precipitates. Only after the econazole was added could the Ca²⁺ be incorporated in the ACSF. The bubbling of the solution could be terminated only when all the Ca²⁺ was added to the solution to reach the final concentration of 2 mM.

H-89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide)

H-89 was purchased from Calbiochem, CA, USA. It was dissolved in DMSO to form a 10 mM stock solution.

Lavendustin A (5-Amino-[(N-2,5-dihydroxybenzyl)-N'-2-hydroxybenzyl]salicylic acid)

Lavendustin A was purchased from Calbiochem, CA, USA. It was dissolved in DMSO to form a 50 mM stock solution.

MAP4 ((S)-2-Amino-2-methyl-4-phosphonobutanoic acid)

MAP4 was purchased from Tocris, UK. It was dissolved in 0.1 M NaOH to form a 125 mM stock solution.

MCPG ((S)-a-Methyl-4-carboxyphenylglycine)

MCPG was purchased from Tocris, UK. It was dissolved in 0.1 M NaOH to form a 60 mM stock solution.

MSOP ((R,S)-a-Methylserine-O-phosphate)

MSOP was purchased from Tocris, UK. It was dissolved in 0.1 M NaOH to form a 125 mM stock solution.

Ni²⁺

Ni²⁺ was purchased from Sigma-Aldrich, Ireland. It was dissolved in H₂O to form a 100 mM stock solution.

Picrotoxin

Picrotoxin was purchased from Sigma-Aldrich, Ireland. It was dissolved in DMSO to form a 400 mM stock solution.

Thapsigargin

Thapsigargin was purchased from Sigma-Aldrich, Ireland. It was dissolved in DMSO to form a 50 mM stock solution.

UPF596 ((2S)-2-(3'-Carboxy-bicyclo[1.1.1]pentyl)glycine)

UPF596 was kindly donated by Prof. Pellicciari, Universita' di Perugia, Istituto di Chimica e Tecnologia del Farmaco, Italy. It was dissolved in H₂O to form a 100 mM stock solution.

2.5 Electrodes

2.5.1 Recording electrodes

The recording electrodes consisted of glass microelectrodes made from borosilicate capillaries (Clark Electromedical Instruments, Reading, UK) with an external diameter of 1.5 mm and an internal diameter of 0.8 mm. Inside the capillaries there was a filament that guaranteed the perfect filling of the tip of the

electrode. The electrodes were pulled using a horizontal flaming/brown micropipette puller (Model P-87, Sutter Instrument Corp., CA, USA). The microelectrodes were filled with ACSF and they had a resistance of 1-3 M Ω . For the electrophysiological recordings a recording electrode filled with ACSF was placed into an electrode holder (QSWA15P, Clark Electromedical Instruments, Reading, UK). The electrode holder was attached to a headstage (CV-4-1/100 Axon Instruments, CA, USA) which was connected to an Axopatch-1D amplifier (Axon Instruments, CA, USA).

2.5.2 Reference electrodes

The reference electrode consisted of a silver chloride electrode (Clark Electromedical Instruments, Reading, UK) . This type of electrode was changed approximately once every six months. The reference electrode was connected to the headstage of the amplifier used for the electrophysiological recordings described above and was immersed in the recording chamber close to the tube from which the solution was entering the chamber.

2.5.3 Stimulating electrodes

Bipolar stimulating electrodes were used for all the experiments. They consisted of two pieces of Teflon-coated tungsten wire (California Wire Company, Grover Beach, CA, USA). The twisted tungsten wires were inserted into a steel syringe needle and connected to the stimulating apparatus. The stimulating electrode was earthed through the stimulus isolation unit SIU5 (Grass Medical Instruments, Quincy, MA, USA) that was connected to it.

2.6 The electrophysiological experimental set-up

2.6.1 The stimulating set-up

The stimulating setup consisted of a stimulator S88 (Grass Medical Instruments, Quincy, MA, USA), a stimulus isolation unit SIU5 (Grass Medical Instruments, Quincy, MA, USA) and a stimulating electrode.

A personal computer (P5-100, Gateway2000, Ireland) was connected to the stimulator via a digital-to-analogue converter (Digidata 1200, Axon Instruments, CA, USA) in order to trigger the stimulation and control its frequency. The amplitude, the duration and the delay of the stimulation relative to the beginning of the trace displayed on the oscilloscope and on the computer screen were set directly on the stimulator. The stimulator was directly connected to the stimulus isolation unit and then to the stimulating electrode.

2.6.2 The electrophysiological recordings

For the electrophysiological recordings, first the stimulating electrode was lowered into the recording chamber until it touched the slice in the stratum moleculare of the dentate gyrus. The fact that the electrode was touching the slice could be determined by a slight movement of the tissue surrounding the tip of the stimulating electrode. The recording electrode was then lowered into the chamber and the offset voltage between the recording electrode and the bath electrode was eliminated with the help of a bridge circuit. The recording electrode was then also positioned in the stratum moleculare of the dentate gyrus. Both the stimulating and the recording electrodes were positioned along the perforant pathway. All the experiments were carried out in the medial perforant pathway. The location of the electrodes in this pathway was determined by giving two stimulations with an interstimulus interval of 40 msec. This type of stimulation in this pathway produces a pair of postsynaptic responses in which the second is of smaller amplitude than

the first one (Kahle and Cotman, 1993b). The recording electrode was then gradually lowered into the slice until the maximal postsynaptic response was obtained while stimulating the slice every 30 seconds.

Field excitatory post-synaptic potentials (EPSP) were recorded for all the experiments. EPSPs were evoked using the stimulation setup described above. The EPSPs were transmitted using an Axopatch-1D amplifier (Axon Instruments, CA, USA) to the analogue-to-digital converter. The data were stored in a personal computer utilising the package software pClamp6 (Axon Instruments, CA, USA).

The stimulus to evoke the postsynaptic response consisted of a squarewave positive voltage pulse of 0.1 msec duration. Before starting each experiment, an input/output curve was determined (stimulus intensity versus EPSP amplitude) by stimulating the slice with increasing positive voltage pulses with a step of 1 volt. The minimal stimulation intensity used was the one that did not induce any post-synaptic response and the maximal intensity was considered the one that produced an EPSP with the largest amplitude whose shape was not contaminated by the presence of population spikes. After this, the amplitude of the stimulation was adjusted to evoke an EPSP with an amplitude of approximately 30% of the maximal value (usually approximately 1 mV).

The first step of the recordings was recording a period of baseline responses to determine that the basal response was stable over a period of time (10-20 min). If changes in the amplitude of the EPSP were detected during the recording of the baseline, the slice was discarded and substituted with a new one. The test stimulation frequency was 0.033Hz when single EPSPs were evoked for baseline responses. When paired-pulse stimulation was given to the slices with an interstimulus interval of 40 msec, the frequency of the paired stimuli was 0.016 Hz. The protocol to induce long-term potentiation (LTP) was high frequency stimulation (HFS) which consisted of 8 trains delivered every 2 sec, each train consisting of 8 pulses given at 200Hz. During this protocol the stimulation intensity was manually increased in order to obtain an EPSP amplitude that was doubled compared to the EPSP evoked for test responses. After the delivery of HFS the

stimulation intensity was manually returned to the value used to evoke the post-synaptic responses for the baseline recordings.

The protocol used to induce long-term depression (LTD) was low frequency stimulation (LFS), which consisted of 900 pulses delivered at 1Hz for 15 min. During this stimulation protocol the stimulation intensity remained unaltered.

In all the plots the normalised EPSP amplitude is plotted against time. Although the measure of the slope of the postsynaptic response would more accurately reflect the synaptic current, the field EPSPs recorded in the experiments in the present thesis did not seem to be contaminated with population spikes or inhibitory post-synaptic potential (IPSPs), since the recordings were made in the presence of picrotoxin, justifying in this way the measurement of the amplitude. The EPSP amplitude was normalised over the period of time that corresponded to the baseline recording.

All the results are expressed as mean \pm SE. For statistical analysis Student's *t*-test, 2 tailed, paired was used to compare values within the same slice and Student's *t*-test, 2 tailed, homoscedastic was used to compare values from different experiments.

2.7 The experimental set-up

The experimental apparatus consisted of a Perspex[®] recording chamber mounted on an anti vibration table (DT 4048M-V, Herz, Optima Research, UK) surrounded by a Faraday cage (Departmental Workshop, Trinity College, Dublin).

A schematic diagram of the recording chamber is given in Fig 2.1.

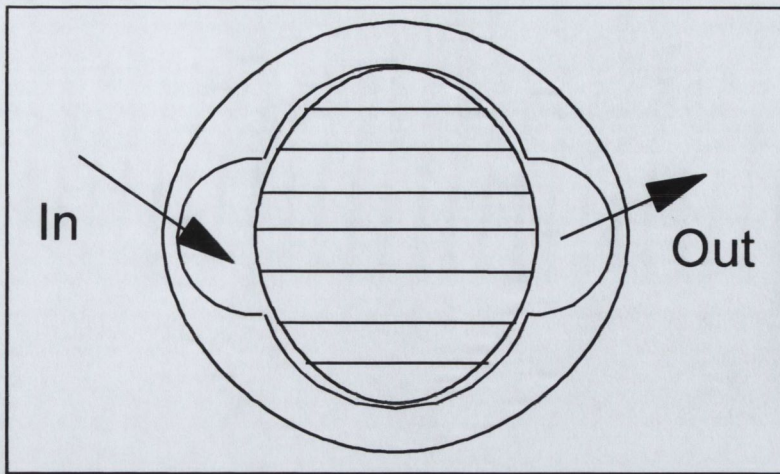


Fig. 2.1 Schematic diagram of the recording chamber.

Schematic diagram of the shape of the recording chamber. In the left semicircle was placed the end of the tubing for conveying the solution (In arrow). This area was also used for eliminating any bubbles present in the solution. In the right semicircle was placed the end of the tubing connected to the peristaltic pump for removing the fluid from the chamber (Out arrow). The brain slice was placed on the net in the central part of the chamber.

This chamber was specially designed to contain a volume of solution of 0.5 ml and allow a perfusion rate up to 10 ml/min.

The perfusate was oxygenated (95% O₂/5% CO₂) at room temperature, and it was warmed before entering the recording chamber, using a gravity fed system. The temperature was kept constant at 32 °C throughout the experiments by using an immersion thermostat (T model, Lauda, Germany). The solution was removed from the chamber with the use of a peristaltic pump (P5100, Spectra Hardware Inc., PA, USA) and recycled into the reservoir that fed the chamber. In experiments requiring the washout of a drug or a special solution, the first 100 ml of ACSF used for the washout were discarded, avoiding large changes in the volume of solution in the feeding reservoir.

A Precision Zoom Stereo Microscope (World Precision Instruments, Inc., Sarasota, FL, USA) was used to view the slices in the recording chamber.

In the recording chamber the slices were placed on a Perspex[®] ring covered by a nylon mesh and they were kept in position by another Perspex[®] ring covered with nylon threads.

An electrode holder (Clark Electromedical Instruments, Reading, UK) was used to connect the recording pipette to the headstage of the amplifier (see 2.5.1). The headstage was mounted on a three-dimensional hydraulic micro-manipulator (MHW-3, Narishige, Tokyo, Japan) which was fixed on a mechanical manipulator (MMN-1, Narishige, Tokyo, Japan). This system allowed adjustments of the recording pipette in three dimensions with a precision of 1mm.

Another electrode holder (Departmental Workshop, Trinity College, Dublin) was mounted on a mechanical micro-manipulator (E905WF, Eclipse, Prior, UK) and used to position the stimulating electrode on the slice.

3 The involvement of mGluR group III in the induction of long-term potentiation (LTP) and long-term depression (LTD) in the medial perforant pathway of the rat dentate gyrus *in vitro*.

3.1 Introduction

The involvement of metabotropic glutamate receptors (mGluRs) in the induction of long-term synaptic plasticity has been studied extensively. It has been demonstrated that pharmacological blocking of mGluRs prevents the induction of long-term potentiation (LTP) and long-term depression (LTD) (Trommer *et al.*, 1996, O'Mara *et al.*, 1995b and Bashir *et al.*, 1993).

mGluRs represent a family of G-protein coupled receptors. They have been classified into three groups (group I, II and III) according to their sequence homology, second messenger coupling and agonist preference. The activation of mGluR group III results in inhibition of cyclic AMP (cAMP) accumulation following forskolin stimulation of adenylyl cyclase. It has been shown that activation of mGluR group III induces a presynaptic depression at many synapses in the CNS (Baskys and Malenka, 1991, Bushell *et al.*, 1996, Macek *et al.*, 1996, Dubé and Marshall, 1997, Vignes *et al.*, 1995 and Jane *et al.*, 1994).

In the present study I have investigated the involvement of mGluR group III in the induction and maintenance of LTP and LTD in the medial perforant pathway of the dentate gyrus in rat hippocampal slices.

L-2-aminophosphonobutyrate (L-AP4) is a potent and selective mGluR group III agonist that has been shown to depress synaptic transmission at a variety of synapses (Kahle and Cotman, 1993a and b, Harris and Cotman, 1983, Baskys

and Malenka, 1991, Salt and Turner, 1996, Bushell *et al.*, 1996, Kemp *et al.*, 1996, Gereau IV and Conn, 1995, Dubé and Marshall, 1997, Koerner and Cotman, 1981, Ugolini and Bordi, 1995, Vignes *et al.*, 1995, Jane *et al.*, 1994 and Dietrich *et al.*, 1997).

(S)-2-methyl-2-amino-4-phosphonobutanoate (MAP4) is a selective mGluR group III antagonist which was found to block the presynaptic depression caused by mGluR group III agonists (Bushell *et al.*, 1996, Kemp *et al.*, 1996, Macek *et al.*, 1996, Salt and Eaton, 1995, Dubé and Marshall, 1997, Ugolini and Bordi, 1995, Vignes *et al.*, 1995 and Jane *et al.*, 1994).

Manahan-Vaughan and Reymann (1995) have demonstrated that *in vivo* the intraventricular injection of L-AP4 in 12 week old rats inhibited LTP and this effect was reversed by (S)- α -methyl-4-carboxyphenylglycine (MCPG), a broad spectrum metabotropic glutamate receptor antagonist (Jane *et al.*, 1994, Vignes *et al.*, 1995, Ugolini and Bordi, 1995 and Watkins and Collingridge, 1994).

3.2 Results.

3.2.1 The effects of activation and inhibition of mGluR group III on the induction of LTP.

When the slices were tested in control solution, high-frequency stimulation (HFS) induced a stable LTP measuring $148\% \pm 4\%$ (Student's *t*-test, 2 tailed paired, $p < 0.001$, $n=6$) at 30 min after delivery of HFS (Fig. 3.1).

Perfusion of the slices with $20 \mu\text{M}$ of the mGluR group III agonist L-AP4 induced a depression of the EPSP amplitude of $35\% \pm 7\%$ (Student's *t*-test, 2 tailed paired, $p < 0.001$, $n=5$) at 40 min after starting perfusion with L-AP4 (Fig. 3.2). This depression of the synaptic response was in agreement with previous studies carried out by other groups in the same pathway (Ugolini and Bordi, 1995).

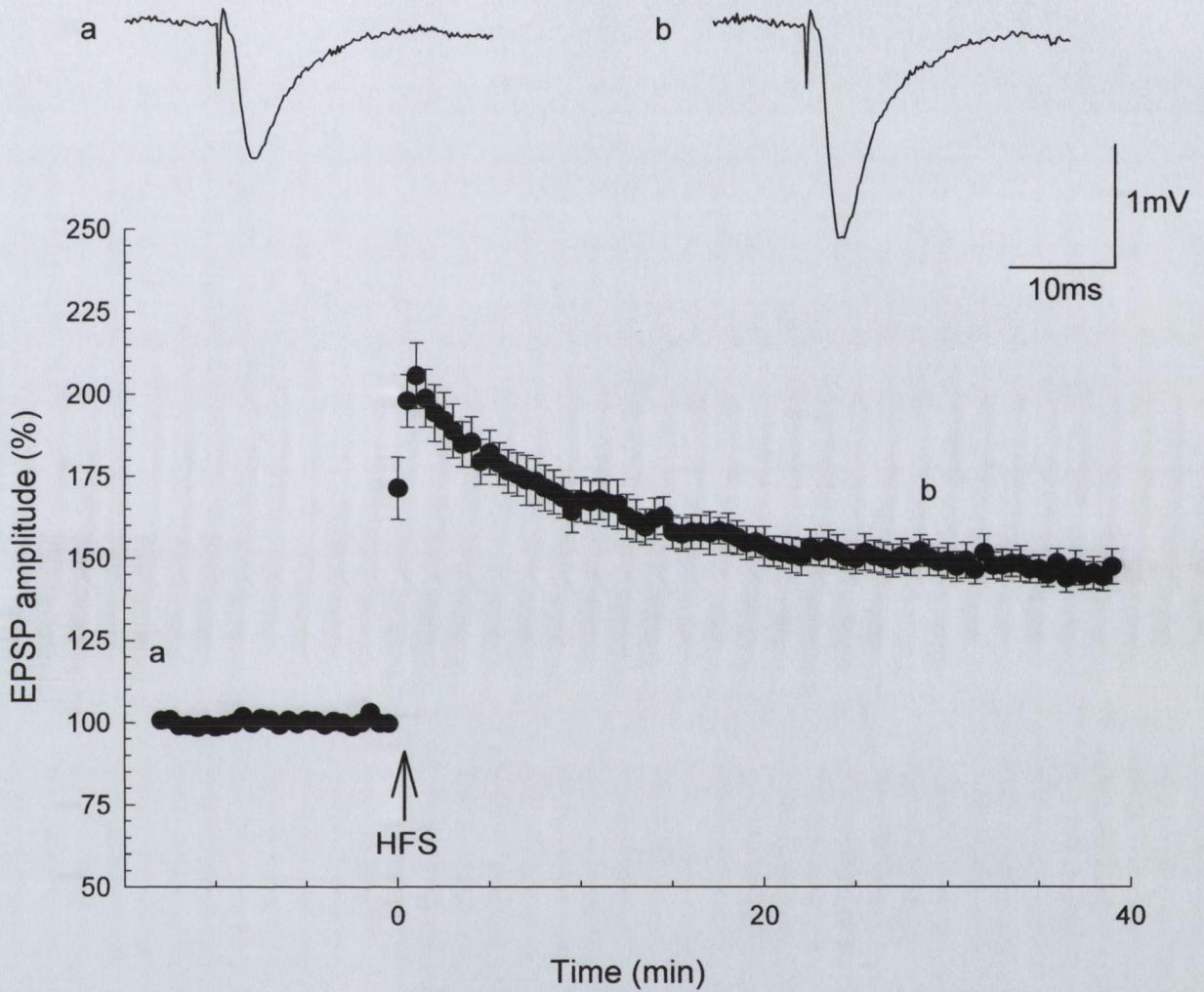


Figure 3.1 LTP in control solution.

When HFS was delivered to slices in control solution (ACSF) an LTP was induced that measured $148\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=6$) at 30 min post HFS. Examples of single EPSPs are shown in **a** (baseline) and **b** (30 min after the induction of LTP).

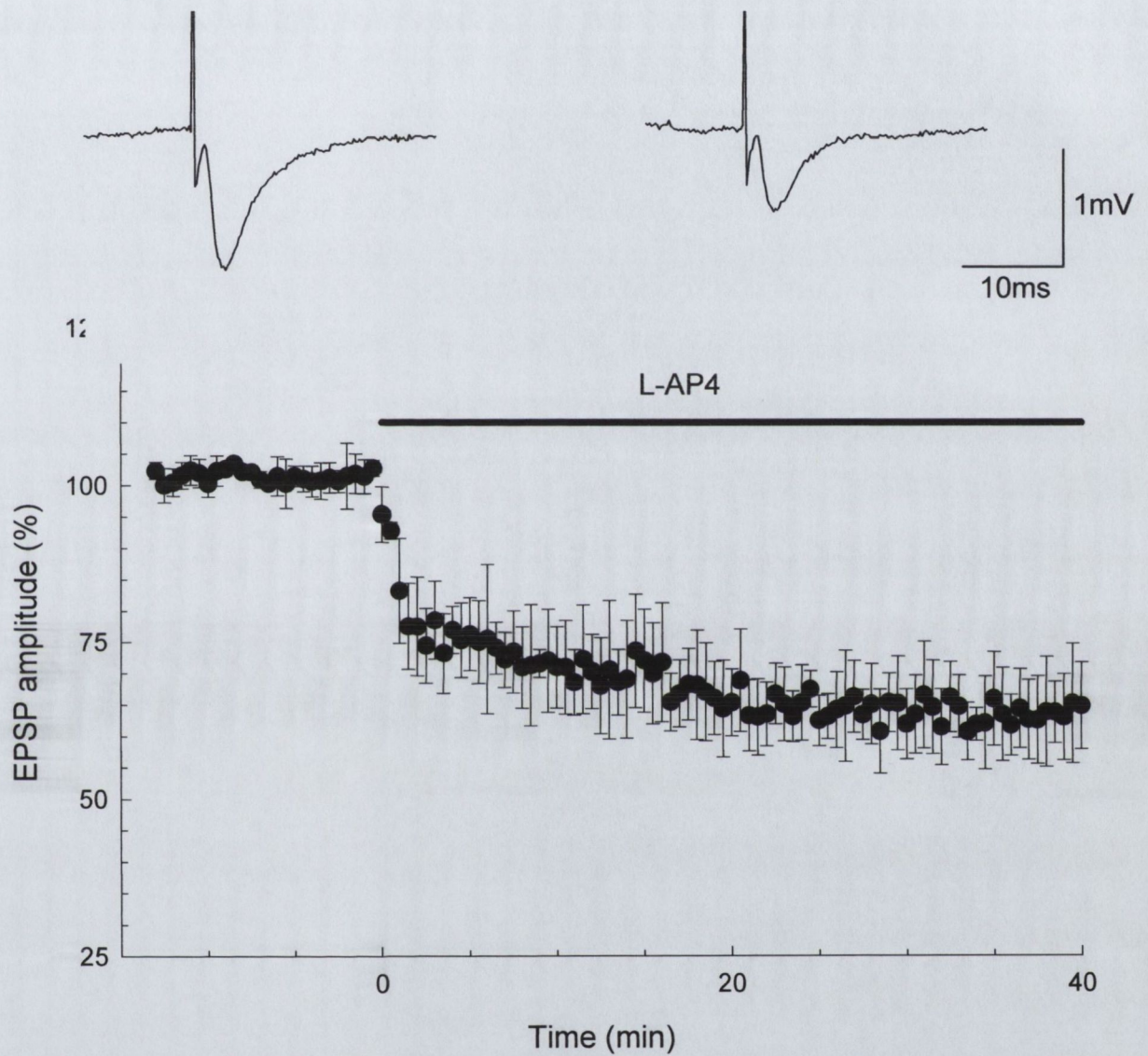


Figure 3.2 Effects of L-AP4 on baseline responses.

The perfusion of slices with the mGluR group III agonist L-AP4 at a concentration of $20 \mu\text{M}$ induced a depression of $35\% \pm 7\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 5$) measured at 40 min after the start of the perfusion. Examples of single EPSPs are shown in **a** (baseline) and **b** (40 min after the perfusion with L-AP4)

When HFS was delivered to slices that were perfused with 20 μ M L-AP4 a stable LTP was induced, measuring 152% \pm 9% (Student's *t*-test, 2 tailed paired, $p < 0.005$, $n = 5$) at 30 min after the delivery of HFS (Fig. 3.3).

LTP induced in the presence of L-AP4 was not significantly different from the LTP induced in the control solution (Student's *t*-test, 2 tailed homoscedastic, $p > 0.05$).

These experiments showed that activation of mGluR group III in the medial perforant pathway with L-AP4 caused a depression of basal synaptic transmission but had no effect on the induction of LTP.

The perfusion of slices with the mGluR group III antagonist MAP4 at the concentration of 500 μ M induced a depression of the EPSP amplitude of 13% \pm 3% (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 5$) measured at 40 min after the start of the perfusion with MAP4 (Fig. 3.4). A more selective mGluR group III antagonist α -methylserine-O-phosphate (MSOP) was also applied at a concentration of 500 μ M but it caused an even greater depression, approximately 30% at 40 min after starting perfusion of MSOP (Fig. 3.5). Because of the greater induced depression, MSOP was not used for any subsequent experiment.

When HFS was delivered to the slices after perfusion of MAP4 for at least 40 min, a stable LTP was induced measuring 155% \pm 10% (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 5$) at 30 min after the delivery of HFS (Fig. 3.6). LTP induced in the presence of MAP4 was not significantly different from the LTP obtained in control solution (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$).

Thus application of MAP4, an antagonist of mGluR group III, in the medial perforant pathway induced a depression in the EPSP amplitude and had no effect on LTP induction.

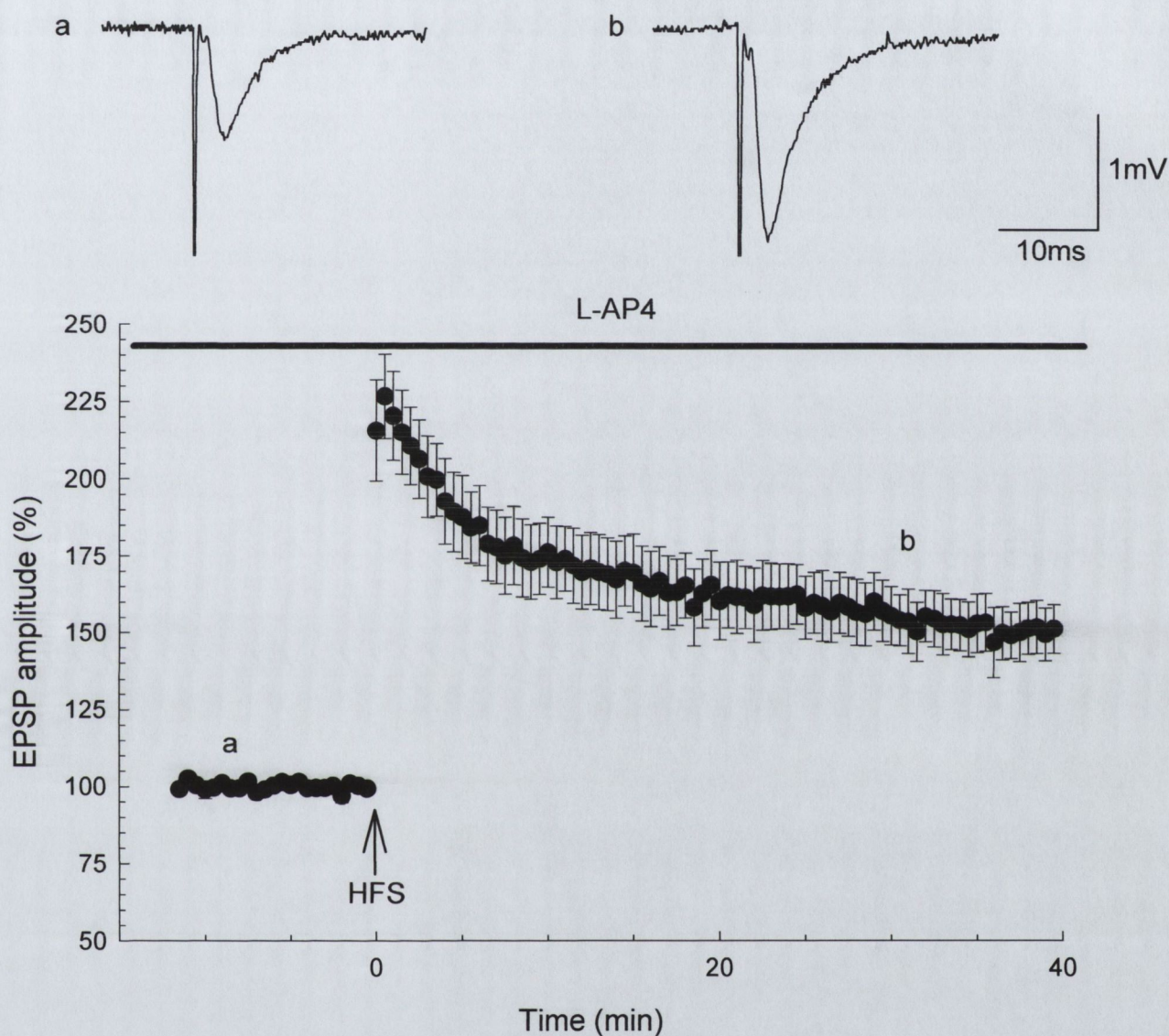


Figure 3.3 LTP in the presence of L-AP4.

HFS induced an LTP measuring $152\% \pm 9\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 5$) in the presence of the mGluR group III agonist L-AP4 at a concentration of $20 \mu\text{M}$. This LTP was not significantly different from the LTP induced in control solution (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Examples of single EPSPs are shown in **a** (baseline) and **b** (30 min after the induction of LTP).

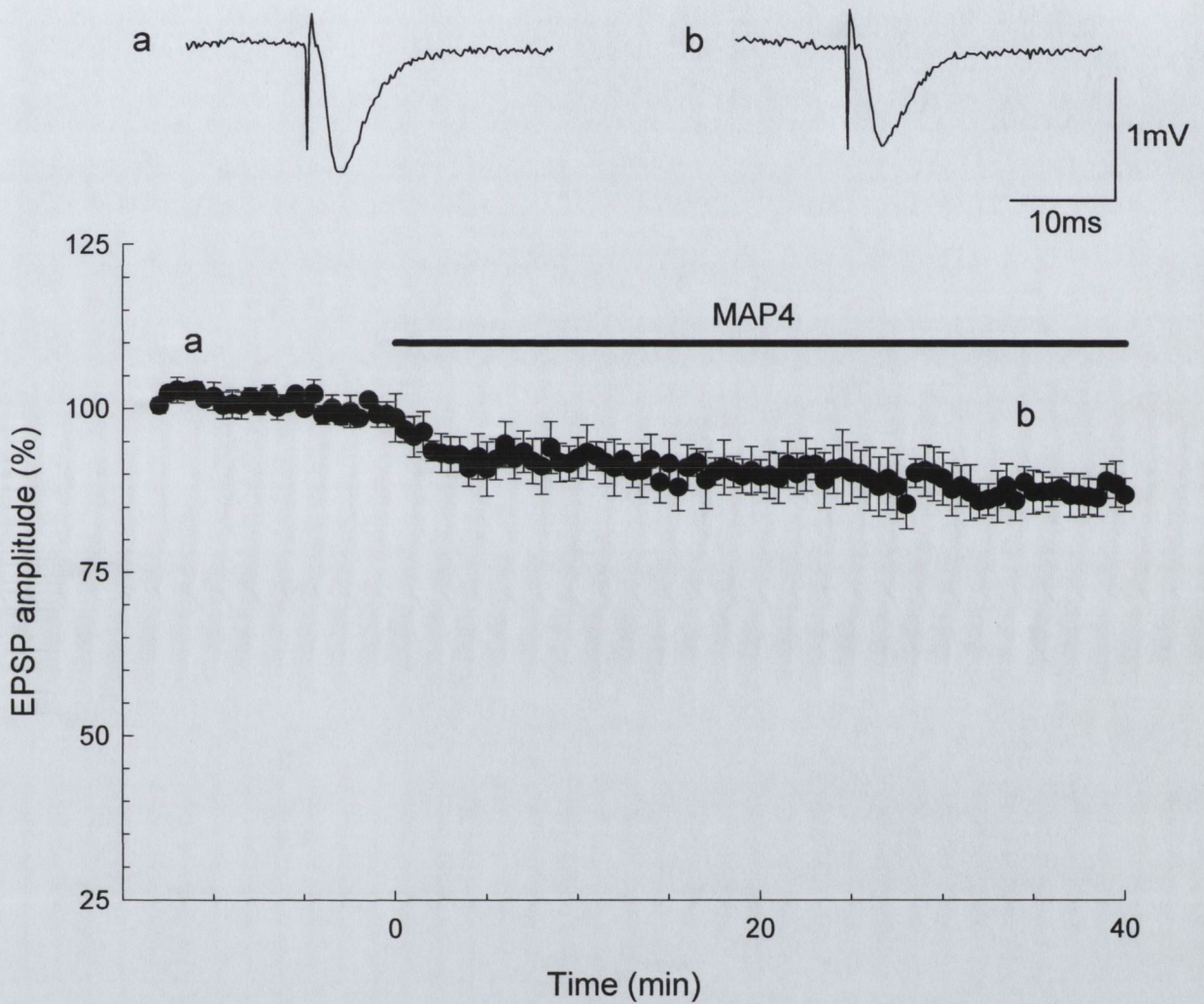


Figure 3.4 Effects of MAP4 on baseline responses.

The perfusion of slices with the mGluR group III antagonist MAP4 at a concentration of $500 \mu\text{M}$ induced a depression of $13\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 5$) measured at 40 min after the start of the perfusion. Examples of single EPSPs are shown in **a** (baseline) and **b** (40 min after the perfusion with MAP4)

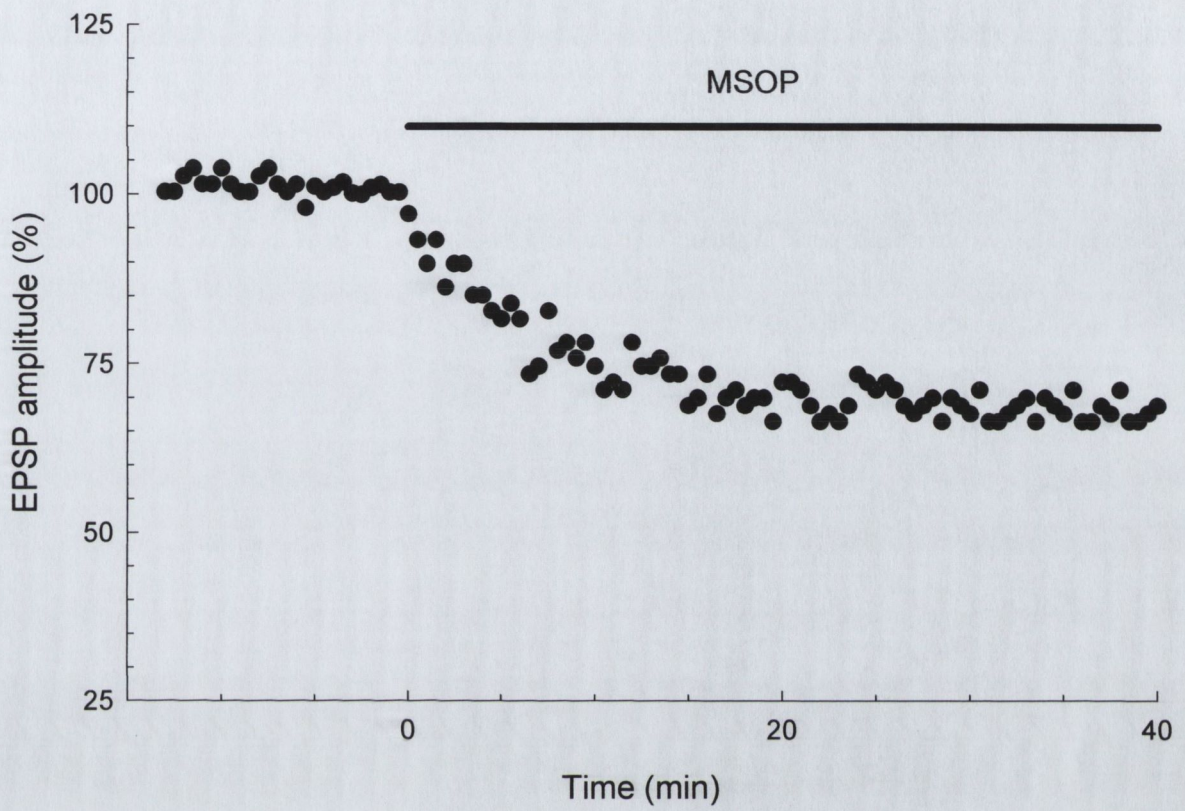


Figure 3.5 Effects of MSOP on baseline responses.

Single experiment in which it is shown that the selective mGluR group III antagonist MSOP at a concentration of 500 μ M induced a depression of approximately 30% of the EPSP amplitude.

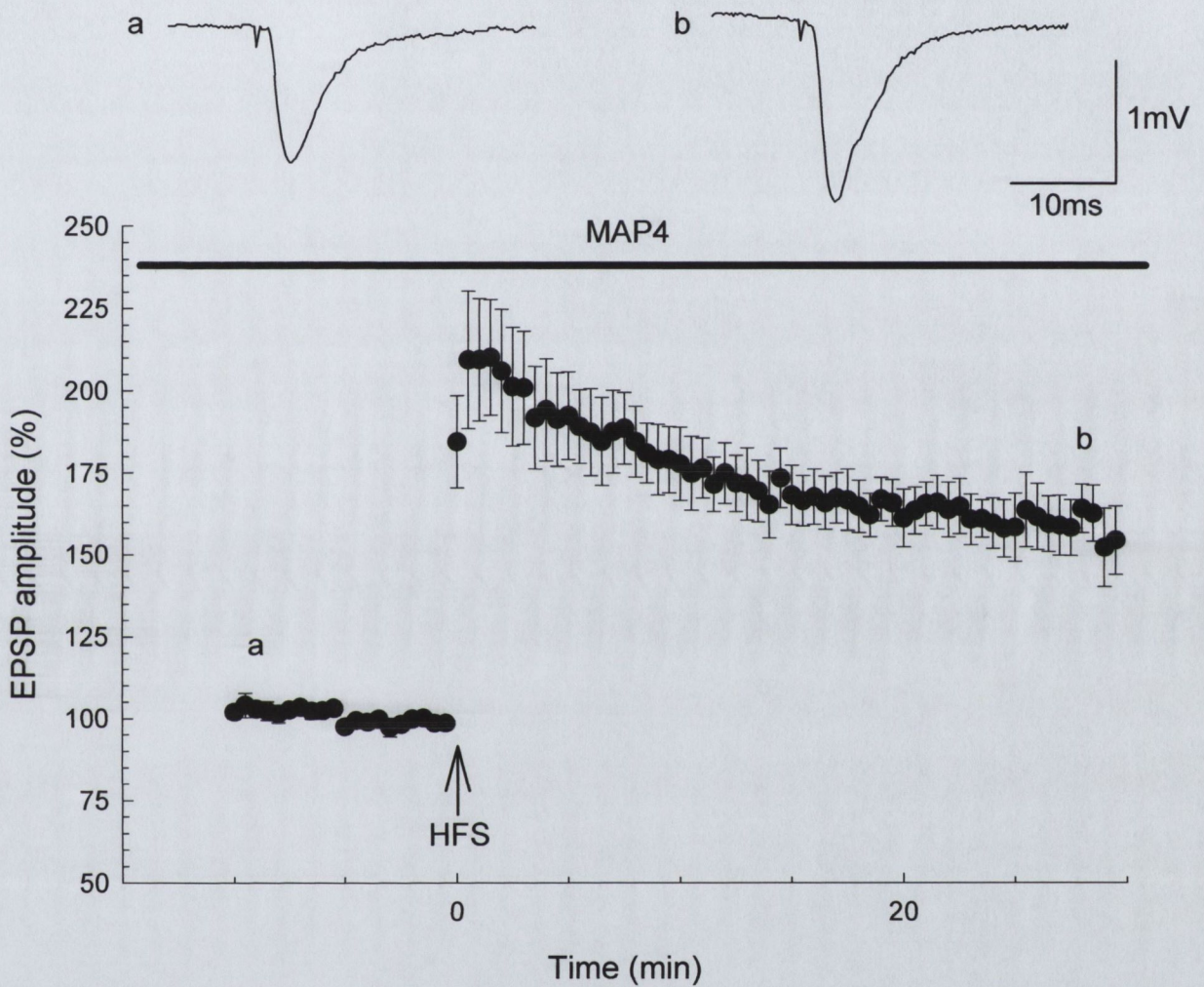


Figure 3.6 LTP in the presence of MAP4.

In the presence of the mGluR group III antagonist MAP4 at a concentration of $500 \mu\text{M}$, HFS induced an LTP measuring $155\% \pm 10\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 5$) at 30 min after the delivery of HFS. This LTP was not significantly different from the LTP induced in control solution (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Examples of single EPSPs are shown in **a** (baseline) and **b** (30 min after the induction of LTP).

3.2.2 The effects of activation and inhibition of mGluR group III on the induction of LTD.

Application of low frequency stimulation (LFS) (1Hz for 15 min) to the slices in control solution resulted in the induction of LTD measuring $31\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=8$) at 30 min after the end of LFS (Fig. 3.7).

When LFS was delivered to slices that were perfused for at least 40 min with $20\mu\text{M}$ L-AP4, it induced an LTD measuring $35\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=5$) at 30 min after the end of the LFS (Fig. 3.8). This LTD was not significantly different from the LTD induced in control solution (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$).

These experiments showed that activation of mGluR group III had no effect on the induction of LTD.

In the presence of $500\mu\text{M}$ MAP4 LFS induced an LTD measuring $18\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n=5$) at 30 min after the end of LFS (Fig. 3.9). This LTD was significantly reduced compared to the LTD induced in control solution (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$).

These results showed that inhibition of mGluR group III partially blocked the induction of LTD.

3.3 Discussion.

These studies have provided evidence for the involvement of mGluR group III in the induction of LTD in the medial perforant pathway of the dentate gyrus, with the group III antagonist MAP4 partially inhibiting LFS induced LTD.

They have also shown that group III mGluRs are not involved in the induction of LTP in the same pathway, since neither L-AP4 nor MAP4 affected the induction of LTP.

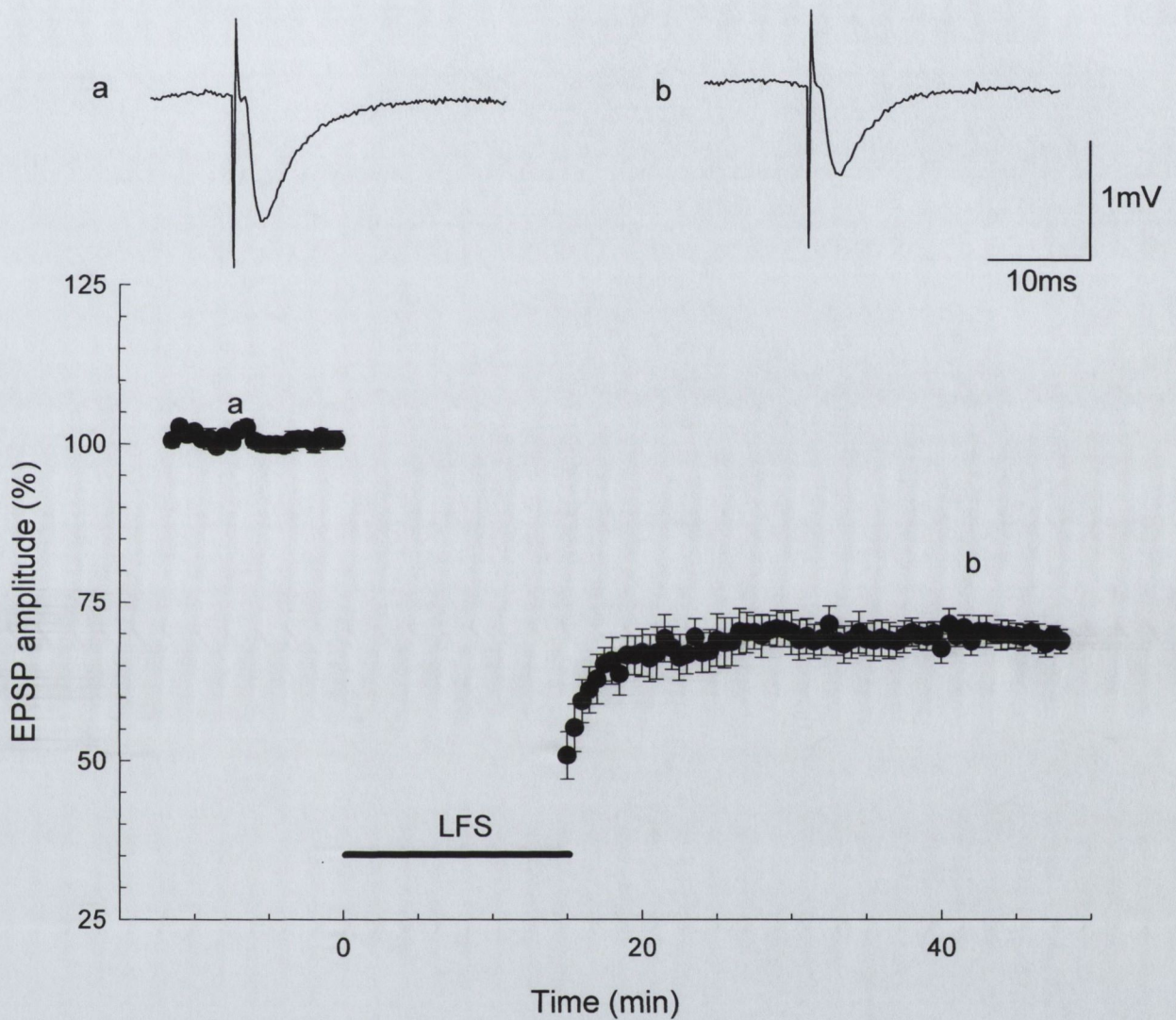


Figure 3.7 LTD in control solution.

When LFS was delivered in slices in control solution an LTD was induced that measured $31\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 8$) at 30 min after the end of LFS. Examples of single EPSPs are shown in **a** (baseline) and **b** (after the induction of LTD).

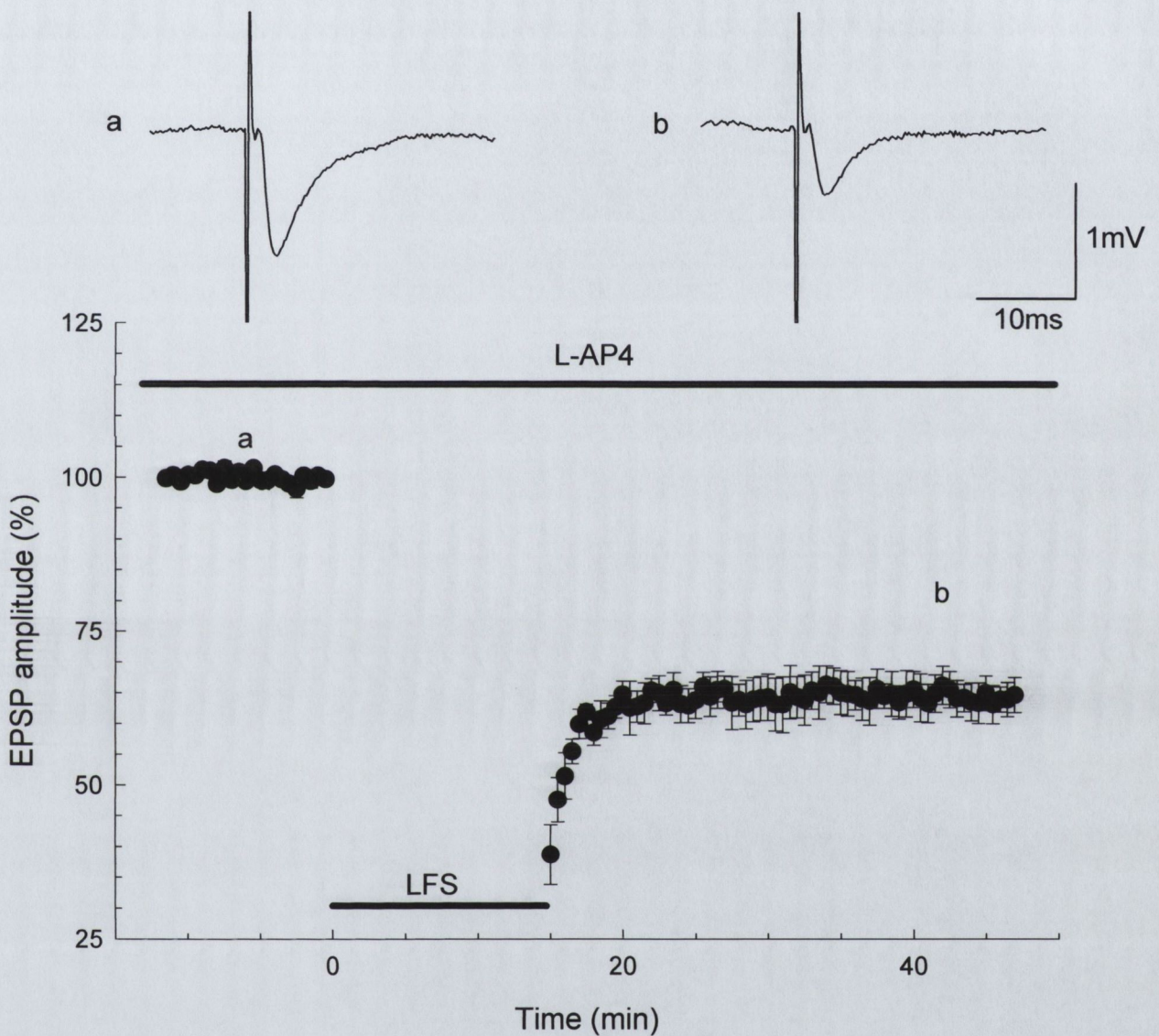


Figure 3.8 LTD in the presence of L-AP4.

In the presence of L-AP4 at a concentration of $20 \mu\text{M}$, LFS induced an LTD measuring $35\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 5$) measured at 30 min after the end of the LFS. This LTD was not significantly different from the LTD obtained in control solution (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Examples of single EPSPs are shown in **a** (baseline) and **b** (after the induction of LTD).

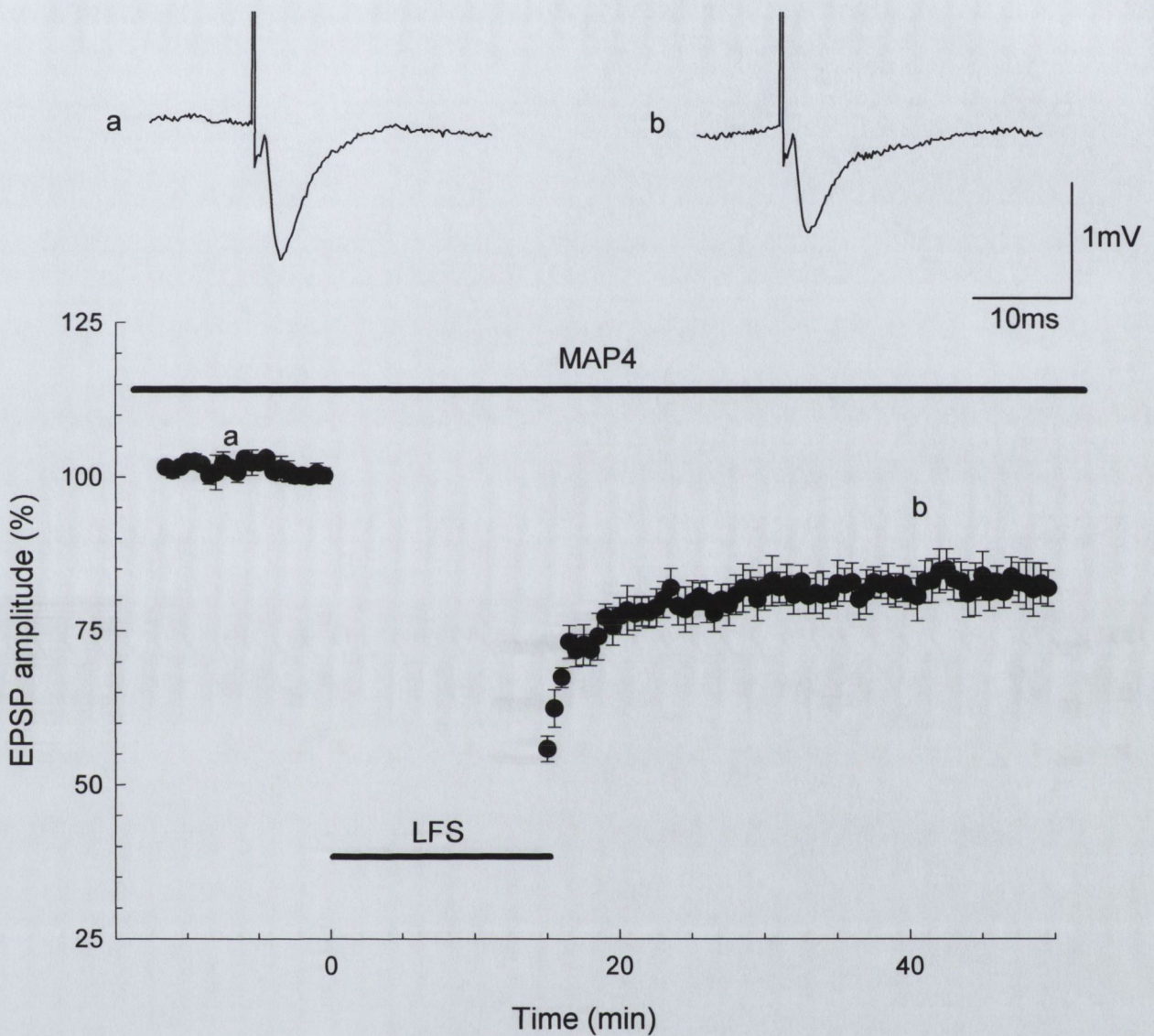


Figure 3.9 LTD in the presence of MAP4.

In the presence of the mGluR group III antagonist MAP4 at a concentration of $500 \mu\text{M}$, LFS induced an LTD of $18\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n=5$) measured at 30 min after the end of LFS. However this LTD was significantly reduced compared to the LTD induced in control solution (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$). Examples of single EPSPs are shown in **a** (baseline) and **b** (after the induction of LTD).

In addition these studies have shown that L-AP4 affects basal synaptic transmission.

Previous studies *in vivo* (Manahan-Vaughan and Reymann, 1995) and in CA1 *in vitro* (Baskys and Malenka, 1991) have demonstrated that L-AP4 affected basal synaptic transmission in an age-dependent manner, causing larger depression in younger animals.

An inhibition in the basal synaptic transmission upon perfusion with L-AP4 has been detected in the medial perforant pathway of the dentate gyrus in the present studies, in agreement with previously reported data (Ugolini and Bordi, 1995). This reduction in baseline transmission could be caused by the activation of a K^+ rectifying outward current, since it has been demonstrated that L-AP4 activates such a current in several preparations (Conn and Pin, 1997 and Cochilla and Alford, 1998).

Moreover, the perfusion of the slices with MAP4 caused a small depression of the EPSP amplitude, which was previously unreported. This depression is likely to be due to partial agonist activity of MAP4 on mGluR group II (Sekiyama *et al.*, 1996), which is abundant in the medial perforant pathway of the dentate gyrus (Shigemoto *et al.*, 1997).

There has been evidence for the involvement of activation of mGluR group III in short term synaptic plasticity in the medial perforant pathway (Kahle and Cotman, 1993b), however, unlike the *in vivo* experiments (Manahan-Vaughan and Reymann, 1995), the present studies showed that L-AP4 did not affect the induction of LTP and LTD in slices.

It has been demonstrated that mGluR group III receptors are located primarily on presynaptic nerve terminals, with the exception of ON bipolar cells in the retina (Nomura *et al.*, 1994), suggesting an action as autoreceptors in controlling glutamate release. There are marked differences in the sensitivity of various synaptic pathways to L-AP4 throughout the central nervous system and this is due to the different distribution of mGluR group III in the brain. In the dentate gyrus the

mGluR group III are distributed differently in the medial perforant pathway compared to the lateral perforant pathway, with a higher density in the lateral pathway (Kinzie *et al.*, 1995, Catania *et al.*, 1994 and Shigemoto *et al.*, 1997).

Activation of mGluR group III is linked to inhibition of the forskolin-stimulated levels of cAMP in the cells, which is an important second messenger involved in the induction of both LTP and LTD (Eriksen and Thomsen, 1995, Kemp *et al.*, 1996, Conn and Pin, 1997, Watkins and Collingridge, 1994, Pin and Duvoisin, 1995 and Sekiyama *et al.*, 1996). Also mGluR group II are linked to inhibition of forskolin stimulated cAMP levels. In addition to this, these receptors have a much higher density than mGluR group III in the medial perforant pathway. Therefore a possible explanation for the lack of effect of L-AP4 on LTP could be the role played by mGluR group II in LTP in the dentate gyrus, since it has already been demonstrated in this laboratory that mGluR group II are involved in the induction of both LTP and LTD in the medial perforant pathway (Huang *et al.*, 1997).

Another possible explanation for the discrepancies between the results reported by Manahan-Vaughan and Reymann (1995) and the results of the present studies could be in the different techniques and preparations used. Using brain slices it is possible to place precisely the electrodes in the medial perforant pathway because of the visual accessibility of the preparation. In the *in vivo* experiments, field potentials were recorded from the dentate granule layer. This does not allow any distinction between the medial and the lateral perforant pathways, leaving the possibility that the results obtained *in vivo* might be a combination of the effects of activation of mGluR group III in different pathways.

Activation of mGluR group III is necessary for the induction of LTD in the medial perforant pathway of the dentate gyrus, since in the presence of MAP4 LFS-induced LTD was greatly reduced. MAP4 is a very selective and potent antagonist at mGluR group III and it has been demonstrated that it blocks the depression of synaptic transmission induced by L-AP4 in several preparations (Salt and Eaton, 1995, Dietrich *et al.*, 1997, Jane *et al.*, 1994 and Vignes *et al.*, 1995). The present

studies did not show a complete block of LTD by MAP4, possibly because of the low density of these receptors in the medial-perforant pathway. This might indicate that other receptors also play a role in the induction of LTD. It has already been shown that mGluR group II antagonists block the induction of LTD in the medial perforant pathway of the dentate gyrus (Huang *et al.*, 1997). In addition to this, it has been demonstrated that activation of T-type Ca^{2+} channels is necessary for the induction of LTD in the dentate gyrus (Wang *et al.*, 1997) and in CA1 (Christie *et al.*, 1997)

In conclusion these studies provided new evidence for the involvement of mGluR group III in the induction of long term synaptic plasticity in the medial perforant pathway of the dentate gyrus.

4 Calcium-induced long-term depression (LTD) in the dentate gyrus *in vitro*.

4.1 Introduction.

An influx of Ca^{2+} from the extracellular medium and the subsequent rise in intracellular Ca^{2+} is a key stage in the induction of both LTP and LTD of excitatory post-synaptic potentials (EPSPs) as post-synaptic injection of Ca^{2+} chelators prevents the induction of LTP in CA1 (Lynch *et al.*, 1983) and LTD in CA1 (Mulkey and Malenka, 1992) and the dentate gyrus (Wang *et al.*, 1997). It has been postulated that the induction of LTP requires a higher influx of Ca^{2+} and a resulting larger increase in the intracellular concentration of Ca^{2+} , than the induction of LTD (Neveu and Zucker, 1996b). In experiments involving direct measurement of intracellular Ca^{2+} , LTD was found to be induced by a smaller increase of Ca^{2+} than LTP in both CA1 (Otani and Connor, 1996) and cortex (Yasuda and Tsumoto, 1996). Thus the induction of LTD is much less sensitive to a lowering of extracellular Ca^{2+} than the induction of LTP in CA1 (Mulkey and Malenka, 1992 and Cummings *et al.*, 1996) and the dentate gyrus (Wang *et al.*, 1997).

LTP is regularly induced by a brief period of high frequency synaptic stimulation (HFS) (Bliss and Lomo, 1973) or by strong intracellular depolarisation paired with brief low frequency synaptic stimulation (LFS) (Abraham *et al.*, 1986, Gustafsson *et al.*, 1987 and Kauer *et al.*, 1988), while LTD is regularly induced by a more prolonged period of low frequency synaptic activity (Mulkey and Malenka, 1992 and Dudek and Bear, 1992) or by weak intracellular depolarisation paired with a brief LFS (Wang *et al.*, 1997). In addition, LTP in CA1 has been found to be induced even by using stimulation at the test frequency if the extracellular Ca^{2+} is raised from the control 2 mM to 4 mM, termed Ca^{2+} -induced LTP (Mody *et al.*,

1984 and Reymann *et al.*, 1986). However the influence of alteration of the Ca^{2+} concentration in the extracellular medium on the induction of LTD had not previously been analysed.

In the present study I have investigated the induction of LTD by altering the Ca^{2+} concentration in the extracellular solution.

4.2 Results

4.2.1 LTD induction by perfusion of Ca^{2+} medium and return to control medium.

The basic phenomenon of the Ca^{2+} -induced LTD is shown in Fig. 4.1. When the extracellular Ca^{2+} concentration was reduced from the control concentration of 2 mM to 1.2 mM, the field excitatory post-synaptic potential (EPSP) was reduced in 5 min to $61\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=8$) of the control baseline value. Following perfusion with the 1.2 mM Ca^{2+} solution for 30 min, a return to the control Ca^{2+} concentration of 2 mM resulted in the generation of the Ca^{2+} -induced LTD, that measured $26\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=8$) at 30 min after the washout of the solution containing 1.2 mM Ca^{2+} . Experiments were carried out to test whether the duration of the application of the low Ca^{2+} solution affected the generation of the LTD. When the 1.2 mM Ca^{2+} solution was applied for 10 min, the EPSP amplitude after washout of the low Ca^{2+} medium was only slightly reduced compared to the baseline value (Fig. 4.2). Moreover, when the 1.2 mM Ca^{2+} solution was applied for 45 min, an LTD was induced that was similar in amplitude to the Ca^{2+} -induced LTD when the 1.2 mM Ca^{2+} solution was applied for 30 min (Fig. 4.3). This implies that a perfusion for 30 min with the low Ca^{2+} solution was necessary and sufficient for the induction of the Ca^{2+} -induced LTD. Therefore, for all the subsequent experiments, a perfusion of

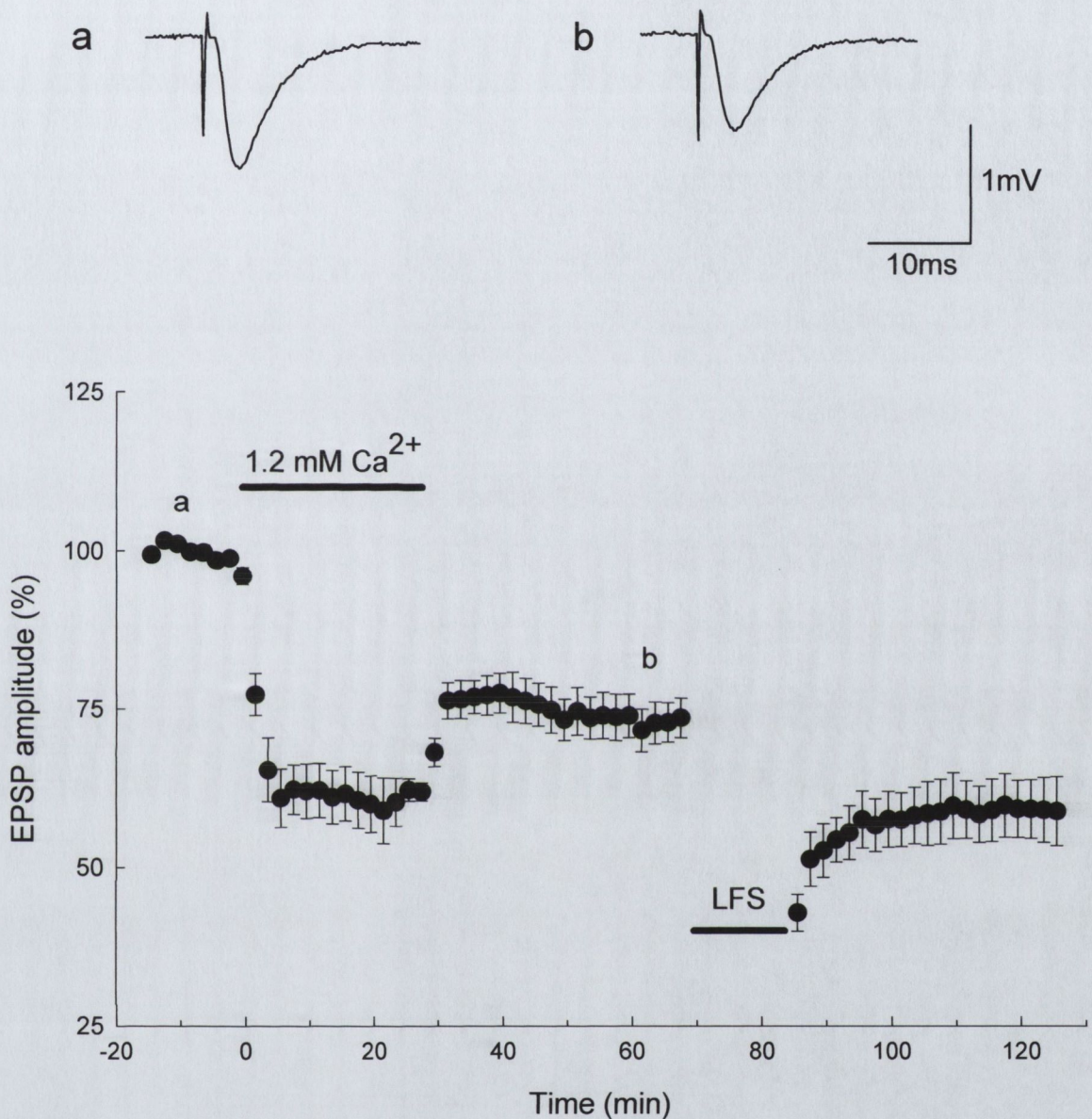


Figure 4.1 Ca²⁺-induced LTD.

When the slices were perfused with the medium containing 1.2 mM Ca²⁺ for 30 min an LTD was generated that measured 26%±2% (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 8$) at 30 min from the washout of the low Ca²⁺ solution. Further LFS induced an LTD of 18%±4% (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 8$) at 40 min post-LFS. Examples of single EPSPs are shown in **a** (baseline) and **b** (30 min after washout).

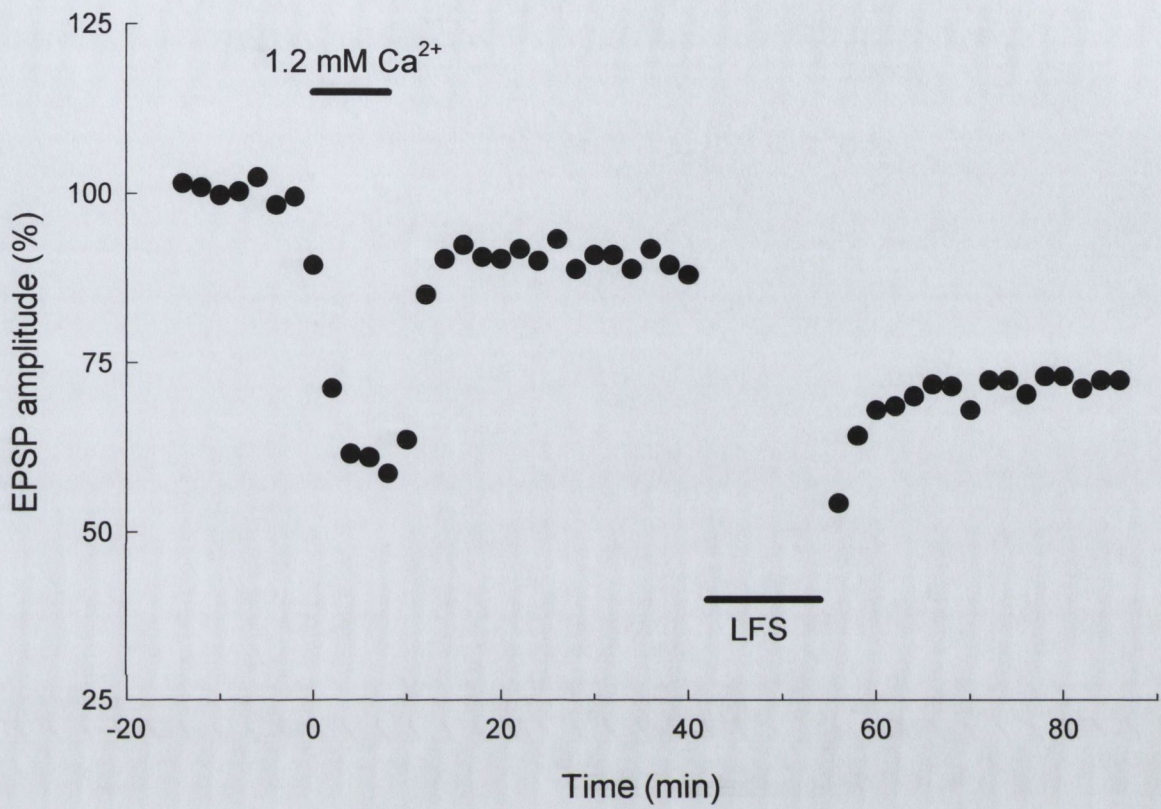


Figure 4.2 Perfusion with the low Ca^{2+} medium for 10 min.

Example of a single slice perfused with the solution containing 1.2 mM Ca^{2+} for 10 min. Upon reperfusion with control solution, the EPSP exhibited a depression of approximately 10%, which was much smaller than the Ca^{2+} -induced LTD obtained when the slices were perfused with 1.2 mM Ca^{2+} for 30 min (see Fig. 4.1).

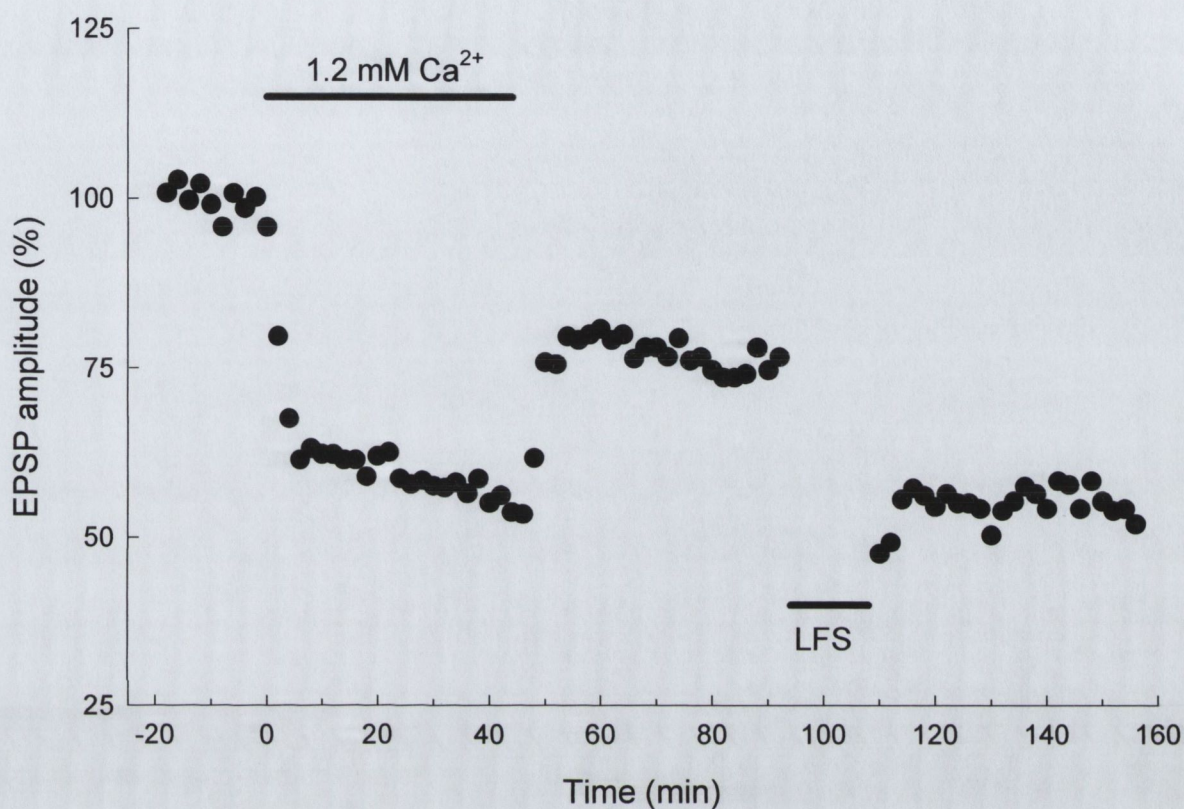


Figure 4.3 Perfusion with low Ca²⁺ solution for 45 min.

Example of a single slice perfused with the medium containing 1.2 mM Ca²⁺ for 45 min. Upon reperfusion with the control solution an LTD was generated similar in amplitude to that obtained when the low Ca²⁺ solution was perfused for 30 min (see Fig. 4.1).

the slices with the solution containing 1.2 mM Ca^{2+} for 30 min was used to induce the Ca^{2+} -induced LTD.

4.2.2 Common intracellular pathways were shared by LFS-induced LTD and Ca^{2+} -induced LTD.

In order to determine whether the Ca^{2+} -induced LTD was produced via the same intracellular mechanisms as those of LFS-induced LTD, occlusion experiments were carried out between the Ca^{2+} -induced LTD and the LFS-induced LTD.

In a first set of experiments, Ca^{2+} -induced LTD was found to significantly occlude the induction of further LTD by LFS. Following Ca^{2+} -induced LTD measuring $26\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=8$), LFS could only induce a further LTD measuring $18\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=8$) (Fig. 4.1) at 40 min after the end of LFS, a value significantly reduced from control LFS-induced LTD of $31\% \pm 2\%$ (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$) (Fig. 3.7). This control LFS-induced LTD was very similar in amplitude to that found previously in the medial perforant pathway (O'Mara *et al.*, 1995b and Wang *et al.*, 1997).

In a second set of experiments, LTD was induced to a near maximal level by application of prolonged LFS. LTD measured $29\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=6$) at 30 min after the end of the second LFS (Fig. 4.4). Upon perfusion of the low Ca^{2+} solution, the amplitude of the EPSP was reduced to $46\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=6$) of the value before the administration of the LFS measured at 30 min after perfusion with the low Ca^{2+} medium. This reduction was similar to that obtained in the non-occluded experiments (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). However, upon return to control medium, no LTD was induced, since the test EPSP returned to a depression of $27\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=6$) from the original baseline measured at 40 min after the start of washout. This value was not

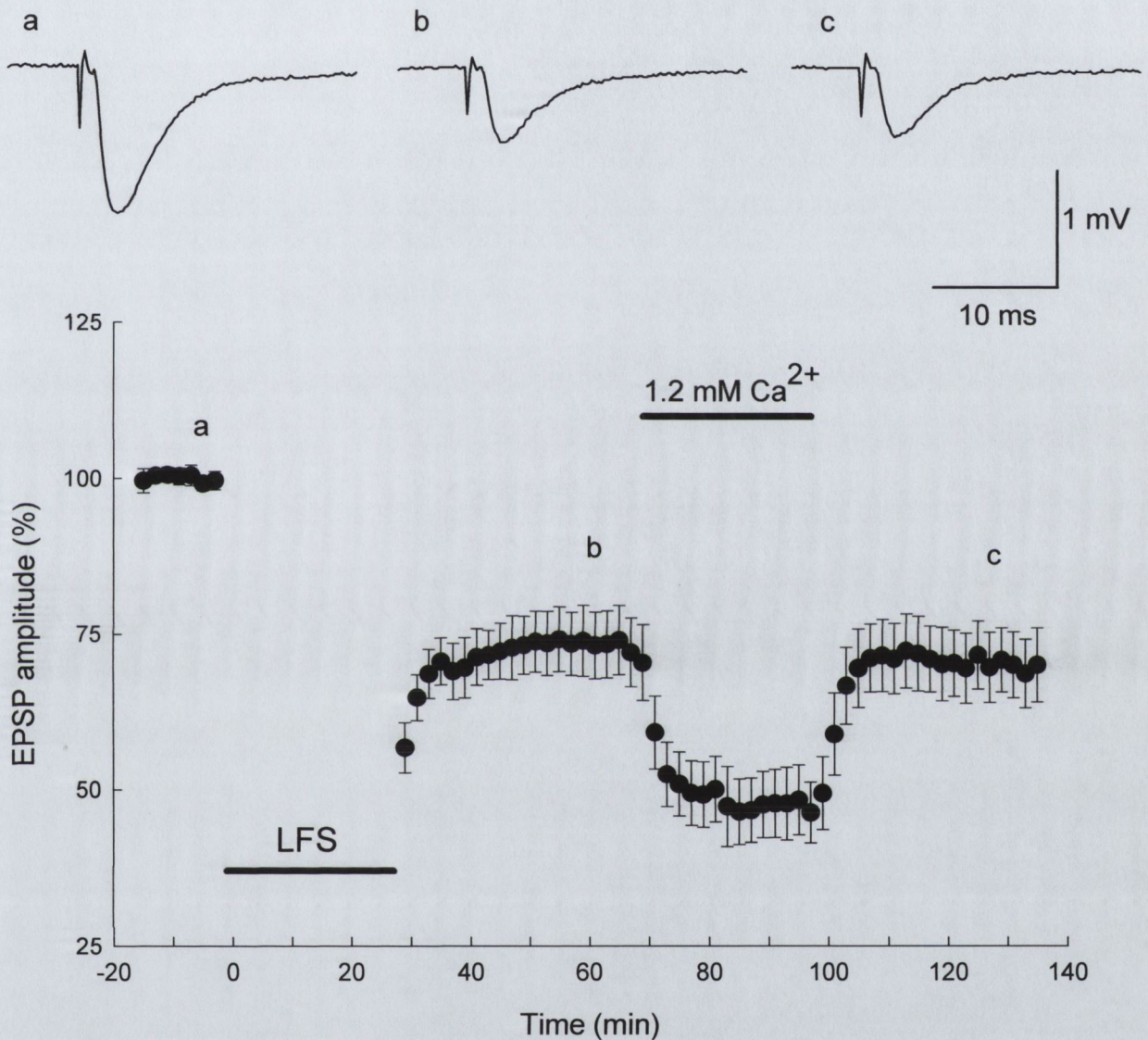


Figure 4.4 LFS-induced LTD occluded Ca²⁺-induced LTD.

After induction of maximal LTD measuring $29\% \pm 5\%$ of control (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 6$) by LFS-application, the perfusion with the solution containing 1.2 mM Ca^{2+} failed to induce any further LTD. Examples of single EPSPs are shown in **a** (baseline), **b** (30 min after the induction of LTD) and **c** (40 min after washout).

significantly different from that reached with LFS prior to the perfusion with low Ca^{2+} solution (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$) (Fig. 4.4).

These occlusion experiments showed that common intracellular pathways are shared by the LFS-induced LTD and the Ca^{2+} -induced LTD.

4.2.3 Ca^{2+} -induced LTD and LFS-induced LTD were not accompanied by a change in the probability of transmitter release.

Another set of experiments was carried out in order to determine whether the Ca^{2+} -induced LTD and the LFS-induced LTD had a presynaptic component by measuring paired-pulse depression throughout the experiments. Any changes in paired-pulse depression would be indicative of changes in the probability of transmitter release.

When LFS was given to slices in control solution an LTD was induced that measured $32\% \pm 1\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 6$) at 30 min post LFS (Fig. 4.5). Paired pulse depression of paired EPSPs (Kahle and Cotman, 1993a), measured at 40 msec interval, was not significantly altered following expression of the LFS induced LTD (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 6$). In the above experiments, paired pulse depression measured $31\% \pm 4\%$, during baseline and $33\% \pm 3\%$ following LFS-induction of LTD.

Similar experiments were carried out for the Ca^{2+} -induced LTD. After washout of the solution containing 1.2 mM Ca^{2+} , LTD was induced measuring $22\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 4$) at 30 min post-washout (Fig. 4.6). Paired pulse depression in these experiments was not significantly altered following the induction of LTD (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 4$), paired pulse depression measuring $24\% \pm 3\%$ during baseline and $23\% \pm 3\%$ following the induction of Ca^{2+} -induced LTD.

These results would indicate that LTD induction was not due to a reduction in the probability of transmitter release, agreeing with previous experiments in CA1 in

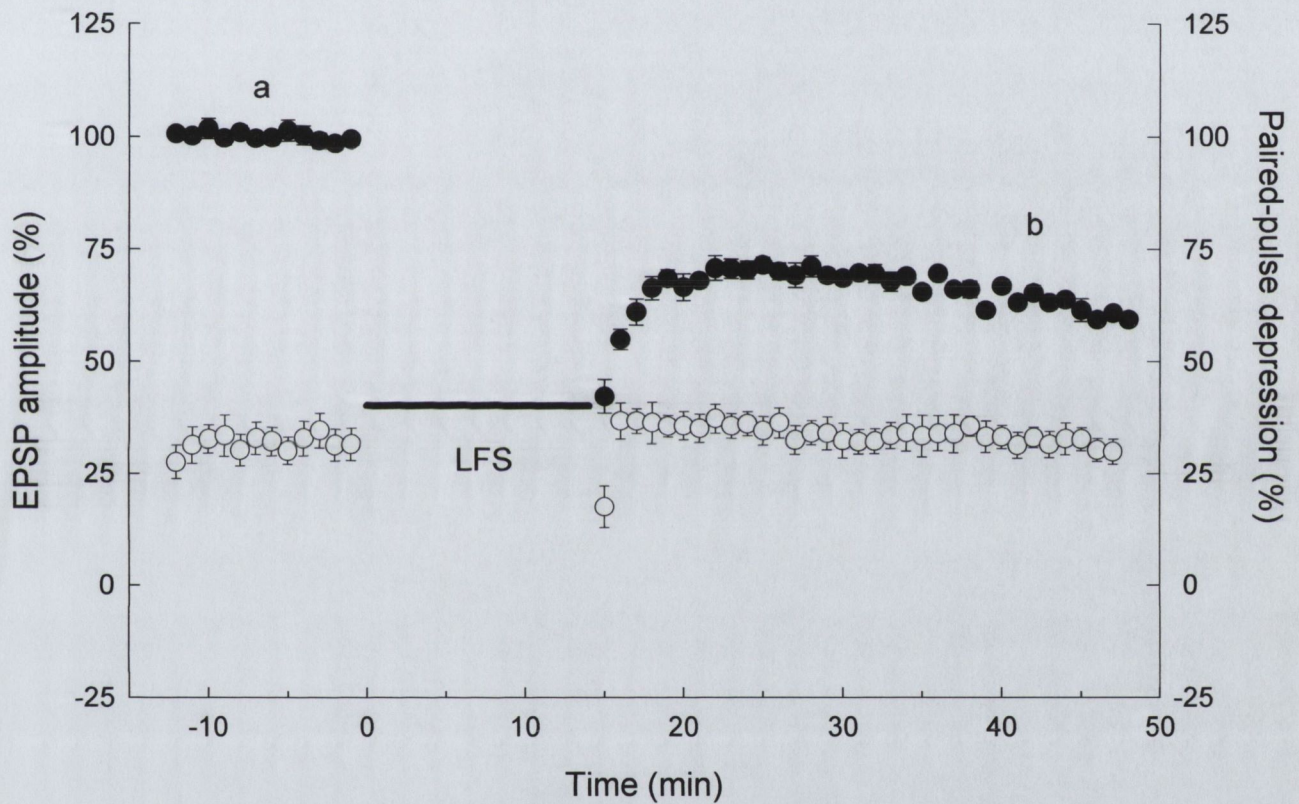
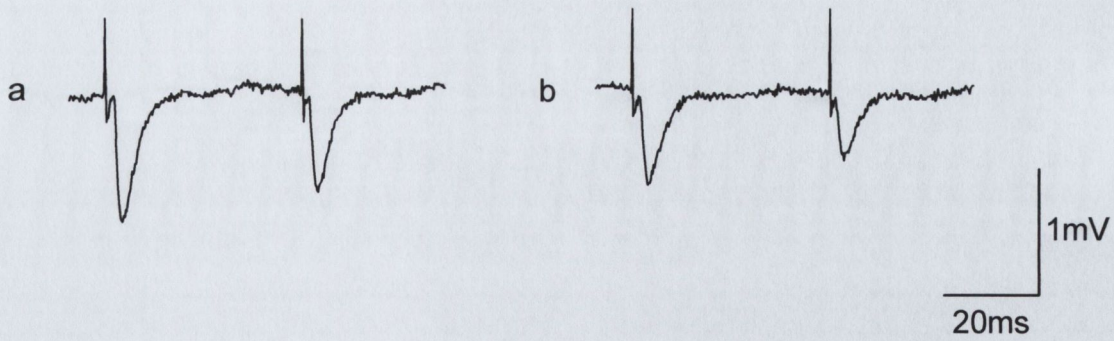


Figure 4.5 Paired-pulse depression and LFS-induced LTD.

LFS induced an LTD of $32\% \pm 1\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 6$) at 30 min post-LFS. This LTD was not accompanied by a change in paired-pulse depression. Filled symbols indicate EPSP amplitude and clear symbols indicate paired-pulse depression. Sample pairs of EPSPs are shown in **a** (baseline) and **b** (30 min after the induction of LTD).

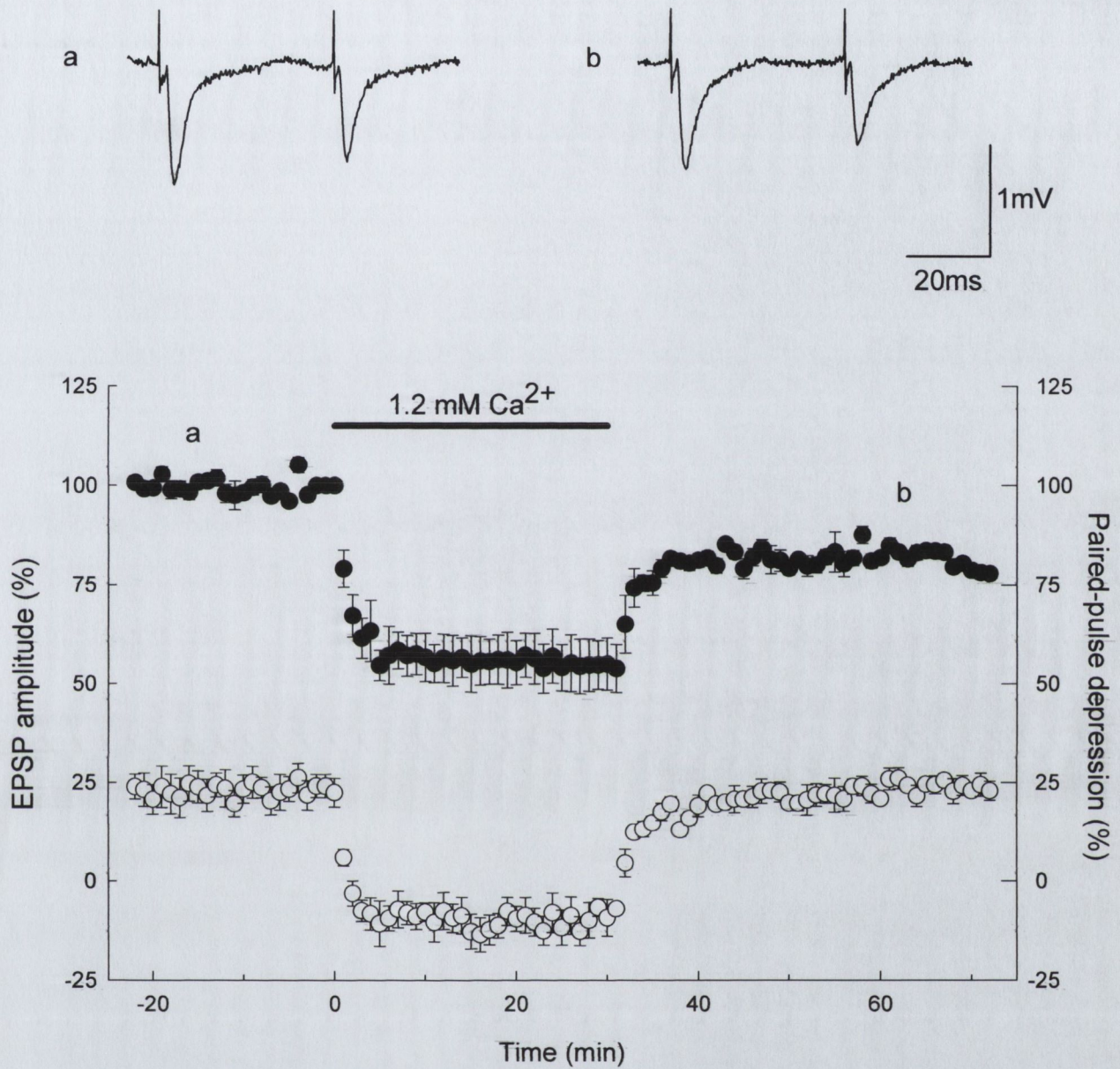


Figure 4.6 Paired-pulse depression and Ca²⁺-induced LTD.

When the slices were perfused with the media containing 1.2 mM Ca²⁺ an LTD was generated that measured 22%±2% (Student's *t*-test, 2 tailed, paired, *p*<0.005, *n*=4) at 30 min from the washout of the low Ca²⁺ solution. This LTD was not accompanied by a change in paired-pulse depression. Filled symbols indicate EPSP amplitude and clear symbols indicate paired-pulse depression. Sample pairs of EPSPs are shown in **a** (baseline) and **b** (30 min after washout).

which no change in paired-pulse facilitation of EPSPs occurred following induction of LTD (Mulkey and Malenka, 1992).

4.2.4 Ca^{2+} -induced LTD was induced by the increase of Ca^{2+} to the control levels from the perfusion with Ca^{2+} medium.

The Ca^{2+} -induced LTD could have been generated either upon the initial perfusion with the low Ca^{2+} medium or, alternatively, upon reperfusion of the control medium. In order to test these alternative theories, LFS was applied during the perfusion with the low Ca^{2+} solution. The idea behind this experiment was that if the Ca^{2+} -induced LTD had been already induced by the perfusion with the low Ca^{2+} medium, then the LFS-induced LTD would have been reduced. As shown in Fig. 4.7, after recording a period of stable baseline responses, the slices were perfused with the low Ca^{2+} solution. After 30 min the EPSP amplitude had reached a stable depressed level measuring $51\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=5$) of the baseline value in the control solution. The stimulation intensity was then increased in order to obtain EPSPs similar in amplitude to the baseline responses. LFS applied in the low Ca^{2+} solution induced an LTD measuring $27\% \pm 3\%$ of the EPSP amplitude recorded after the increase in the stimulation intensity in the presence of the 1.2 mM Ca^{2+} medium (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=5$). This value was not significantly different from LFS-induced LTD induced in the control medium (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$).

These experiments demonstrated that the Ca^{2+} -induced LTD was not induced by the perfusion with the low Ca^{2+} solution alone and the transfer from the low Ca^{2+} medium to the control Ca^{2+} medium was essential for the induction of the Ca^{2+} -induced LTD.

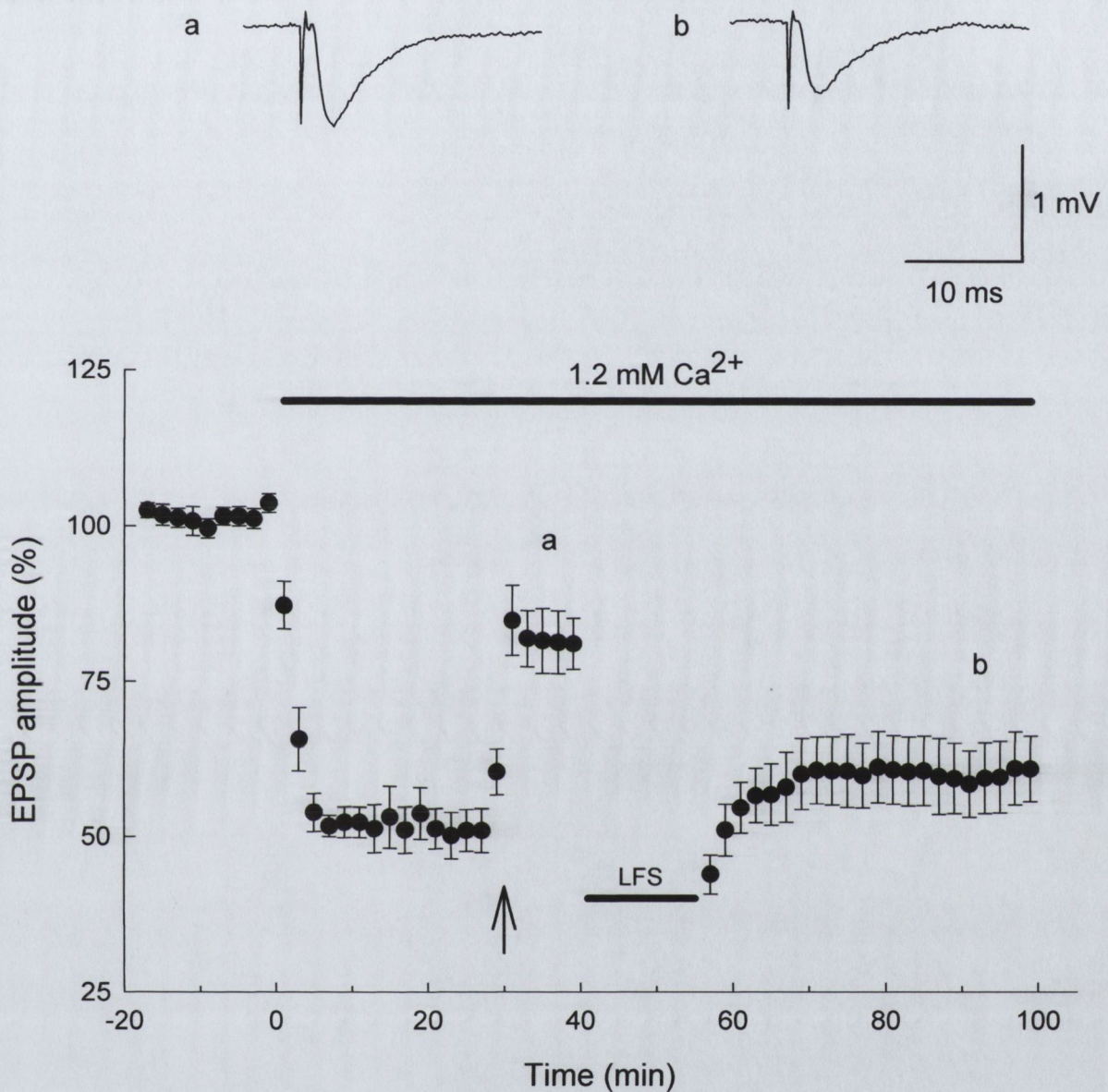


Figure 4.7 LFS-induced LTD during perfusion with low Ca²⁺ solution.

After perfusion with the low Ca²⁺ medium for 30 min, the stimulus intensity was increased (arrow). After 10 min at the increased stimulus intensity, LFS was delivered, inducing an LTD measuring 27%±3% of the of the EPSP amplitude recorded after the increase in the stimulation intensity (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 5$) at 30 min post LFS. Examples of single EPSPs are shown in **a** (baseline) and **b** (30 min after the induction of LTD).

4.2.5 The increase of the Ca^{2+} concentration in the control medium alone was not sufficient to induce the Ca^{2+} -induced LTD.

Since it has been shown that the Ca^{2+} -induced LTD was not induced by lowering the Ca^{2+} concentration in the external medium alone, it was postulated that a rise in the Ca^{2+} concentration was responsible for the LTD induction. It was therefore of interest to determine whether raising Ca^{2+} directly from the concentration of 2 mM in the control solution could also result in the induction of LTD.

As shown in Fig. 4.8, after recording EPSPs in control solution with 2 mM Ca^{2+} , the slices were perfused with a medium containing 4 mM Ca^{2+} for 30 min. During the perfusion with the solution with 4 mM Ca^{2+} a slight increase of the EPSP amplitude could be detected, the EPSP measuring $106\% \pm 6\%$ ($n=7$) at 30 min after the start of the perfusion with the solution with the increased Ca^{2+} concentration. However this increase was not significant when compared with the baseline EPSP amplitude (Student's t -test, 2 tailed, paired, $p > 0.05$, $n=7$). This was in agreement with what was found previously by Turner and Miller (1982).

Moreover, the washout of the solution with 4 mM Ca^{2+} with the control solution failed to induce any LTD, as the EPSP amplitude measured $95\% \pm 4\%$ (Student's t -test, 2 tailed, paired, $p > 0.05$, $n=7$) at 30 min after the start of the reperfusion with the solution containing 2 mM Ca^{2+} .

Subsequent application of LFS to these slices resulted in the induction of LTD that measured $24\% \pm 3\%$ (Student's t -test, 2 tailed, paired, $p < 0.005$, $n=7$) at 30 min after the end of the LFS. Thus the perfusion with the solution containing 4 mM Ca^{2+} did not affect the induction of the LFS-induced LTD. This LTD was not significantly different from the LTD induced in control solution (Student's t -test, 2 tailed, homoscedastic, $p > 0.05$).

These experiments showed that a direct rise in the Ca^{2+} concentration from the control level failed to induce LTD, implying that the reduction of the Ca^{2+} concentration to 1.2 mM prior to the reperfusion with the control Ca^{2+} solution was necessary for the induction of the Ca^{2+} -induced LTD.

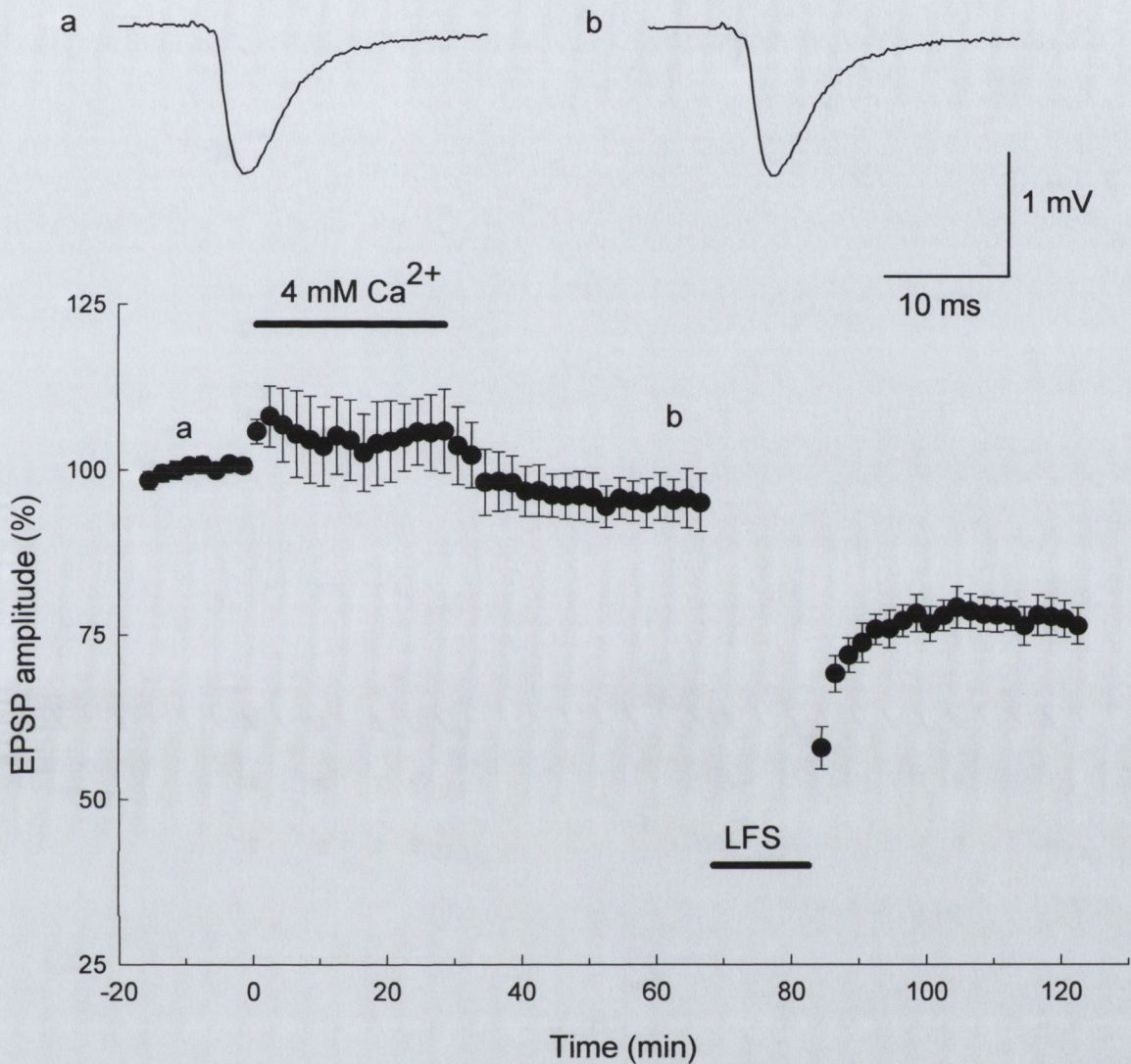


Figure 4.8 Perfusion with 4 mM Ca²⁺ solution.

When the slices were perfused with a solution containing 4 mM Ca²⁺ no LTD was generated, the EPSP measuring 95%±4% (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 7$) at 30 min after the start of the reperfusion with the solution containing 2 mM Ca²⁺. Subsequent LFS induced LTD that measured 24%±3% (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 7$) at 30 min after the end of the LFS. Examples of single EPSPs are shown in **a** (baseline) and **b** (30 min after washout).

4.2.6 Synaptic stimulation was required for the induction of Ca²⁺ the induced LTD.

From the preliminary experiments described up to this point it can be seen that two major phases were necessary for the induction of the Ca²⁺-induced LTD:

- 1) Perfusion for 30 min in a solution with a lower Ca²⁺ concentration (1.2 mM) compared to the control solution (2 mM).
- 2) Increase of the extracellular Ca²⁺ concentration to 2mM upon reperfusion with control medium.

The next step was therefore to assess whether synaptic stimulation was necessary during both phases (1) and (2) in order to induce the Ca²⁺-induced LTD.

In these experiments after recording EPSPs at the test frequency of 0.033 Hz in control solution, the slices were perfused with the medium containing 1.2 mM Ca²⁺ for 30 min during which the stimulation was ceased. After the usual period in the low Ca²⁺ solution, the slices were then reperfused with the control solution, but the stimulation was reinitiated only 15 minutes after the start of the reperfusion. As shown in Fig. 4.9 no LTD was induced with this protocol, the EPSP measuring 91%±7% (Student's *t*-test, 2 tailed, paired, *p*>0.05, *n*=10) at 30 min after the stimulation was reinitiated. It can be noted also that when the stimulation was reinitiated in control solution, a short-term potentiation was induced, with a maximum amplitude of 114%±9% (*n*=10) which decayed to baseline levels over a period of 10 to 15 min.

Moreover, as shown in Fig. 4.10, when the same stimulation protocol was applied to a slice perfused for the whole length of the experiment with only control solution, a similar STP could be detected.

These experiments demonstrated that synaptic stimulation during the perfusion with the low Ca²⁺ solution and then reperfusion with the control solution was essential for the induction of the Ca²⁺-induced LTD.

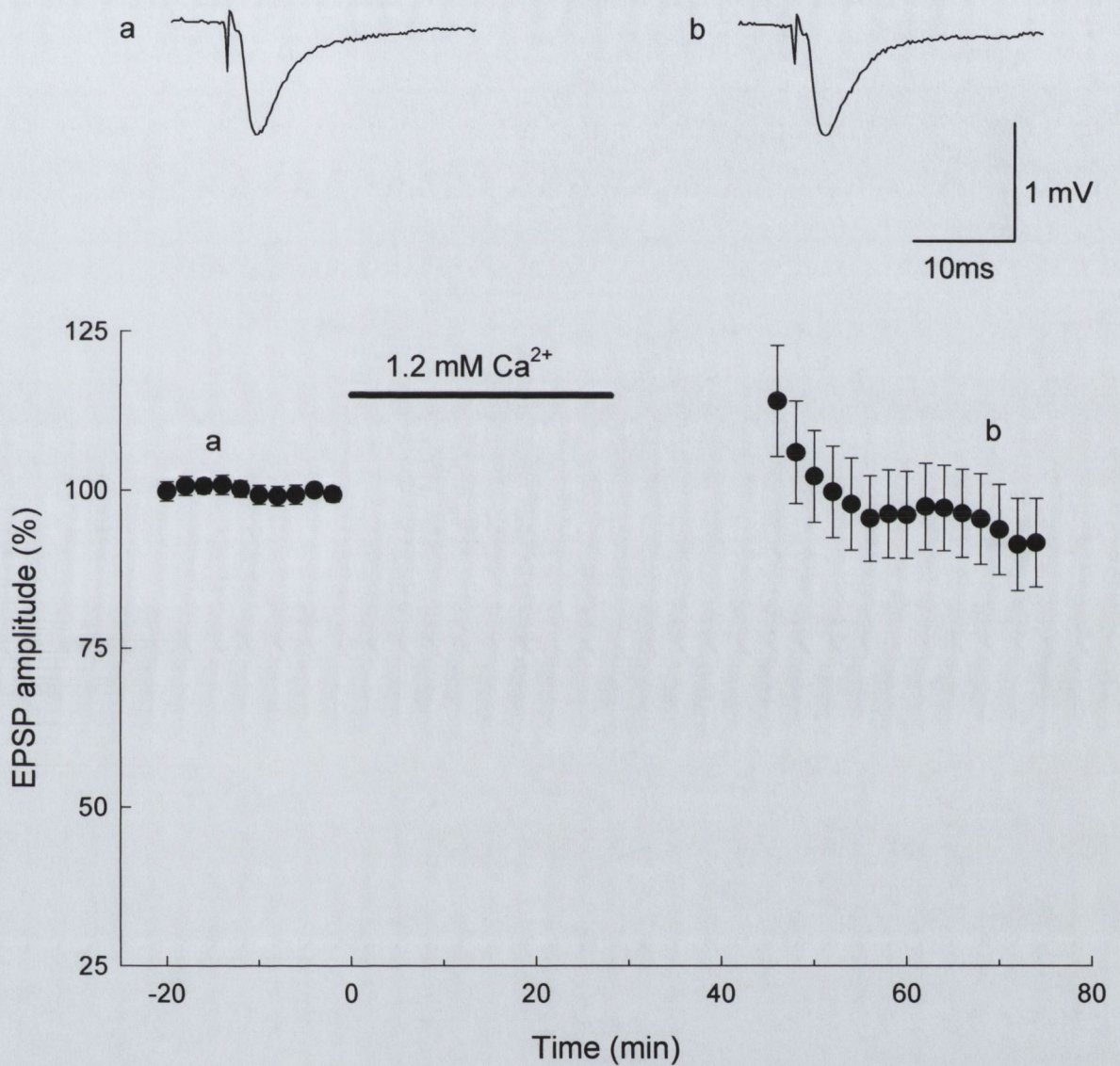


Figure 4.9 Synaptic stimulation was required for Ca²⁺-induced LTD.

When the stimulation was stopped during perfusion with the media containing 1.2 mM Ca²⁺ and for the first 15 min of the washout, no LTD was induced, the EPSP measuring 91%±7% (Student's *t*-test, 2 tailed, paired, *p*>0.05, *n*=10) at 30 min after the stimulation was reinitiated. Examples of single EPSPs are shown in **a** (baseline) and **b** (30 min after the reinitiation of stimulation).

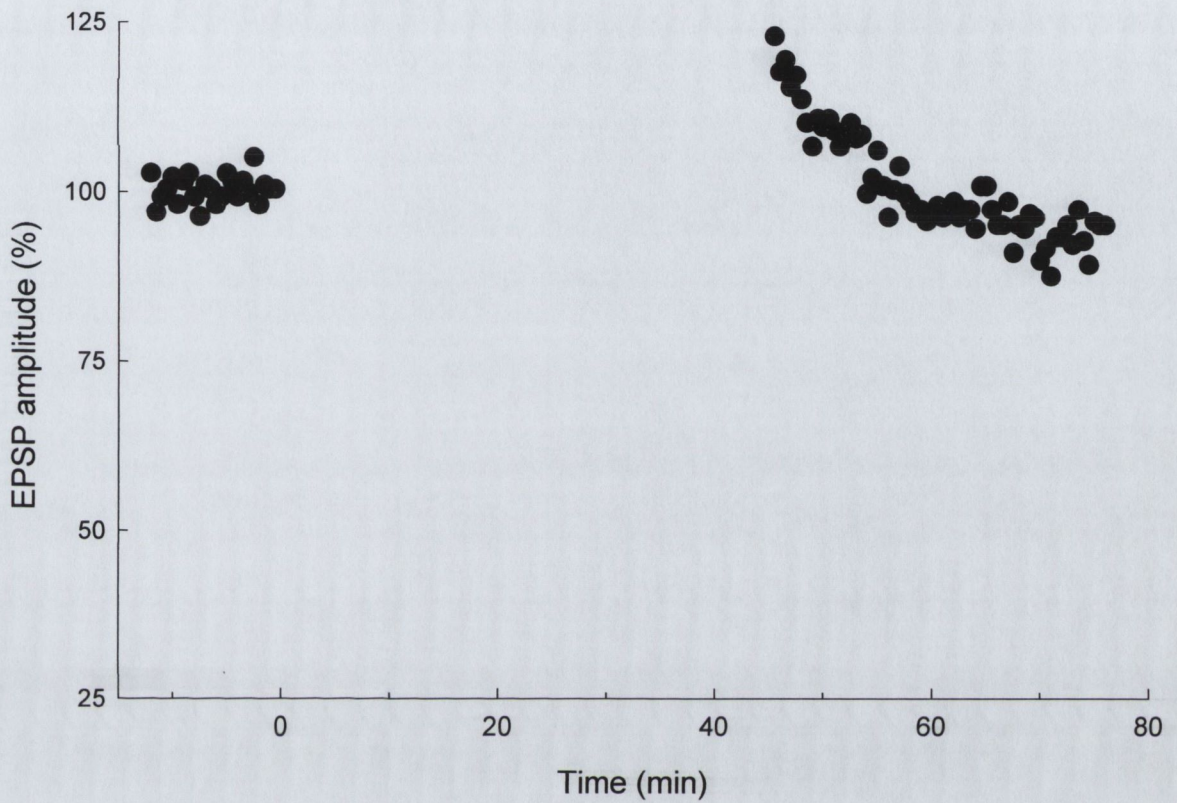


Figure 4.10 Effects of ceasing synaptic stimulation in control solution.

A slice perfused with control solution without being stimulated for 45 min exhibited an STP when the stimulation was recommenced, like the slices in which the stimulation was stopped during perfusion with the low Ca^{2+} solution (see Fig. 4.9).

4.2.7 Ca²⁺ influx via NMDA receptors (NMDAR) and Ni²⁺ sensitive voltage activated Ca²⁺ channels was necessary for the induction of the Ca²⁺-induced LTD.

Since synaptic stimulation and a rise in Ca²⁺ concentration from a lower level were necessary for the induction of the Ca²⁺-induced LTD, this suggested that the synaptic stimulation resulted in the opening of channels permeable to Ca²⁺ responsible for the Ca²⁺ influx in the neurones. Two possible candidates for this role would be the NMDAR and the low voltage activated (LVA) Ca²⁺ channels.

Thus two sets of experiments were carried out, one in the presence of the NMDAR antagonist D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) and the other with the LVA Ca²⁺-channel blocker Ni²⁺ in order to determine which channels were responsible for the induction of the Ca²⁺-induced LTD.

4.2.7.1 The NMDAR antagonist D-AP5 partially blocked the induction of the Ca²⁺-induced LTD.

To determine whether the influx of Ca²⁺ took place through activation of NMDAR, experiments were carried out in the presence of the competitive NMDAR antagonist D-AP5 at a concentration of 50 µM.

Perfusion of the slices with 50 µM D-AP5 resulted in a small but significant depression of the EPSP amplitude of 6%±3% (Student's *t*-test, 2 tailed, paired, *p*<0.001, *n*=6) measured at 40 min after the start of the perfusion with the antagonist (Fig. 4.11).

All the following experiments were carried out perfusing the slices with D-AP5 for at least 40 min prior to the start of the recordings.

As shown in Fig, 4.11, after recording EPSPs in the control solution in the presence of 50 µM D-AP5, the slices were perfused with the medium containing 1.2 mM Ca²⁺ and 50 µM D-AP5, during which the EPSP amplitude was reduced to 51%±4% (Student's *t*-test, 2 tailed, paired, *p*<0.001, *n*=6) of the baseline value.

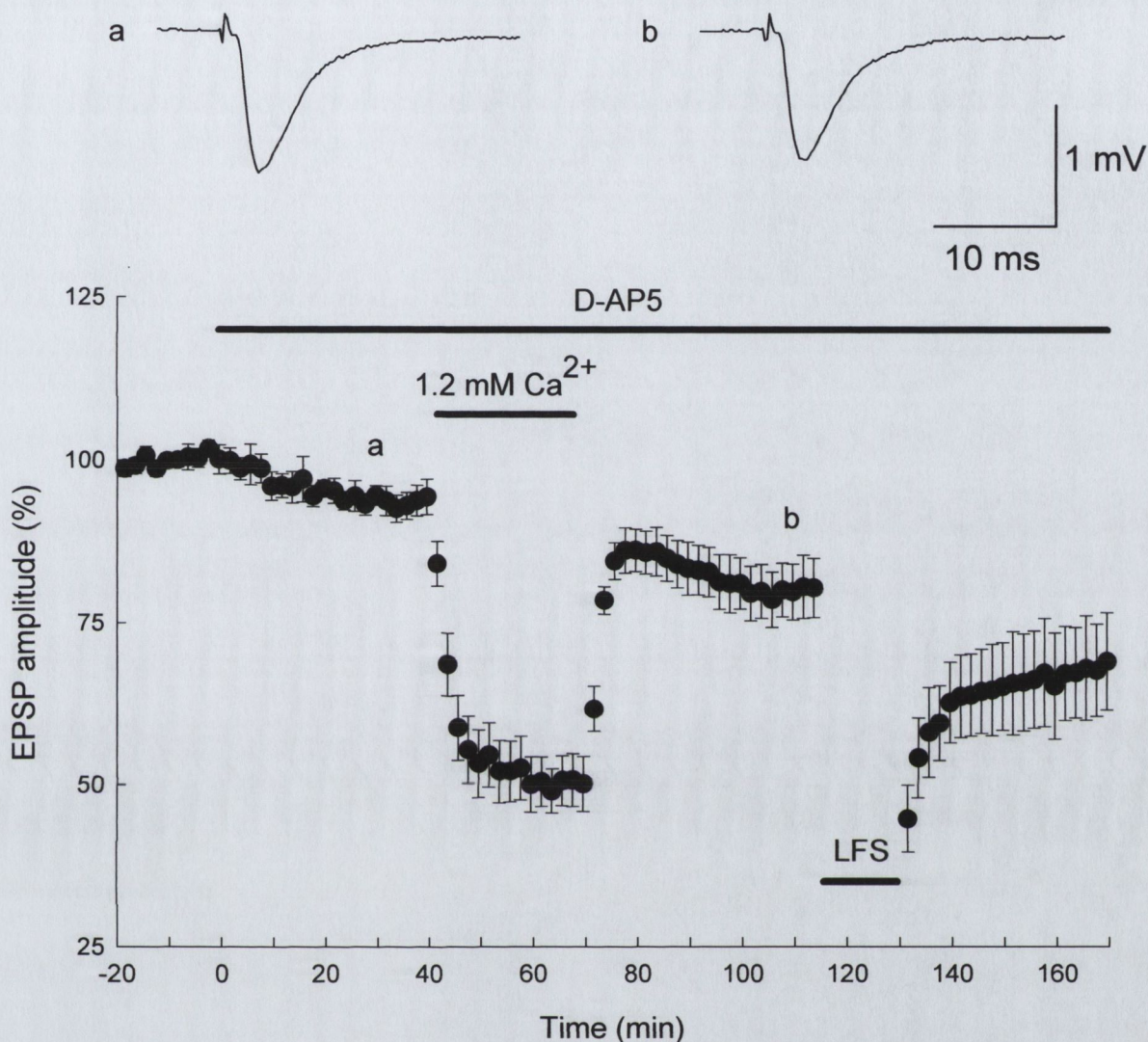


Figure 4.11 Effects of D-AP5 on Ca²⁺-induced LTD.

In the presence of 50 μM D-AP5, perfusion with the media containing 1.2 mM Ca²⁺ generated an LTD of $13\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 6$) at 30 min from the washout of the low Ca²⁺ solution, which was significantly reduced compared to that induced in the absence of D-AP5 (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.01$). Subsequent LFS induced further significant LTD measuring $13\% \pm 6\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 6$). Examples of single EPSPs are shown in **a** (baseline in D-AP5) and **b** (30 min after washout in D-AP5).

This depression was similar (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$) to the depression obtained in all the other experiments in the presence of the medium containing 1.2 mM Ca^{2+} without D-AP5. Upon reperfusion with the control solution containing 50 μM D-AP5 a Ca^{2+} -induced LTD was generated measuring $13\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 6$) at 30 min from the start of the reperfusion. However this LTD was significantly reduced compared to the LTD obtained in the absence of D-AP5 (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.01$).

Subsequent application of LFS to these slices in the presence of 50 μM D-AP5 was able to induce further LTD measuring $13\% \pm 6\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 6$). This residual LTD was not statistically different from that obtained in the same conditions in the absence of D-AP5 (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$).

This latter result was consistent with previous studies by Wang *et al.* (1997).

As can be seen in Fig. 4.12, application of the medium containing 1.2 mM Ca^{2+} after washout of 100 μM D-AP5 induced a normal LTD.

These experiments demonstrated that the influx of Ca^{2+} through activation of NMDAR played a role in the generation of the Ca^{2+} -induced LTD.

4.2.7.2 The LVA Ca^{2+} channel blocker Ni^{2+} inhibited the Ca^{2+} -induced LTD.

To determine whether the Ca^{2+} -induced LTD was dependent upon influx of Ca^{2+} through LVA Ca^{2+} channels, experiments were carried out in the presence of Ni^{2+} , a selective inhibitor of LVA Ca^{2+} channels at concentrations between 25 μM and 50 μM (Avery and Johnston, 1996).

When Ni^{2+} was applied in the medial perforant pathway at a concentration of 50 μM it caused a large depression, the EPSP amplitude measuring 21% as can be seen in Fig. 4.13. It was therefore decided to reduce the Ni^{2+} concentration to 25 μM for all the following experiments.

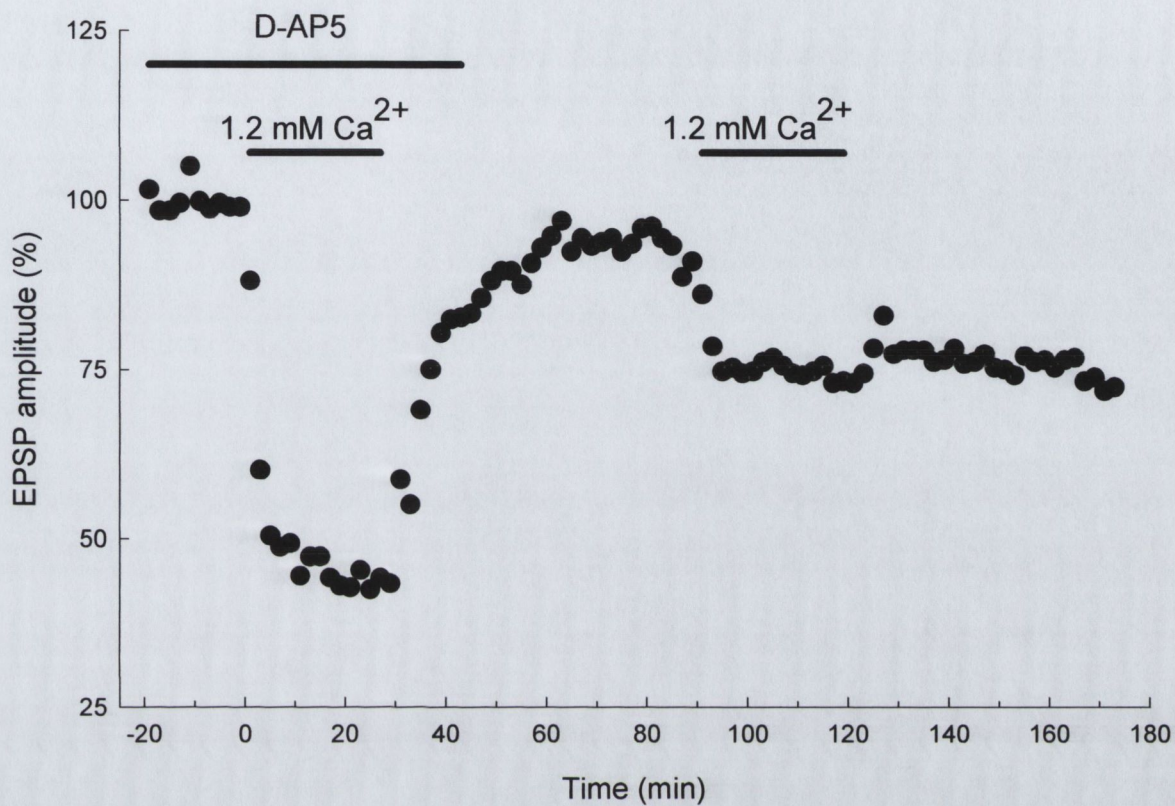


Figure 4.12 Reversible effects of D-AP5 on Ca²⁺-induced LTD.

Example of a single slice in which application of the 1.2 mM Ca²⁺ solution after washout of 100 μM D-AP5 induced a normal LTD.

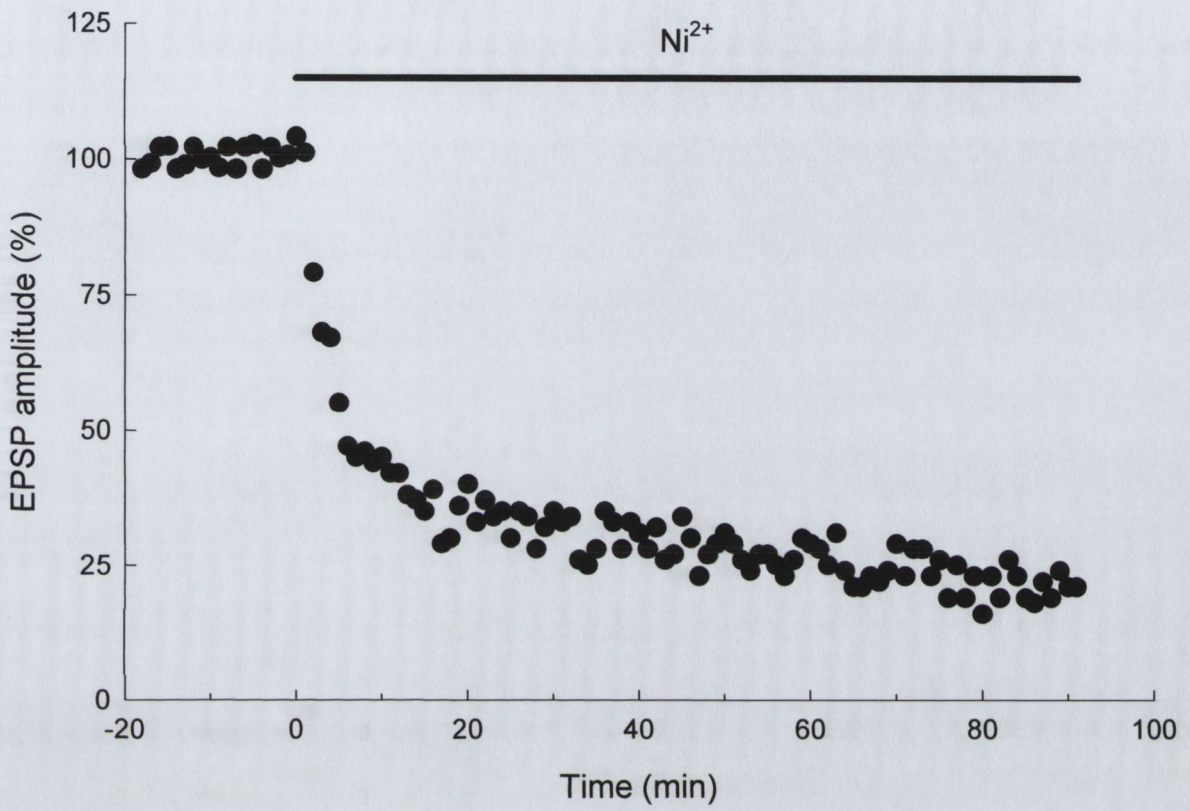


Figure 4.13 Effects of 50 μM Ni^{2+} on baseline responses.

Example of a single slice in which application of 50 μM Ni^{2+} induced a large depression of the EPSP amplitude.

Before the start of the experiments the slices were perfused with control solution containing also 25 μM Ni^{2+} for at least 40 min.

After recording EPSPs in the control solution in the presence of 25 μM Ni^{2+} , the slices were perfused with the medium containing 1.2 mM Ca^{2+} and 25 μM Ni^{2+} , during which the EPSP amplitude was reduced to $55\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=6$) as shown in Fig. 4.14. This value was similar to that reached in the presence of the solution containing 1.2 mM Ca^{2+} in all the previous experiments. Upon reperfusion with the control solution in the presence of 25 μM Ni^{2+} , no LTD was generated, since the EPSP amplitude returned almost to the value before the perfusion with the Ca^{2+} medium, measuring $96\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=6$) at 40 min after the start of the reperfusion with the solution containing 2 mM Ca^{2+} (see Fig. 4.14).

Subsequent application of LFS induced a further LTD measuring $19\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=6$) at 40 min after the end of LFS. This LTD was not significantly different from the further LTD obtained by application of LFS after the generation of the Ca^{2+} -induced LTD in the absence of Ni^{2+} (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). In previous experiments by Wang *et al.* (1997) a higher concentration of Ni^{2+} (50 μM) was required to completely inhibit the LFS-induced LTD.

These experiments showed that the influx of Ca^{2+} through low voltage sensitive Ca^{2+} channels contributes toward the induction of the Ca^{2+} -induced LTD.

4.2.8 Antagonising of metabotropic glutamate receptors (mGluRs) had no effect on the induction of the Ca^{2+} -induced LTD.

As it has been shown that Ca^{2+} influx through activation of NMDAR and low voltage sensitive Ca^{2+} channels was necessary for the induction of the Ca^{2+} -induced LTD, the next step was to assess whether activation of mGluRs was necessary for the generation of the Ca^{2+} -induced LTD. For this purpose experiments were carried out in the presence of (S)- α -methyl-4-

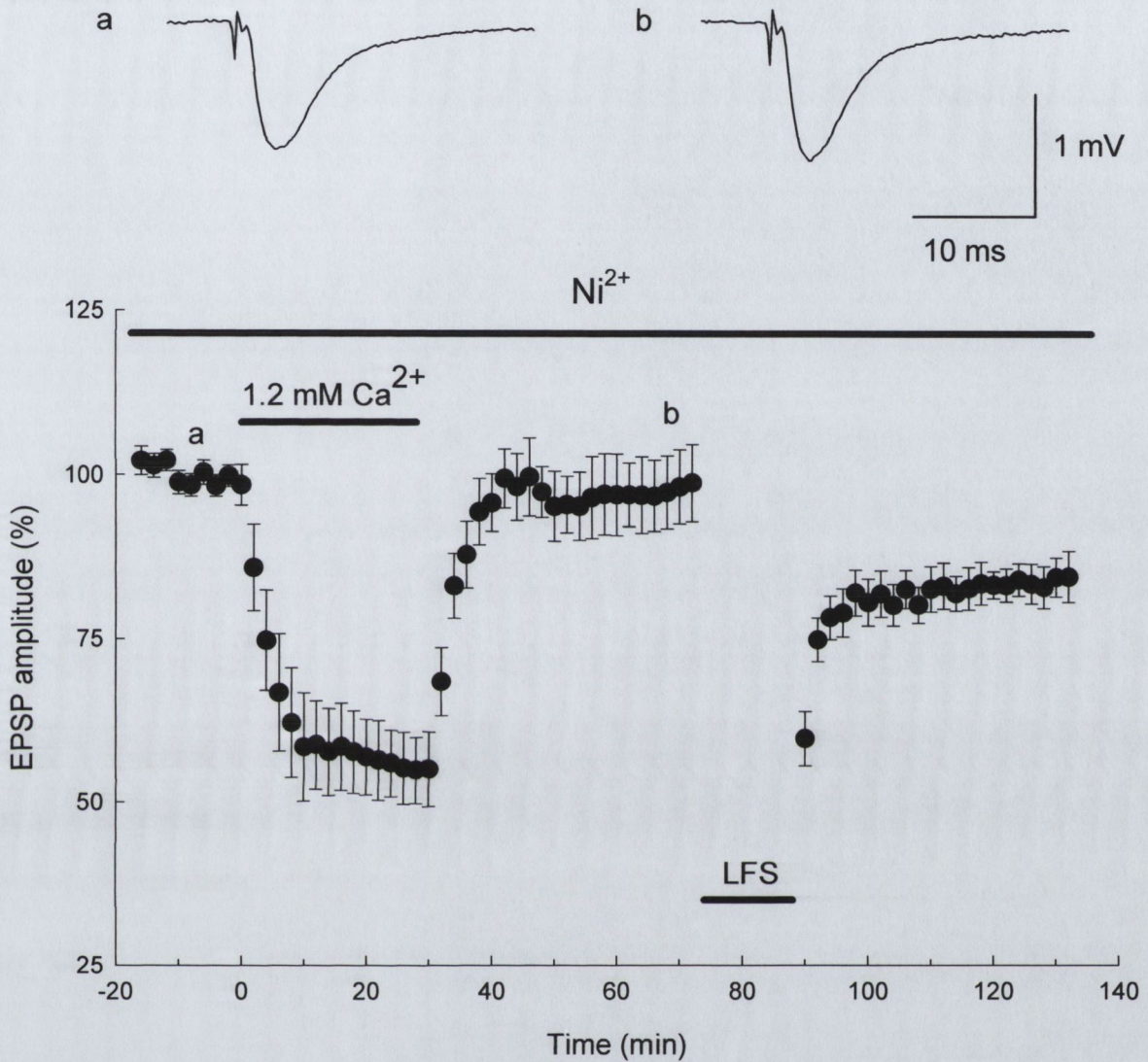


Figure 4.14 Effects of Ni^{2+} on the Ca^{2+} -induced LTD.

In the presence of $25 \mu\text{M Ni}^{2+}$, perfusion with the low Ca^{2+} media failed to induce any LTD, the EPSP measuring $96\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 6$) at 40 min after the start of the reperfusion with the solution containing 2 mM Ca^{2+} . Subsequent LFS induced a further LTD measuring $19\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 6$) at 40 min after the end of LFS. Examples of single EPSPs are shown in **a** (baseline in the presence of Ni^{2+}) and **b** (30 min after washout in the presence of Ni^{2+}).

carboxyphenylglycine (MCPG), a broad spectrum metabotropic glutamate receptor antagonist (Jane *et al.*, 1994, Vignes *et al.*, 1995, Ugolini and Bordi, 1995 and Watkins and Collingridge, 1994).

Perfusion of slices with 250 μM MCPG induced a small but significant depression of the EPSP amplitude measuring $9\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n=5$) at 30 min after the start of the perfusion with MCPG, as can be seen in Fig. 4.15.

After recording EPSPs in the presence of 250 μM MCPG, the slices were perfused with the low Ca^{2+} medium in the presence of 250 μM MCPG. This caused a depression of the EPSP amplitude of $70\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=5$) measured at 30 min from the start of the perfusion with the medium containing 1.2 mM Ca^{2+} (see Fig. 4.15). This value was significantly different from the amplitude measured in any other experiment in the presence of the low Ca^{2+} medium (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$). However, upon washout with the control solution in the presence of 250 μM MCPG a Ca^{2+} -induced LTD was generated measuring $25\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=5$) at 40 min from the start of the washout. This LTD was not significantly different from the Ca^{2+} -induced LTD generated in the absence of MCPG (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$).

Subsequent LFS in the presence of 250 μM MCPG failed to induce further significant depression, the LTD measuring only $10\% \pm 7\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=5$) at 30 min after the end of LFS.

These experiments demonstrated that the antagonistic action of MCPG on mGluRs had no effects on the generation of the Ca^{2+} -induced LTD.

4.2.9 The release of Ca^{2+} from intracellular Ca^{2+} stores was necessary for the generation of the Ca^{2+} -induced LTD.

In previous studies carried out by O'Mara *et al.* (1995a) and Wang *et al.* (1997) it was demonstrated that the release of Ca^{2+} from intracellular Ca^{2+} stores was

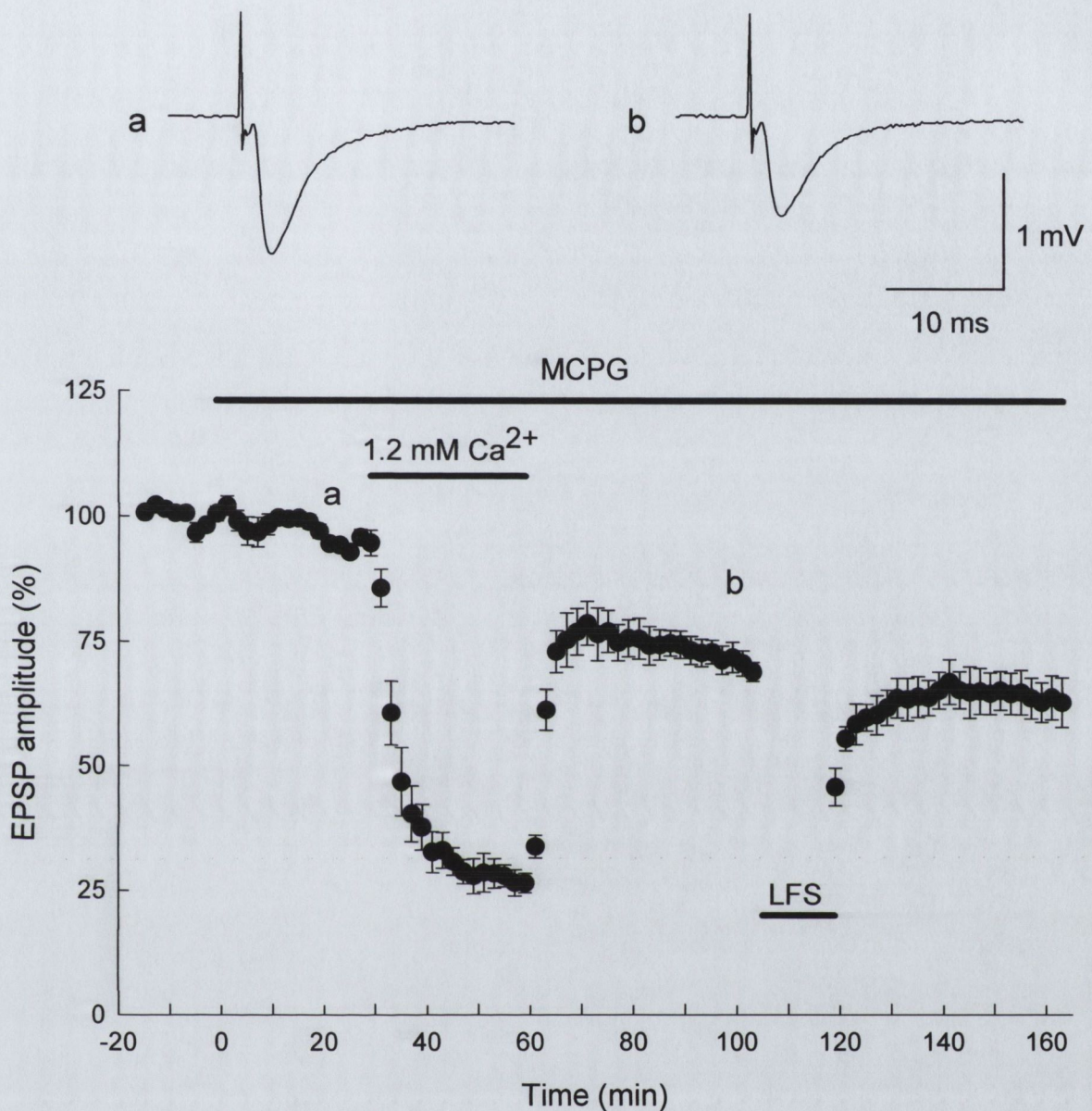


Figure 4.15 Effects of MCPG on the Ca²⁺-induced LTD.

In the presence of 250 μM MCPG, perfusion with the low Ca²⁺ media induced an LTD of $25\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 5$) at 40 min from the washout of the solution with 1.2 mM Ca²⁺. Further application of LFS failed to induce any significant LTD, the depression measuring only $10\% \pm 7\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 5$) at 30 min after the end of LFS. Examples of single EPSPs are shown in **a** (baseline in the presence of MCPG) and **b** (30 min after washout in the presence of MCPG).

necessary for the induction of LTD generated by application of LFS in the dentate gyrus.

In order to investigate the role of intracellular Ca^{2+} stores in the induction of the Ca^{2+} -induced LTD two sets of experiments were carried out.

The first set of experiments involved the use of thapsigargin, an agent known to deplete the intracellular Ca^{2+} stores by acting as an antagonist at the ATP-dependent Ca^{2+} uptake pump on the membrane of the intracellular Ca^{2+} stores (Thastrup *et al.*, 1990).

The second set of experiments involved the use of econazole, an imidazole antimycotic that has been used extensively as a blocker of the calcium-release-activated-calcium current (I_{CRAC}) responsible for the refilling of the intracellular Ca^{2+} stores (Villalobos *et al.*, 1992 and Vostal and Fratantoni, 1993).

4.2.9.1 Thapsigargin partially blocked the induction of the Ca^{2+} -induced LTD.

To determine whether the depletion of the intracellular Ca^{2+} stores by blocking the ATP-dependent Ca^{2+} pump was essential for the generation of the Ca^{2+} -induced LTD, experiments were carried out in the presence of 5 μM thapsigargin, a concentration known to deplete completely the intracellular Ca^{2+} stores in several preparations (Thastrup *et al.*, 1990).

Perfusion of the slices with 5 μM thapsigargin induced a slight but not significant depression of the EPSP amplitude measuring $5\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=6$) at 60 min after the start of the perfusion with thapsigargin (Fig. 4.16).

As shown in Fig. 4.16, perfusion of the slices with the low Ca^{2+} solution still in the presence of 5 μM thapsigargin caused a depression of the EPSP value to $41\% \pm 11\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n=6$), a value not significantly different from the depression reached in the presence of the medium containing 1.2 mM Ca^{2+} in the absence of thapsigargin (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Upon reperfusion with the solution containing 2 mM Ca^{2+}

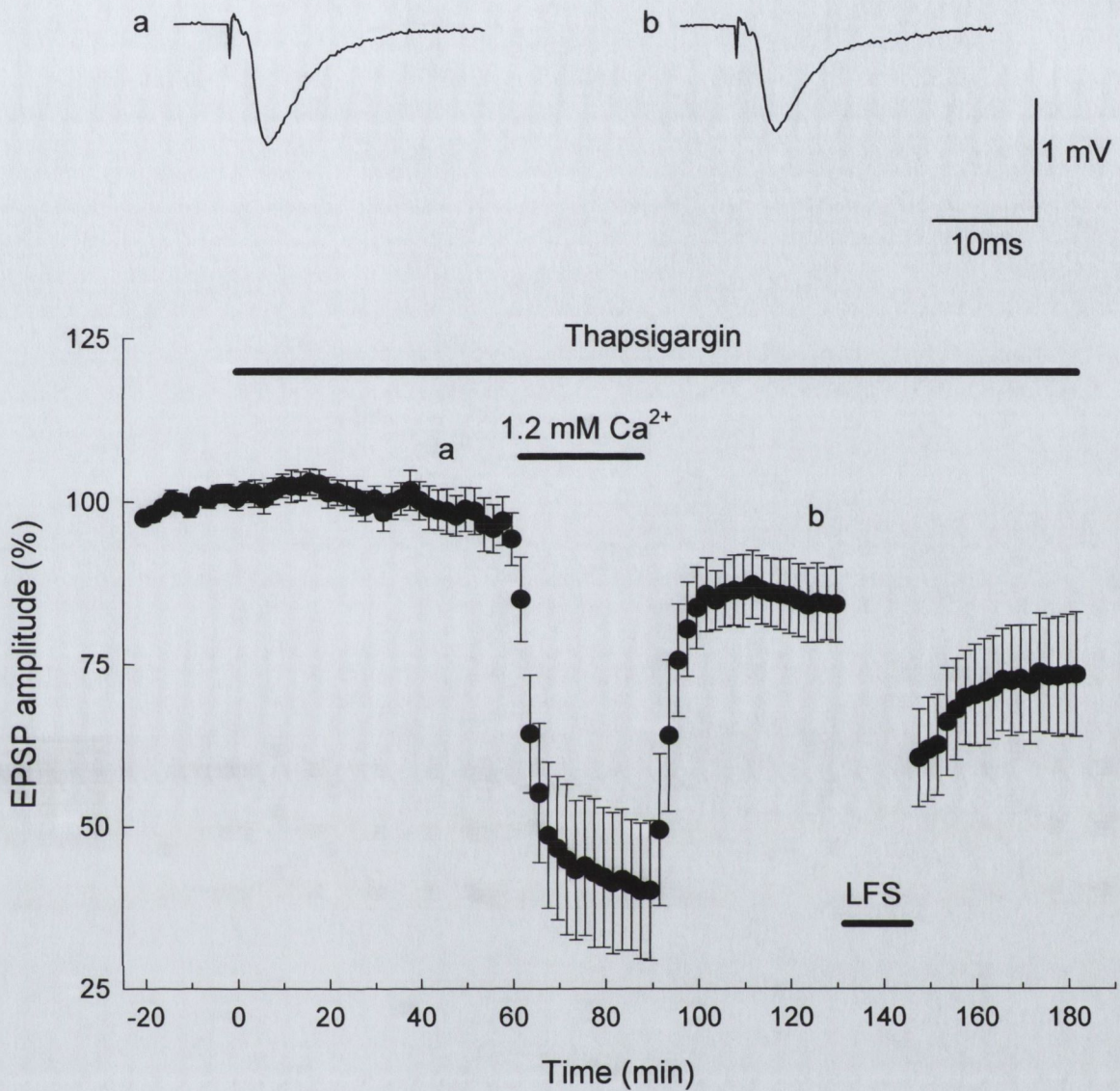


Figure 4.16 Effects of thapsigargin on the Ca^{2+} -induced LTD.

In the presence of $5\mu\text{M}$ thapsigargin, perfusion with the 1.2 mM Ca^{2+} medium induced an LTD of $12\%\pm 6\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 6$) at 30 min from the washout of the low Ca^{2+} solution. This value was significantly smaller than that obtained in the absence of thapsigargin (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.005$). Subsequent LFS failed to induce any further LTD, the depression measuring $5\%\pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 6$) at 40 min after the end of the LFS. Examples of single EPSPs are shown in **a** (baseline in thapsigargin) and **b** (30 min after washout in thapsigargin).

with 5 μM thapsigargin, a Ca^{2+} -induced LTD was generated measuring $12\% \pm 6\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=6$) at 40 min after the start of the reperfusion with the control solution in the presence of 5 μM thapsigargin. This Ca^{2+} -induced LTD in the presence of 5 μM was greatly reduced compared to the Ca^{2+} -induced LTD obtained in the absence of 5 μM thapsigargin (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.005$).

Subsequent administration of LFS failed to induce any further LTD, the depression measuring $5\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=6$) at 40 min after the end of the LFS.

These experiments showed that the depletion of the intracellular Ca^{2+} stores by blocking the ATP-dependent Ca^{2+} pump with thapsigargin affected the generation of the Ca^{2+} -induced LTD.

4.2.9.2 Econazole blocked the Ca^{2+} -induced LTD.

To determine whether the refilling of the intracellular Ca^{2+} stores through the I_{CRAC} channels was necessary for the generation of the Ca^{2+} -induced LTD, experiments were carried out in the presence of 20 μM econazole, a concentration known to block the I_{CRAC} (Vostal and Fratantoni, 1993).

Before the start of the experiments the slices were perfused with control solution containing also 20 μM econazole for at least 40 min.

As shown in Fig. 4.17, after recording EPSPs in the control solution in the presence of 20 μM econazole, the slices were perfused with the medium containing 1.2 mM Ca^{2+} and 20 μM econazole, during which the EPSP amplitude was reduced to $65\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=5$), a value similar to that reached in the presence of the solution containing 1.2 mM Ca^{2+} in all the previous experiments. Upon reperfusion with the control solution containing 20 μM econazole, no significant LTD was induced, the depression measuring only $6\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=5$) at 40 min after the start of the reperfusion with the solution containing 2 mM Ca^{2+} and 20 μM econazole. This

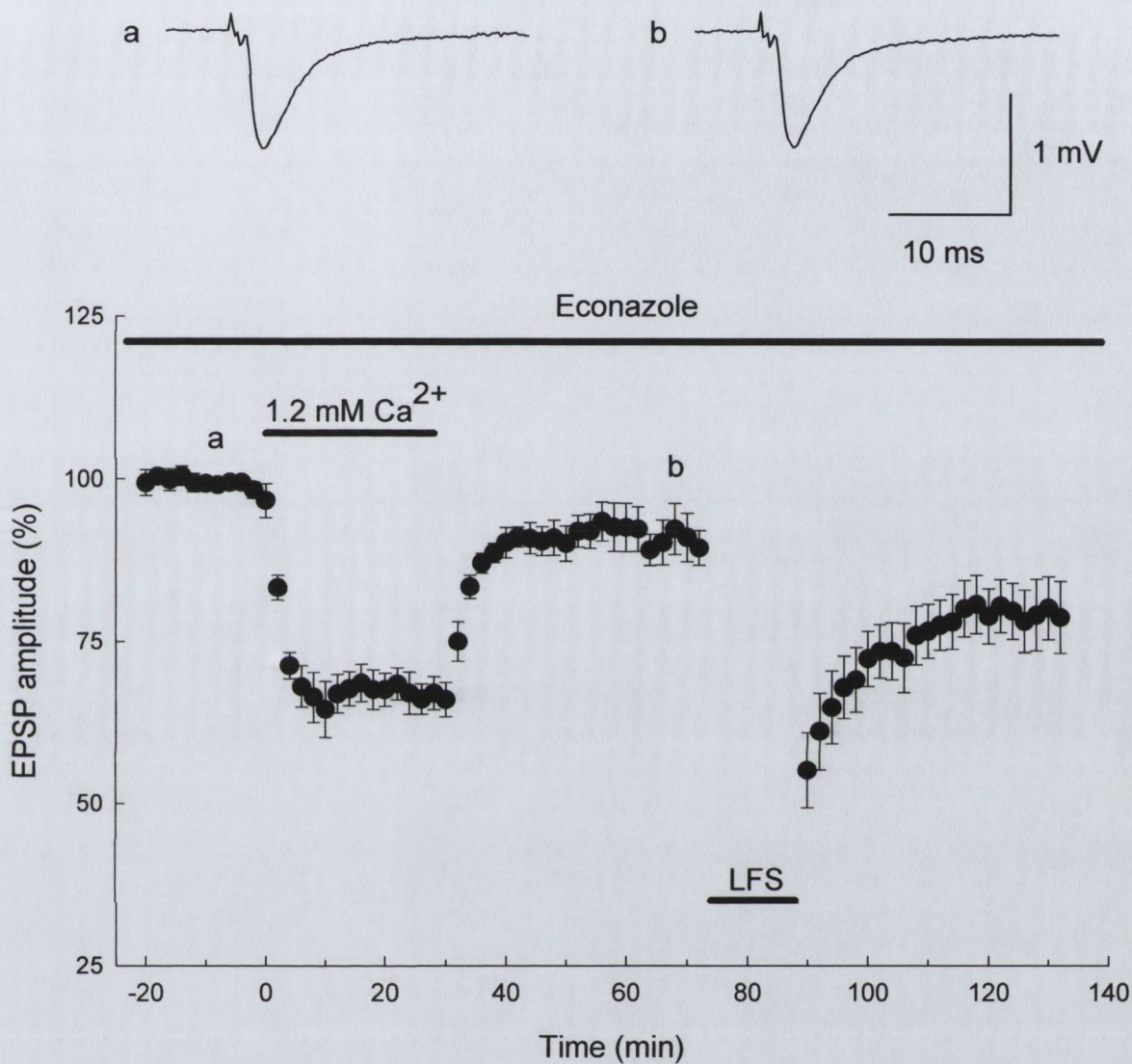


Figure 4.17 Effects of econazole on Ca^{2+} -induced LTD.

In the presence of $20 \mu\text{M}$ econazole, perfusion with the media containing 1.2 mM Ca^{2+} failed to induce any significant LTD, the depression measuring $6\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 5$) at 40 min after the start of the reperfusion. Subsequent LFS induced an LTD that measured only $10\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 5$) at 40 min post-LFS which was significantly reduced compared to the LTD in Fig 4.1 (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$). Examples of single EPSPs are shown in **a** (baseline in econazole) and **b** (30 min after washout in the presence of econazole).

Ca²⁺-induced LTD was significantly reduced when compared with the Ca²⁺-induced LTD generated in the absence of 20 μM econazole (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.005$).

Subsequent application of LFS induced a further LTD measuring 10%±5% (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 5$) at 40 min after the end of LFS, which was also significantly reduced compared to the further LTD obtained by application of LFS after the generation of the Ca²⁺-induced LTD in the absence of 20 μM econazole (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$).

These experiments showed that the refilling of the intracellular Ca²⁺ stores through the CRAC channels is essential for the induction of the Ca²⁺-induced LTD.

4.3 Discussion

It had been shown previously that LTD can be induced by increasing the frequency of stimulation from the test frequency (usually between 0.1 Hz and 0.05 Hz) to 1-10 Hz for a period of several minutes (the duration varying with the frequency chosen for the induction protocol). This type of stimulation was termed LFS and the most commonly used protocol for the induction of LTD was 900 pulses at 1 Hz (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Bear and Abraham, 1996 and Linden, 1994).

With the present study it has been demonstrated that in the medial perforant pathway of the dentate gyrus LTD can be induced also at the normal stimulation frequency of 0.033 Hz by first decreasing the Ca²⁺ concentration in the extracellular medium for a period of 30 min, and then increasing it again to the control value, without the administration of LFS. This LTD has been termed "Ca²⁺-induced LTD".

One important feature of the Ca²⁺-induced LTD was that it was completely occluded by the LFS-induced LTD. This indicated that these two differently induced LTDs share some common induction/maintenance mechanisms. However the fact that the LFS-induced LTD was not completely occluded by the Ca²⁺-

induced LTD (since application of LFS after the induction of the Ca^{2+} -induced LTD gave rise to a further LTD) suggested that more pathways may be activated by the LFS protocol which could be responsible for the induction of the additional LTD.

Both the Ca^{2+} -induced LTD and the LFS-induced LTD were not accompanied by a change in the paired-pulse depression, which indicated both types of LTD are not accompanied by a change in the probability of transmitter release. In this way, even though a presynaptic involvement in the generation of LTD could not be completely excluded, these experiments provided strong evidence that both the Ca^{2+} -induced LTD and the LFS-induced LTD were induced and/or expressed post-synaptically, in agreement with what was found in cultured pyramidal cells by Goda and Stevens (1996)

The Ca^{2+} -induced LTD was generated by reperfusion of the control medium containing 2 mM Ca^{2+} following a period in which the slices were in the presence of the solution containing only 1.2 mM Ca^{2+} . If the lowering of the external Ca^{2+} concentration by itself was necessary for the induction of the LTD, administration of LFS in the presence of the medium containing 1.2 mM Ca^{2+} should have given rise only to a residual LTD of amplitude similar to that obtained in the occlusion experiments after the generation of the Ca^{2+} -induced LTD. However, in the presence of the medium containing 1.2 mM Ca^{2+} LFS was able to induce LTD similar to that obtained in the control solution. Similar results were obtained previously by Wang *et al.* (1997). Therefore the subsequent increase in the external Ca^{2+} concentration was crucial for the induction of the Ca^{2+} -induced LTD.

It has been demonstrated that LFS-induced LTD is induced by a post-synaptic rise of the intracellular Ca^{2+} concentration (Mulkey and Malenka, 1992, Wang *et al.*, 1997 and Neveu and Zucker, 1996b).

Mulkey and Malenka (1992) showed that chelation of intracellular Ca^{2+} prevented the induction of LTD in the CA1 area, while Wang *et al.* (1997) proved similar

results in the dentate gyrus. Furthermore Neveu and Zucker (1996a) gave evidence that post-synaptic photolysis of a caged- Ca^{2+} compound directly induced LTD, demonstrating that pre-synaptic activity was not required for the induction of this LTD.

However the influx of Ca^{2+} that is required for the induction of LTD was found to be relatively smaller than that required for the induction of LTP (Otani and Connor, 1996, Neveu and Zucker, 1996b). According to the studies by (Otani and Connor, 1996), the intracellular Ca^{2+} has to increase by a moderate amount to induce LTD (approximately 500 nM), while for the induction of LTP the intracellular Ca^{2+} had to increase by a higher amount ($>1 \mu\text{M}$). Similar values (between 300 and 1000 nM) for the intracellular Ca^{2+} concentration to be reached for the induction of LTD were found by Neveu and Zucker (1996b).

The present studies and those by Wang *et al.* (1997) also showed that a reduction in the extracellular Ca^{2+} concentration had very little effect on the induction of LFS-induced LTD, unlike the effect that a similar reduction in the extracellular Ca^{2+} concentration had on the induction of LTP (Mulkeen *et al.*, 1988).

These results therefore reinforced the previous findings that a smaller influx of Ca^{2+} was required for the induction of LTD than for the induction of LTP.

However, simply increasing the extracellular Ca^{2+} concentration was not sufficient to induce LTD, without the previous perfusion in low Ca^{2+} medium. A possible explanation for this could be that the Ca^{2+} influx responsible for the induction of the Ca^{2+} -induced LTD is effective because of the changes that had been caused in the intracellular Ca^{2+} homeostasis by the perfusion with the low Ca^{2+} medium for the previous 30 min.

In the present studies it was found that synaptic stimulation was necessary for the induction of the Ca^{2+} -induced LTD. However when the stimulation was recommenced after being stopped for 45 min, the EPSP amplitude was greater for a brief interval. It is possible that this STP was caused by accumulation of Ca^{2+} in the vicinity of the synapses previously stimulated, since the same phenomenon

happened when the stimulation was interrupted for the same length of time in slices that were always perfused only with the medium containing 2 mM Ca^{2+} .

It is clear from these experiments that an influx of Ca^{2+} was necessary for the generation of the Ca^{2+} -induced LTD. With the present experiments it was shown that the influx of Ca^{2+} responsible for the Ca^{2+} -induced LTD was most likely to occur via both the activation of NMDAR and T or R-type voltage-gated Ca^{2+} channels.

In the presence of D-AP5, a potent inhibitor of NMDAR (Watkins and Olverman, 1987) known to block a major proportion of these receptors at the concentration used (50 μM), the Ca^{2+} -induced LTD was partially blocked. To support the fact that the Ca^{2+} -induced LTD is partially dependent upon activation of NMDAR, Thiels *et al.* (1996) proved that LTD in the dentate gyrus both *in vivo* and *in vitro* was partially dependent upon activation of NMDAR. In addition to this, however, a substantial component of LFS-induced LTD is NMDAR independent as shown by O'Mara *et al.* (1995b), Wang *et al.* (1996) and Wang *et al.* (1997). Similarly, LFS-induced LTD was initially found to be completely dependent upon activation of NMDAR in the area CA1 (Dudek and Bear, 1992 and Mulkey and Malenka, 1992). However, more recently it has also been shown that in the area CA1 of the hippocampus two forms of LTD are present, one NMDAR-dependent and the other mGluR-dependent (Oliet *et al.*, 1997).

When the slices were perfused with 50 μM D-AP5, a small but significant depression was detected, possibly the expression of the blockage of the NMDARs.

Another route for the influx of Ca^{2+} necessary for the induction of the Ca^{2+} -induced LTD was through the LVA Ca^{2+} channels, since in the presence of Ni^{2+} the Ca^{2+} -induced LTD was inhibited. It has been previously shown that Ni^{2+} at concentrations similar to those used in the present studies (25-50 μM), preferentially inhibited T-type Ca^{2+} channels in granule cells of the dentate gyrus (Blaxter *et al.*, 1989) and of the area CA1 in the hippocampus (Avery and

Johnston, 1996). The same concentrations of Ni^{2+} have also been shown to inhibit R-type Ca^{2+} channels (Christie *et al.*, 1997).

Ni^{2+} was first shown to strongly inhibit LFS-induced LTD in the dentate gyrus by Wang *et al.* (1996) at a concentration of 50 μM . However in the experiments presented here, the perfusion of the slices with 50 μM Ni^{2+} gave rise to a large depression, possibly due to a less selective blocking action of Ni^{2+} at such concentration. Therefore for the Ca^{2+} -induced LTD experiments a lower Ni^{2+} concentration (25 μM) was used in order to maximise the selectivity of this blocker towards the R/T-type Ca^{2+} channels, as supported also by Avery and Johnston (1996).

Antagonising of mGluRs did not affect the induction of the Ca^{2+} -induced LTD, since the mGluR antagonist MCPG was not able to block it. However, a relatively low concentration of MCPG was used for these experiments (250 μM), because of the depression it caused. MCPG has been used widely as a broad spectrum mGluR antagonist at higher concentrations than the one used in the present experiments (Jane *et al.*, 1994, Vignes *et al.*, 1995, Ugolini and Bordi, 1995 and Watkins and Collingridge, 1994). Therefore the lack of effect of MCPG on the Ca^{2+} -induced LTD did not completely rule out the involvement of mGluRs in the induction of this type of LTD, because of the low concentration of MCPG used. In addition to this it has been demonstrated that mGluRs can function as Ca^{2+} sensing receptors (Kubo *et al.*, 1998). Kubo *et al.* (1998) have shown that in particular mGluR group I, which are widely expressed in the dentate gyrus (Shigemoto *et al.*, 1997), could be easily activated by increasing the extracellular Ca^{2+} concentration. Activation of mGluR group I is linked to increase in the intracellular Ca^{2+} concentration, supporting the possibility that mGluRs may play a role in the generation of the Ca^{2+} -induced LTD. In support of this hypothesis, Huber *et al.* (1998) have demonstrated that in the visual cortex MCPG failed to antagonise the action of mGluR group I.

In the presence of MCPG the application of LFS to the slices after the generation of the Ca^{2+} -induced LTD failed to induce any additional LTD. This could be due to the antagonistic action of MCPG, since it has been previously demonstrated that MCPG was able to block LFS-induced LTD (O'Mara *et al.*, 1995b). Another explanation for these results, however, could be based on the role of MCPG as an agonist (Breakwell *et al.*, 1998 and Huang *et al.*, 1999). It has been recently demonstrated that MCPG was able to induce LTD in the dentate gyrus which mutually occluded LFS-induced LTD (Huang *et al.*, 1999). Therefore the lack of further LTD following LFS in the presence of MCPG could be due to occlusion of the LFS-induced LTD by the MCPG-generated LTD.

A simple mechanism for the generation of the Ca^{2+} -induced LTD could be that the influx of Ca^{2+} directly activates intracellular messengers leading to the induction of LTD. However, it was shown in the current studies that in the presence of thapsigargin, the inhibitor of the ATP-dependent Ca^{2+} pump responsible for the refilling of the stores, which depletes the Ca^{2+} stores, the Ca^{2+} -induced LTD was partially inhibited. This suggested that the release of Ca^{2+} from the intracellular stores was an essential step for the generation of the Ca^{2+} -induced LTD. The involvement of intracellular Ca^{2+} stores in the induction of LTD had already been demonstrated in the dentate gyrus (Wang *et al.*, 1997), in the CA1 area (Stanton and Gage, 1996) and in the cerebellum (Kohda *et al.*, 1995).

It is possible therefore to conclude that the increase in the intracellular Ca^{2+} concentration did not happen only because of the direct influx of Ca^{2+} through the NMDAR channels and through the LVA Ca^{2+} channels, but Ca^{2+} needed to be released from the stores to allow the generation of the Ca^{2+} -induced LTD.

Additionally, in the presence of econazole, the inhibitor of the CRAC channel (Villalobos *et al.*, 1992 and Vostal and Fratantoni, 1993), the Ca^{2+} -induced LTD was blocked, supporting the hypothesis that intracellular Ca^{2+} stores played a role in the increase of the intracellular Ca^{2+} concentration for the generation of the Ca^{2+} -induced LTD.

It had previously been shown that econazole prevented the induction of LTP in CA1 (Forghani and Krnjevic, 1995), giving evidence for a role of intracellular Ca^{2+} stores in the induction of LTP. Previous studies in the dentate gyrus by O'Mara *et al.* (1995a) demonstrated that intracellular Ca^{2+} stores play a role in the induction of the LFS-induced LTD, since in the presence of dantrolene, an agent known to block Ca^{2+} release via the ryanodine receptor, LFS-induced LTD was inhibited. Similarly Wang *et al.* (1997) showed that application of 20 μM ryanodine, a concentration at which this compound locks the channel in a subconductance state (Meissner, 1986), was able to block the LFS-induced LTD, a further proof of the involvement of intracellular Ca^{2+} stores in the induction of LTD.

From all this evidence it can be postulated that when the extracellular Ca^{2+} concentration was lowered the intracellular Ca^{2+} stores were depleted to maintain cytosolic Ca^{2+} homeostasis. Therefore when the Ca^{2+} concentration was returned to control level, the intracellular Ca^{2+} concentration was increased thanks to the influx of Ca^{2+} through the NMDAR channels and the LVA Ca^{2+} channels. At first, this increase in the Ca^{2+} concentration promoted the refilling of the stores via stores-regulated Ca^{2+} channels. Then the continuing influx of Ca^{2+} through the various channels increased the concentration to such a level that it triggered Ca^{2+} release from the stores via the Ca^{2+} -induced Ca^{2+} release (CICR). At this point the Ca^{2+} released from the stores by CICR could initiate the intracellular mechanisms leading to LTD.

Another possible mechanism for the induction of this LTD could involve the activation of G-protein coupled receptors which could start the enzymatic cascade leading to the induction of LTD. A possible candidate for this role among the G-protein coupled receptors could be mGluR group I. The activation of these receptors is linked to activation of phospholipase C (PLC) which leads both to increase of the intracellular Ca^{2+} concentration by production of inositol triphosphate (IP_3) and also to the activation of protein kinase C (PKC) which has been shown to be involved in the induction of LTD in the dentate gyrus (Wang *et al.*, 1998) and in CA1 (Oliet *et al.*, 1997).

In conclusion these studies provided an alternative protocol for the induction of LTD in the medial perforant pathway of the dentate gyrus giving further evidence for the role played by Ca^{2+} in the induction of this form of synaptic plasticity.

5. The role of metabotropic glutamate receptors (mGluRs) group I in the induction of long-term depression (LTD) in the medial perforant pathway of the dentate gyrus *in vitro*.

5.1 Introduction

Initial studies on the involvement of mGluR in the induction of LTD in the hippocampus led to controversy. While certain studies showed that the induction of LTD was mGluR-dependent, with a block of the induction of LTD by (S)- α -methyl-4-carboxyphenylglycine (MCPG) in both CA1 (Bolshakov and Siegelbaum, 1994) and in the dentate gyrus (O'Mara *et al.*, 1995b), other studies did not find a block of LTD induction by MCPG in CA1 (Selig *et al.*, 1995). One possible resolution of the controversy was the finding of an mGluR-dependent and an mGluR-independent form of LTD in both CA1 (Oliet *et al.*, 1997) and dentate gyrus (O'Mara *et al.*, 1995b and Wang *et al.*, 1997) which also required different induction paradigms. In the dentate gyrus, mGluR-dependent LTD can be induced in "normal" medium (O'Mara *et al.*, 1995b), but in CA1 a high $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration (4 mM/4 mM) was required for the induction of the mGluR-dependent LTD (Oliet *et al.*, 1997). Further evidence for the involvement of mGluRs in LTD generation was the induction of LTD by the mGluR group I and group II agonist (1S, 3R)-1-Aminocyclopentane-1,3-dicarboxylic acid (ACPD). This compound was able to induce LTD both in CA1 (Bolshakov and Siegelbaum, 1994 and 1995) and in the dentate gyrus (O'Mara *et al.*, 1995b). However in the CA1 area, the ACPD application had to be accompanied by a train of post-synaptic depolarising pulses (Bolshakov and Siegelbaum, 1994), or by release of Ca^{2+} from a caged compound (Bolshakov and Siegelbaum, 1995).

The studies investigating the effects of MCPG and ACPD did not reveal which group of mGluRs was involved in LTD induction, as both these compounds are non-selective mGluR ligands. Some evidence has been found for the involvement of group I mGluRs in the induction of LTD in CA1 (Palmer *et al.*, 1997). The selective mGluR group I antagonist (CRS)-1-aminoindan-1,5-dicarboxylic acid (AIDA) prevented the induction of the mGluR-mediated component of LTD (Oliet *et al.*, 1997). Moreover, the mGluR group I agonist (RS)-3,5-Dihydroxyphenylglycine ((R,S)-DHPG) induced LTD, particularly under hyperexcitable conditions such as low Mg^{2+} (which removes the block of NMDAR channels by Mg^{2+}) or in the presence of picrotoxin (to block the GABA_A-mediated responses) (Palmer *et al.*, 1997), although such LTD did not occlude with LFS-induced LTD.

5.2 Results.

5.2.1 The mGluR group I agonist DHPG induced LTD in the medial perforant pathway of the dentate gyrus.

3,5-Dihydroxyphenylglycine (DHPG) is a selective agonist of mGluR group I (Ito *et al.*, 1992). Two different enantiomers of this compound have been synthesised and they have different potency (Baker *et al.*, 1995). The (S) enantiomer has an EC₅₀ of 11 μ M whereas the (R) enantiomer has an EC₅₀ of 106 μ M (Baker *et al.*, 1995) when tested on hippocampal slices .

The first set of experiments was carried out using the racemic mixture of DHPG ((R,S)-DHPG) at a concentration of 30 μ M. As can be seen in Fig.5.1, the application of 30 μ M (R,S)-DHPG induced a transient depression measuring 20% \pm 5% (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n = 4$) after 20 min in the presence of the compound. Upon washout of the agonist, an LTD was generated that

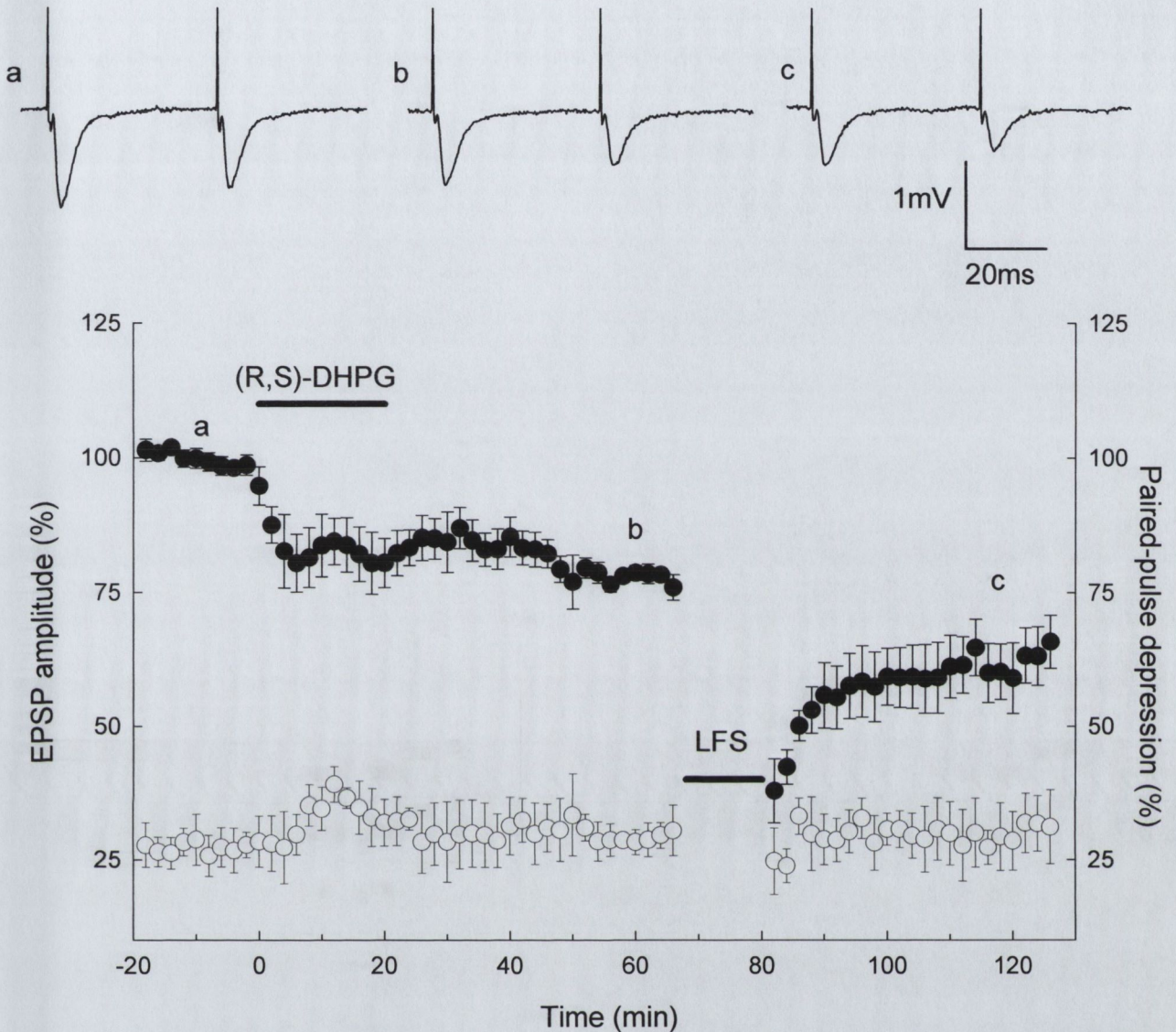


Figure 5.1 LTD induced by 30 μM (R,S)-DHPG.

Application of 30 μM (R,S)-DHPG for 20 min induced an LTD of $23\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 4$) at 40 min post-washout. Subsequent LFS induced a further LTD of $10\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n = 4$) at 40 min post-LFS. Neither LTD was accompanied by a change in paired-pulse depression. Filled symbols indicate EPSP amplitude and clear symbols indicate paired-pulse depression. Sample pairs of EPSPs are shown in **a** (baseline), **b** (30 min after washout) and **c** (40 min post-LFS).

measured $23\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=4$) at 40 min from the start of the washout of (R,S)-DHPG.

In order to determine the optimal concentration of (R,S)-DHPG to obtain the maximal LTD, several experiments were performed using concentrations of (R,S)-DHPG ranging from 3 μM to 100 μM .

Fig. 5.2 shows the depression of the EPSP amplitude for each concentration of (R,S)-DHPG. Each bar of the histogram represents an average of data between 30 and 40 min after the start of washout of (R,S)-DHPG. One slice was used for each concentration of (R,S)-DHPG tested.

Table 5.1 shows the amount of depression induced by various concentrations of (R,S)-DHPG measured at $t=40$ min after washout of (R,S)-DHPG.

Table 5.1 LTD induced by various concentrations of (R,S)-DHPG.

(R,S)-DHPG concentration	LTD
3 μM	13%
10 μM	20%
30 μM	23%
50 μM	7%
100 μM	13%

From the results shown in Fig.5.1, Fig.5.2 and Table 5.1 it can be seen that the optimal concentration for the induction of the maximal LTD using the racemic mixture (R,S)-DHPG was 30 μM .

Another set of experiments was carried out using only the more potent enantiomer (S)-DHPG. When the slices were perfused with 20 μM (S)-DHPG for 20 min a small transient depression could be detected measuring $10\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=6$) (Fig. 5.3). Subsequent reperfusion with the control solution resulted in an LTD measuring $26\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n=6$) at 40 min from the start of the washout of the agonist.

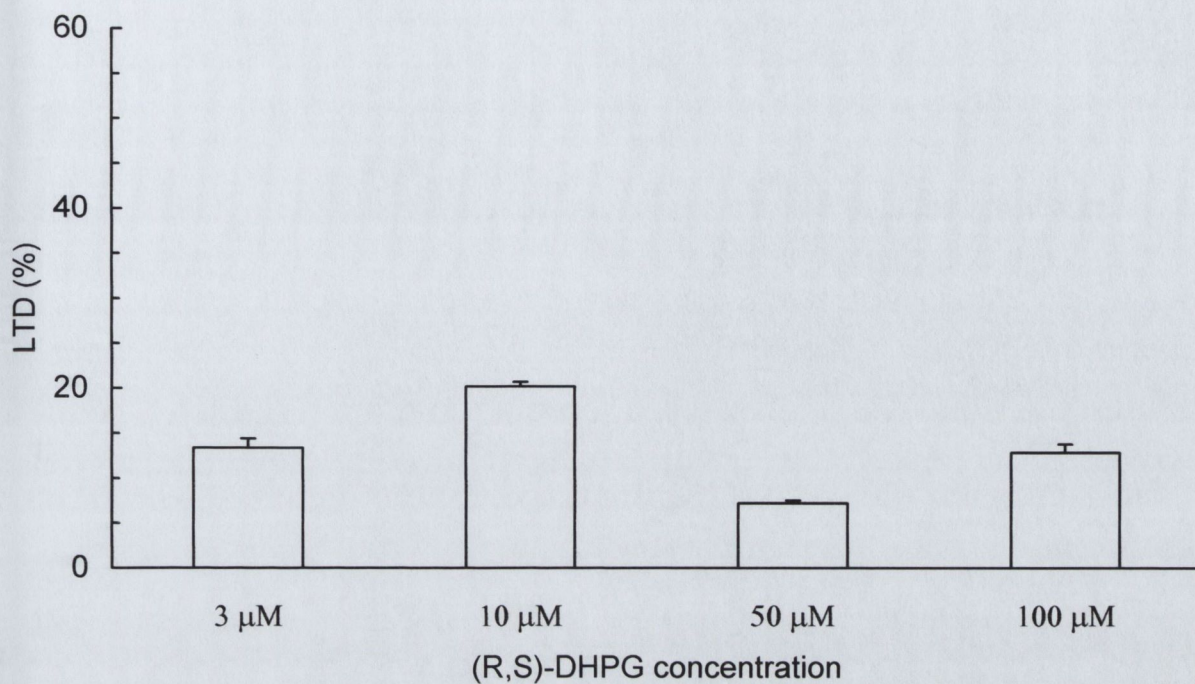


Figure 5.2 LTD generated by different concentrations of (R,S)-DHPG.

Several concentrations of (R,S)-DHPG were tested on single slices. For each concentration tested one slice was used. Each bar in the histogram represents an average of data (mean \pm SE) between 30 and 40 min after the start of washout of (R,S)-DHPG.

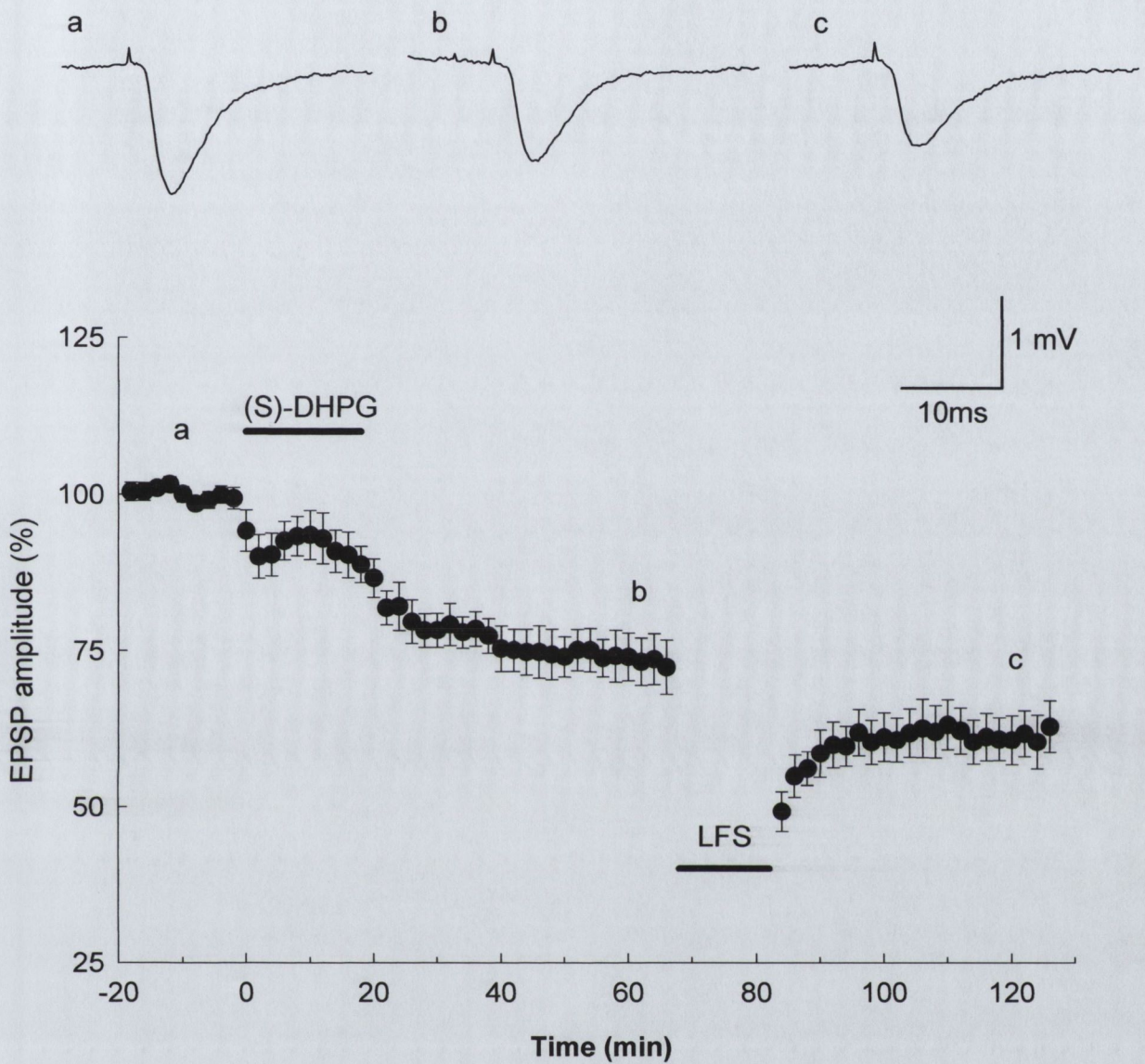


Figure 5.3 LTD induced by 20 μ M (S)-DHPG.

Application of 20 μ M (S)-DHPG induced an LTD of $26\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 6$) at 40 min after washout. Further LFS induced an LTD of $10\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 6$) at 40 min post-LFS. Examples of single EPSPs are shown in **a** (baseline), **b** (40 min after washout) and **c** (40 min post-LFS).

As for the studies with the racemic mixture (RS)-DHPG, experiments were carried out using different concentrations of (S)-DHPG between 10 μ M and 100 μ M in order to establish the optimal concentration of this agonist to allow the induction of the maximal LTD.

As can be seen in Fig. 5.4, the perfusion of the slices with 10 μ M (S)-DHPG caused a small, non significant depression measuring 10% \pm 4% (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 4$). When the (S)-DHPG was washed out a slow onset LTD was induced measuring 19% \pm 6% (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n = 4$) at 40 min after the start of reperfusion with the control solution.

Fig.5.5 shows the depression of the EPSP amplitude for concentrations of (S)-DHPG of 15 μ M, 50 μ M and 100 μ M. Each bar of the histogram represents an average of data between 30 and 40 min after the start of washout of (S)-DHPG. One slice was used for each concentration of (S)-DHPG tested.

Table 5.2 shows the amount of depression induced by various concentrations of (S)-DHPG measured at $t = 40$ min after washout of (S)-DHPG.

Table 5.2 LTD induced by various concentrations of (S)-DHPG.

(S)-DHPG concentration	LTD
10 μ M	19%
15 μ M	23%
20 μ M	26%
50 μ M	12%
100 μ M	20%

From the results presented in Fig. 5.3, Fig. 5.4, Fig 5.5 and Table 5.2 it can be seen that 20 μ M was the optimal concentration of (S)-DHPG to induce the maximal LTD.

An additional experiment was carried out to determine whether a second application of (S)-DHPG could induce more LTD. As shown in Fig. 5.6, after the first application of (S)-DHPG an LTD of approximately 30% was induced. The

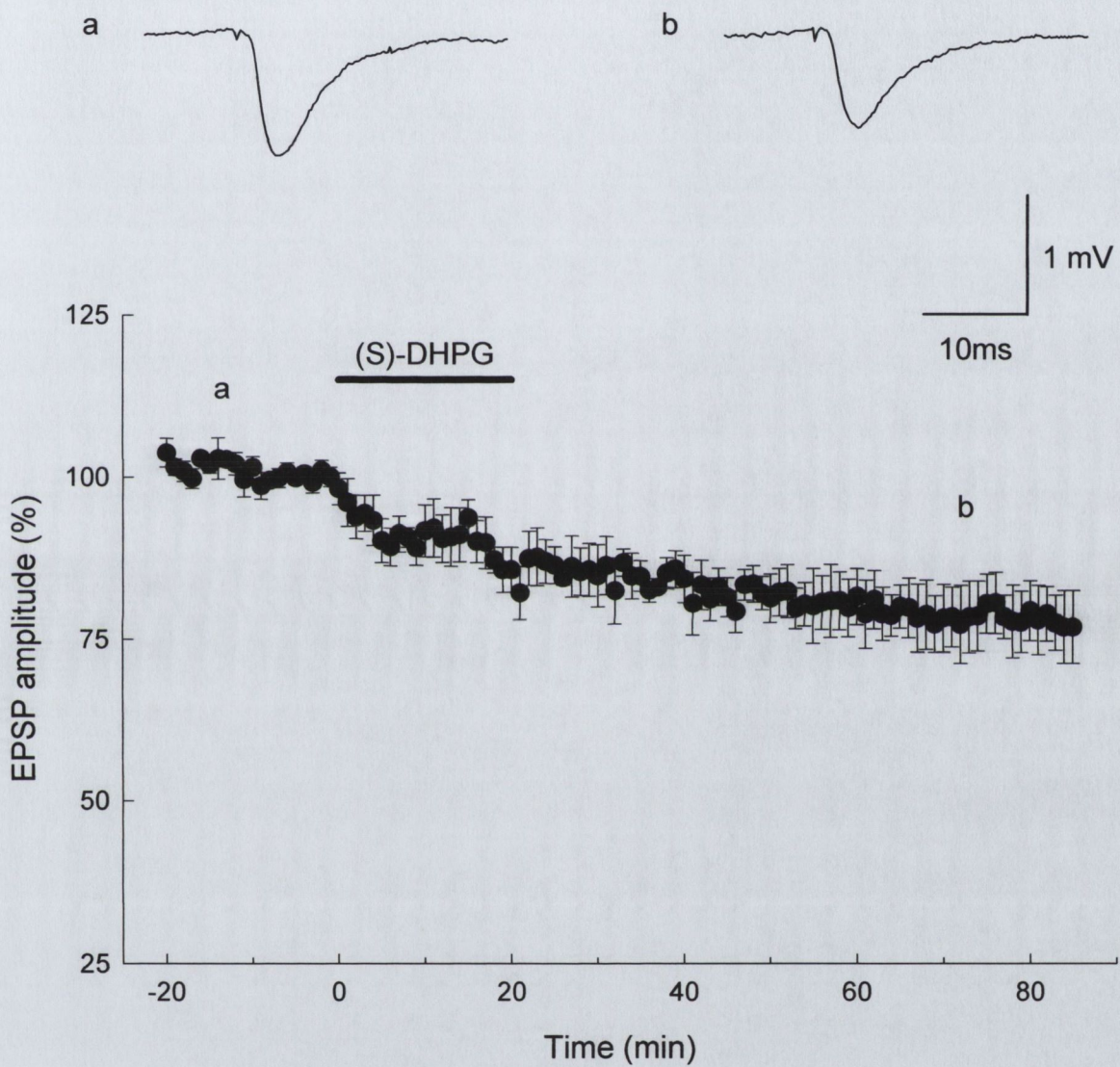


Figure 5.4 LTD induced by 10 μ M (S)-DHPG.

Perfusion with 10 μ M (S)-DHPG for 20 min generated an LTD of $19\% \pm 6\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n = 4$) at 40 min after washout. Examples of single EPSPs are shown in **a** (baseline) and **b** (40 min after washout).

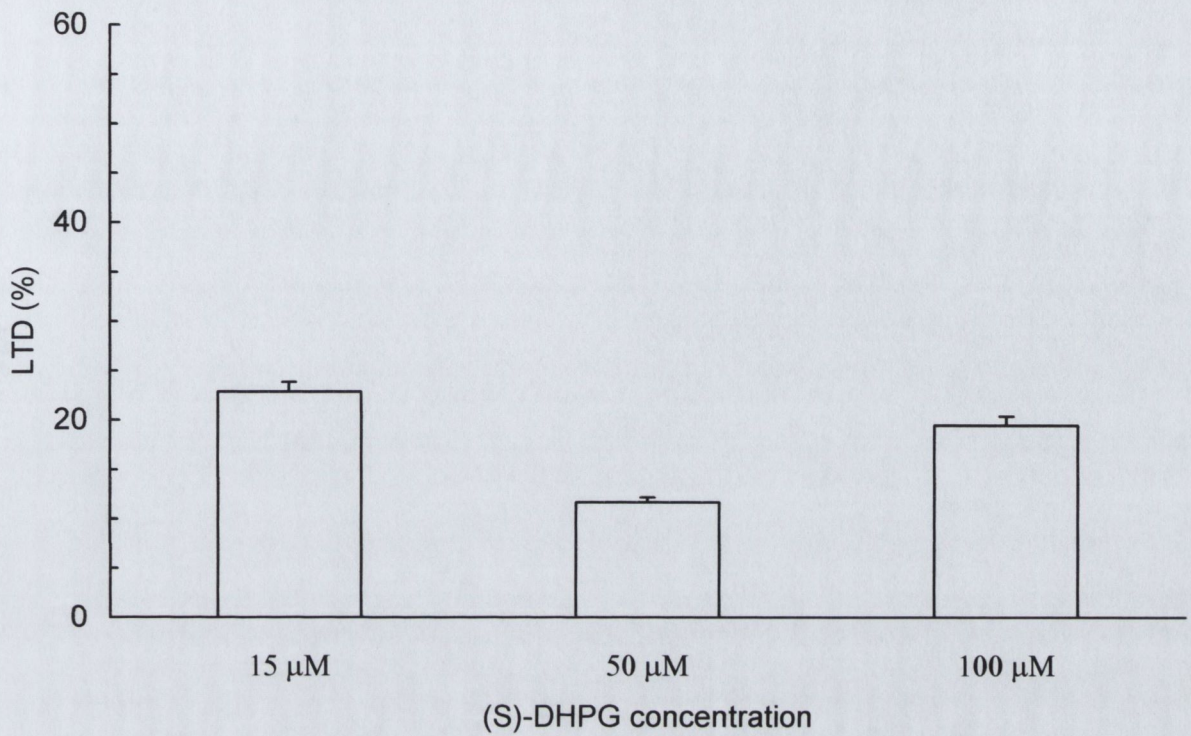


Figure 5.5 LTD generated by different concentrations of (S)-DHPG.

Several concentrations of (S)-DHPG were tested on single slices. For each concentration tested one slice was used. Each bar in the histogram represents an average of data (mean \pm SE) between 30 and 40 min after the start of washout of (S)-DHPG.

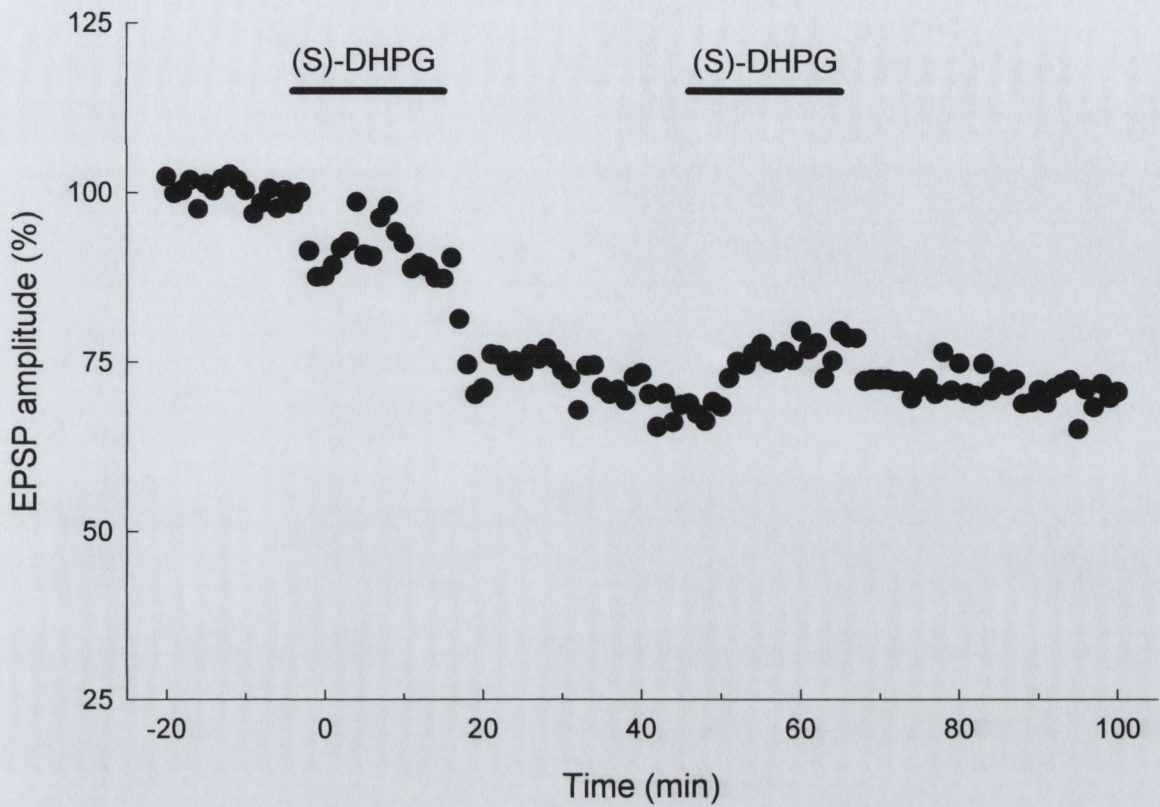


Figure 5.6 Effects of repeated application of 20 μ M (S)-DHPG.

Application of 20 μ M (S)-DHPG for 20 min generated an LTD of approximately 30% at 30 min after washout. Repeating the application of 20 μ M (S)-DHPG failed to induce any further LTD. One slice was used for this experiment.

second application of (S)-DHPG failed to induce any further LTD, since the EPSP was depressed by roughly 30% of its original value after 30 min from the start of the second washout.

The LTD generated after perfusion with 30 μ M (R,S)-DHPG and the LTD obtained after perfusion with 20 μ M (S)-DHPG were not statistically different (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$), consequently all the subsequent experiments were carried out using the (S) enantiomer at a concentration of 20 μ M and the LTD generated will be referred to as DHPG-induced LTD.

These experiments showed that the activation of mGluR group I by perfusion with the agonist DHPG induced LTD in the medial perforant pathway of the dentate gyrus.

5.2.2 Common intracellular pathways were shared by the DHPG-induced LTD and LFS-induced LTD.

In order to determine whether the DHPG-induced LTD and the LFS induced LTD shared common intracellular pathways, occlusion experiments were carried out.

In a first set of experiments, shown in Fig. 5.3, the DHPG-induced LTD was found to significantly occlude the induction of further LTD by LFS. Following the DHPG-induced LTD measuring $26\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n=6$), LFS could only induce a further LTD measuring $10\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=6$) at 40 min after the end of LFS. This LTD was significantly reduced (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$) from the LFS-induced LTD in control solution (See Fig. 3.7).

Similar experiments were carried out using the racemic mixture (RS)-DHPG. As shown in Fig. 5.1, subsequent to the LTD induced by the application of 30 μ M (RS)-DHPG, application of LFS generated an LTD that measured only $17\% \pm 6\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n=4$) at 40 min after the end of LFS. This

LTD, as well, was significantly reduced (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$) from the LTD induced by LFS in control solution (See Fig. 3.7).

In a second set of experiments, application of LFS induced an LTD measuring $33\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=5$) (Fig. 5.7). Subsequent perfusion of DHPG did not induce any further LTD ($1\% \pm 2\%$, Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=5$).

These occlusion experiments showed that common intracellular pathways are shared by DHPG-induced LTD and LFS-induced LTD.

5.2.3 DHPG-induced LTD and LFS-induced LTD were not accompanied by a change in the probability of transmitter release.

Another set of experiments was carried out in order to determine whether the DHPG-induced LTD and LFS-induced LTD had a pre-synaptic component by measuring paired pulse depression throughout the experiments. Any changes in paired-pulse depression would indicate a change in the probability of transmitter release.

As can be seen in Fig. 5.1, paired-pulse depression of paired EPSPs measured at 40 msec interval was not significantly altered (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=4$) following the induction of DHPG-induced LTD, measuring $28\% \pm 2\%$ before the perfusion with DHPG and $25\% \pm 3\%$ after the induction of DHPG-induced LTD.

As shown in Fig. 5.7, paired-pulse depression was not significantly changed (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=5$) after the induction of LFS-induced LTD, measuring $30\% \pm 5\%$ before the application of LFS and $29\% \pm 3\%$ after the induction of LTD.

These results would indicate that the DHPG-induced LTD, like LFS-induced LTD, was not accompanied by a change in the probability of transmitter release.

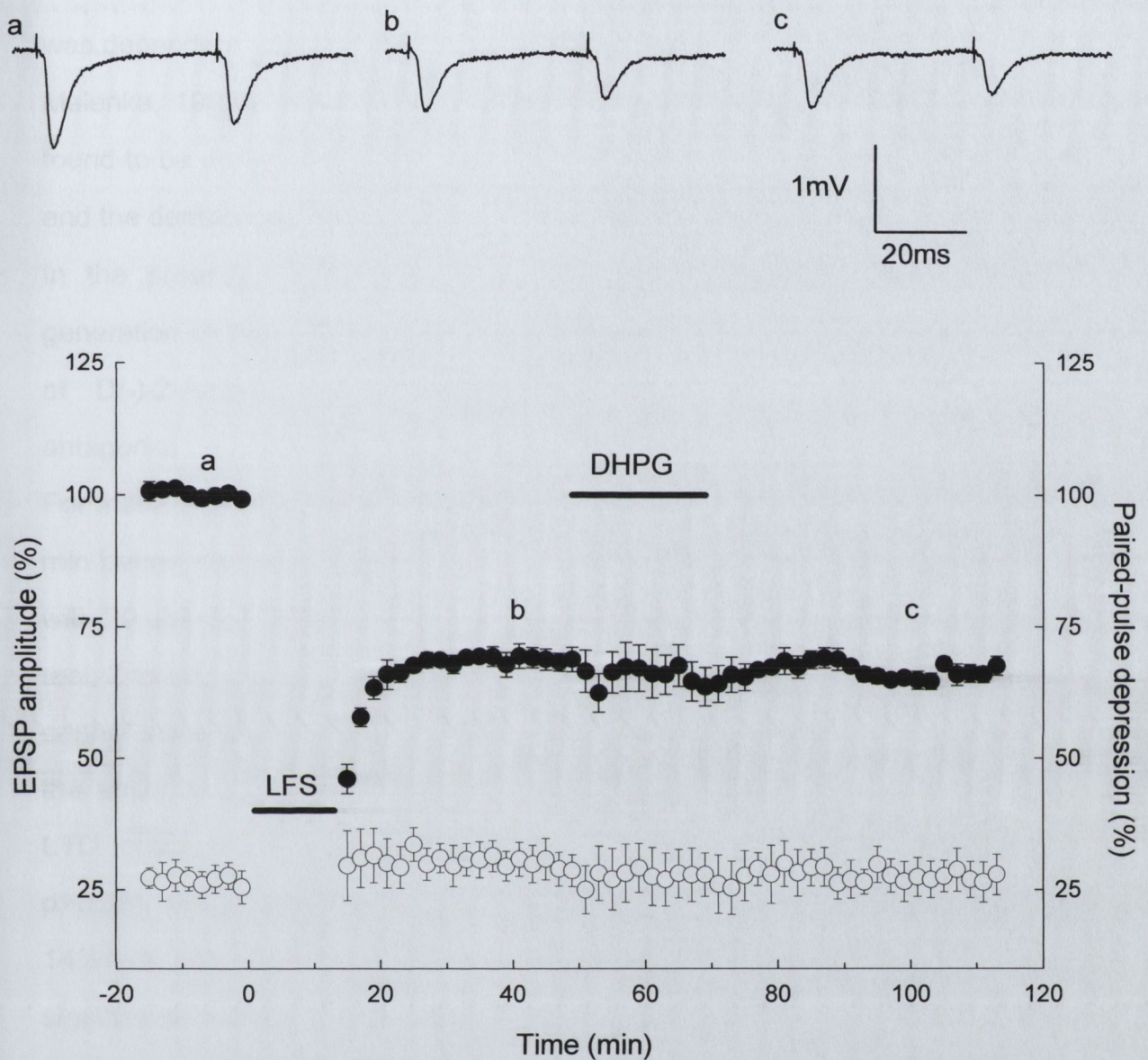


Figure 5.7 LFS-induced LTD occluded DHPG-induced LTD.

After induction of LTD by LFS-application, the perfusion with 20 μM (S)-DHPG failed to induce any further LTD (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 5$). No change in paired-pulse depression could be detected throughout the experiments. Filled symbols indicate EPSP amplitude and clear symbols indicate paired-pulse depression. Sample pairs of EPSPs are shown in **a** (baseline), **b** (40 min post-LFS) and **c** (40 min after washout).

5.2.4 DHPG-induced LTD did not require the activation of NMDAR.

Initial studies on the induction of LTD in CA1 showed that the induction of LTD was dependent upon activation of mGluR (Dudek and Bear, 1992 and Mulkey and Malenka, 1992). However, in later studies, a component of LTD induction was found to be dependent upon activation of NMDAR in both CA1 (Oliet *et al.*, 1997) and the dentate gyrus (O'Mara *et al.*, 1995b and Wang *et al.*, 1997).

In the present studies the involvement of the activation of NMDAR in the generation of the DHPG-induced LTD was investigated by application of 100 μ M of D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) a selective NMDAR antagonist.

For these experiments the slices were perfused with 100 μ M D-AP5 for at least 40 min before starting the recordings. As can be seen in Fig. 5.8, after the perfusion with 20 μ M (S)-DHPG, an LTD was induced that measured $18\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n=5$) at 40 min after the start of the perfusion with the control solution. Although this LTD was slightly smaller than the LTD obtained in the absence of D-AP5, it was not significantly different from the DHPG-induced LTD obtained in control solution (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Application of subsequent LFS induced a further LTD that measured $14\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=5$). This LTD was not significantly different (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$) from the residual LFS-induced LTD obtained after the perfusion with DHPG in the absence of D-AP5.

These experiments showed that the generation of the DHPG-induced LTD was not dependent upon activation of NMDAR.

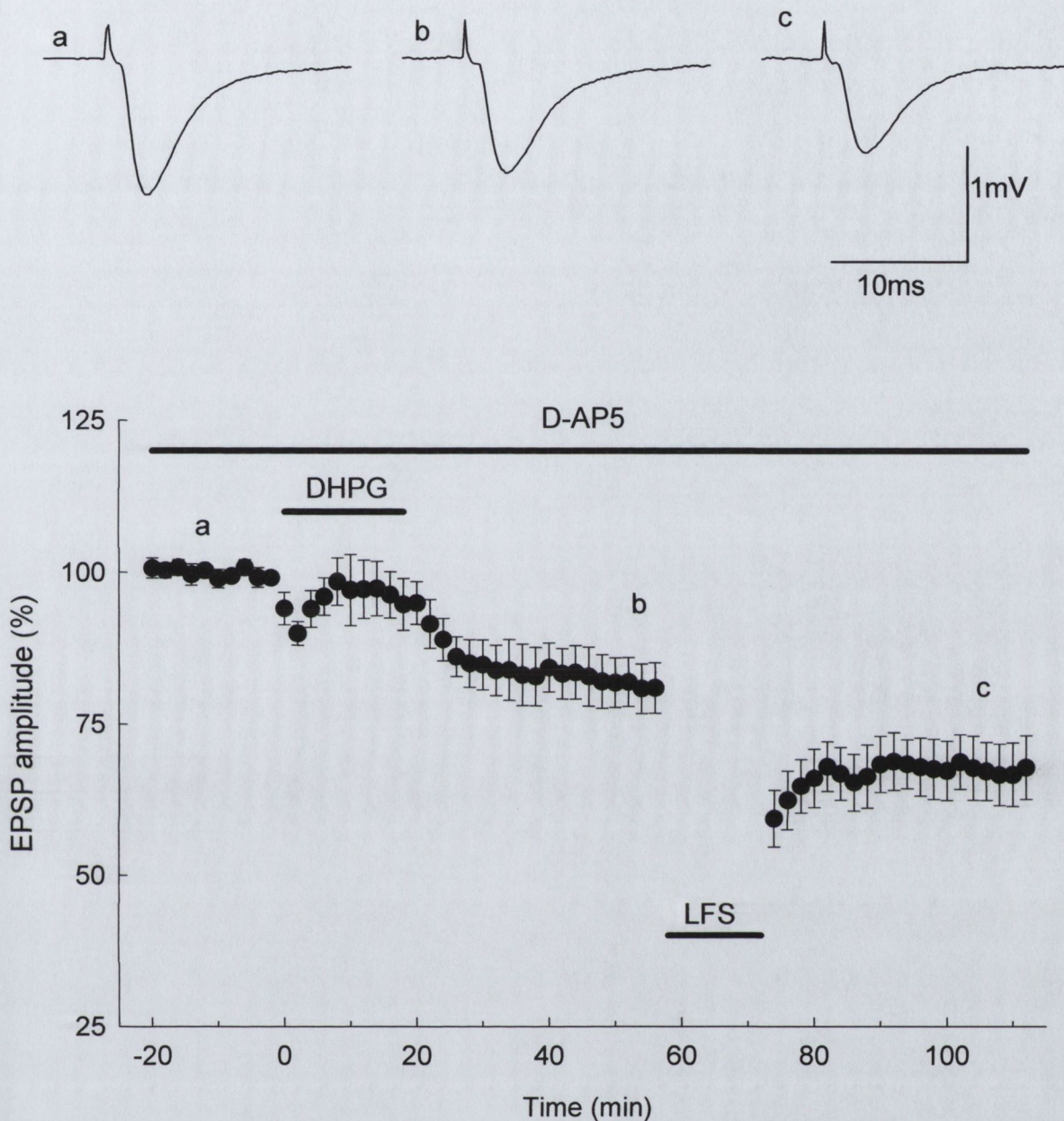


Figure 5.8 Effects of D-AP5 on DHPG-induced LTD.

In the presence of 100 μM D-AP5, application of 20 μM (S)-DHPG induced an LTD of $18\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 5$) at 40 min post-washout, which was not significantly different from that obtained in the absence of D-AP5 (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Subsequent LFS induced a further LTD of $14\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 5$) at 40 min post-LFS. Examples of single EPSPs are shown in **a** (baseline), **b** (40 min after washout) and **c** (40 min post-LFS).

5.2.5 GABA_A activity was not required for the induction of the DHPG-induced LTD.

Palmer *et al.* (1997) have shown that in adult CA1, DHPG induced only a small LTD in the control medium and 40% of the slices tested in those studies were not exhibiting any LTD. However, if the excitability of the slices was increased by either the presence of picrotoxin, a blocker of the GABA_A-mediated activity, or by using a solution containing no Mg²⁺, in order to remove the block of the NMDAR by the extracellular Mg²⁺, the DHPG-induced LTD was facilitated (Palmer *et al.*, 1997).

As explained in the Materials and Methods section, in the present experiments 50 μM picrotoxin were regularly added to every experimental solution in order to relieve some of the strong inhibition present in the dentate gyrus. It should be noted, however, that in the presence of 50 μM picrotoxin the EPSPs in the dentate gyrus did not show epileptiform spiking as in CA1.

In order to determine whether the DHPG-induced LTD generated in the dentate gyrus was influenced by the increased excitation in the slices because of the presence of 50 μM picrotoxin, experiments were carried out in the absence of picrotoxin. As shown in Fig. 5.9, after the perfusion with 20 μM (S)-DHPG an LTD was induced that measured 27%±3% (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 5$) at 30 min after the start of reperfusion with the control solution without the 50 μM picrotoxin. This DHPG-induced LTD was not significantly different (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$) from the DHPG-induced LTD obtained in control solution in the presence of 50 μM picrotoxin. Subsequent LFS applied after the DHPG-induced LTD in the absence of picrotoxin generated an LTD that measured 11%±3% (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 5$) at 30 min after the end of LFS. This further LFS-induced LTD was not significantly different (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$) from the residual LFS-induced LTD obtained after the DHPG-induced LTD in control solution in the presence of 50 μM picrotoxin.

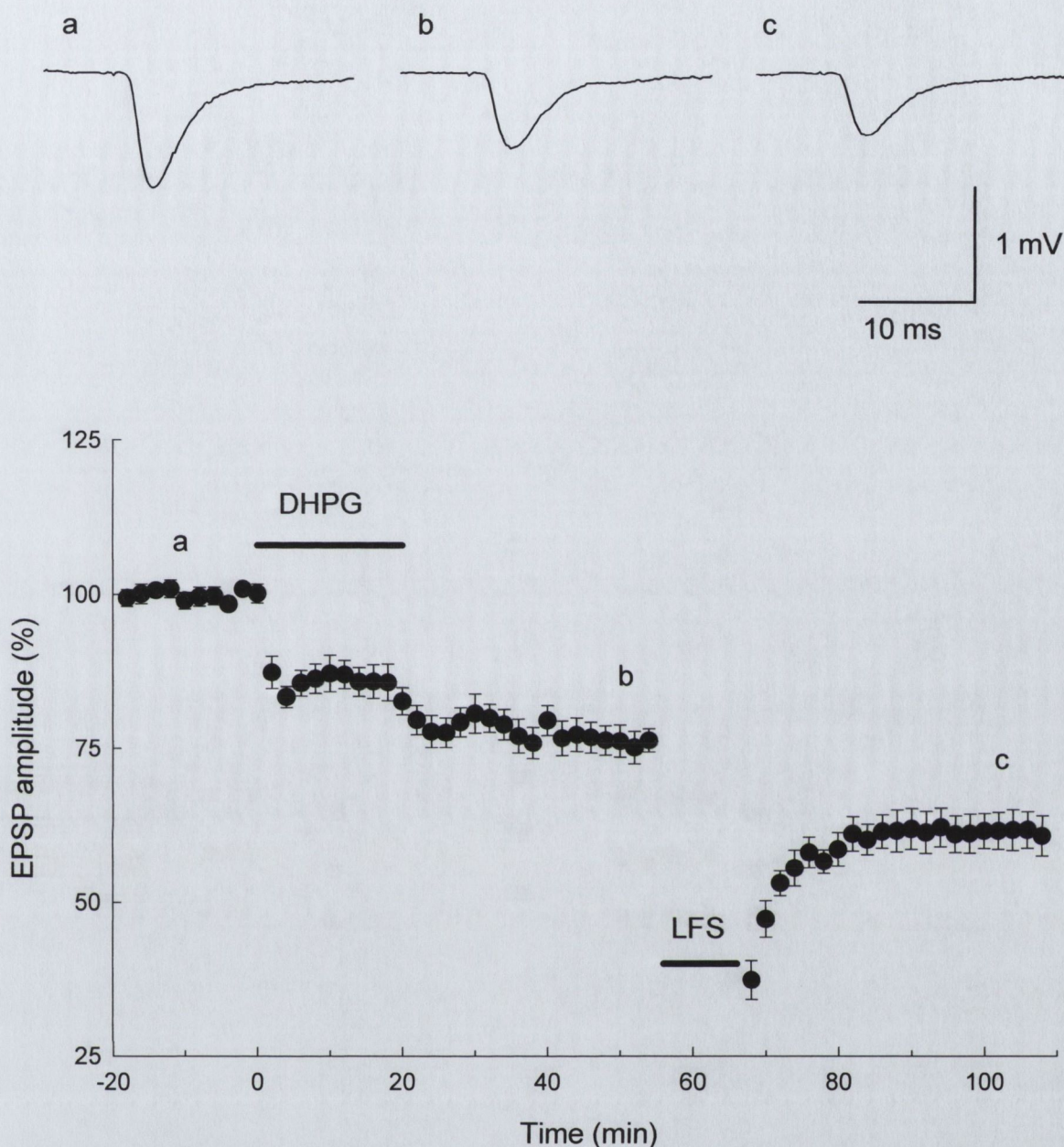


Figure 5.9 The absence of picrotoxin did not affect DHPG-induced LTD.

In the absence of 50 μM picrotoxin, application of 20 μM (S)-DHPG induced an LTD of $27\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 5$) at 30 min after washout, which was not significantly different from that obtained in the presence of picrotoxin (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Subsequent LFS induced a further LTD of $11\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 5$) at 30 min post-LFS. Examples of single EPSPs are shown in **a** (baseline), **b** (30 min after washout) and **c** (30 min post-LFS).

These experiments showed that the presence of picrotoxin did not affect the generation of the DHPG-induced LTD, indicating that in the dentate gyrus the induction of LTD by DHPG did not require GABA_A activity.

5.2.6 The mGluR ligand (S)-(+)-2-(3'-Carboxybicyclo[1.1.1]pentyl)-glycine (UPF 596) induced LTD in the medial perforant pathway of the dentate gyrus.

In order to determine whether both mGluRs belonging to group I (mGluR1 and mGluR5) were involved in the generation of the DHPG-induced LTD or only one of them was responsible for the induction of this LTD, experiments were carried out in the presence of UPF 596. This compound is a recently synthesised mGluR group I ligand, with partial agonistic properties at mGluR5 (EC_{50} $103 \pm 33 \mu\text{M}$) as well as antagonistic properties at mGluR1 (IC_{50} $25 \pm 6 \mu\text{M}$) (Pellicciari *et al.*, 1996). As shown in Fig. 5.10, when $150 \mu\text{M}$ UPF 596 was applied to the slices a depression of the EPSP of $35\% \pm 6\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n=5$) measured at 20 min after the start of perfusion with the compound. This depression was significantly greater than the depression generated in the presence of (S)-DHPG (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$).

Upon washout with the control solution an LTD was induced that measured $22\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=5$) at 60 min after the start of the washout of the UPF 596. This LTD generated by UPF 596 was not significantly different from the DHPG-induced LTD (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Subsequent application of LFS failed to induce any further significant LTD, the depression generated measuring $8\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=5$).

These experiments showed that the binding of UPF 596 to mGluR group I, which led to the activation of mGluR5 and the blocking of mGluR1, induced an LTD in the dentate gyrus similar to the DHPG-induced LTD.

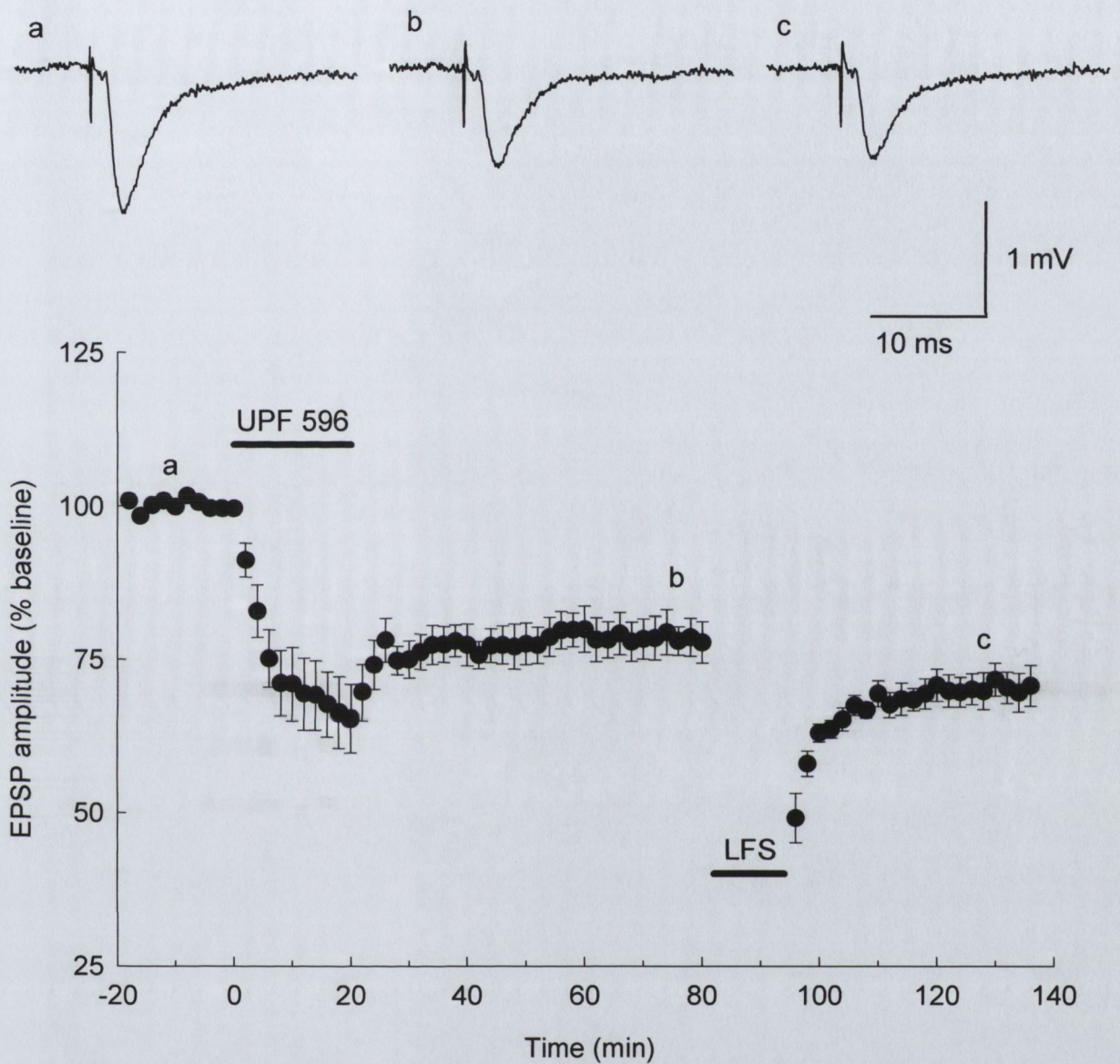


Figure 5.10 LTD induced by UPF 596.

Application of 150 μ M UPF 596 for 20 min induced an LTD of $22\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 5$) at 60 min after washout, which was similar to the DHPG-induced LTD (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Subsequent LFS failed to induce any further significant LTD (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 5$). Examples of single EPSPs are shown in **a** (baseline), **b** (60 min after washout) and **c** (40 min post-LFS).

5.2.7 The mGluR5 agonist (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) induced LTD in the medial perforant pathway of the dentate gyrus.

Because the perfusion of UPF 596, a partial mGluR5 agonist, induced LTD in the dentate gyrus, CHPG, a more selective mGluR5 agonist, was used in order to further identify the role of mGluR5 in the induction of the LTD by activation of mGluR group I. CHPG is a novel phenylglycine derivative that has been shown to selectively activate mGluR5 but not mGluR1 in transfected CHO cells (Doherty *et al.*, 1997). Doherty *et al.* (1997) found that the EC₅₀ for CHPG in this preparation was 750±86µM, hence the following experiments were carried out in the presence of 1 mM of CHPG.

When the slices were perfused with 1 mM CHPG a transient depression was generated that measured 11%±3% (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 7$) at 20 min after the start of perfusion with the agonist (Fig. 5.11). This depression was not significantly different (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$) from that obtained in the presence of 20 µM (S)-DHPG. Upon washout of 1 mM CHPG an LTD was induced which measured 14%±3% (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 7$) at 40 min after the start of reperfusion with control solution. This CHPG-induced LTD was not significantly different (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$) from the DHPG-induced LTD.

Subsequent LFS induced further LTD measuring 12%±3% (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 7$) at 40 min after the end of LFS. This residual LTD was not significantly different (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$) from the LFS-induced LTD obtained after the DHPG-induced LTD.

These experiments demonstrated that 1 mM CHPG induced an LTD that was similar to the DHPG-induced LTD.

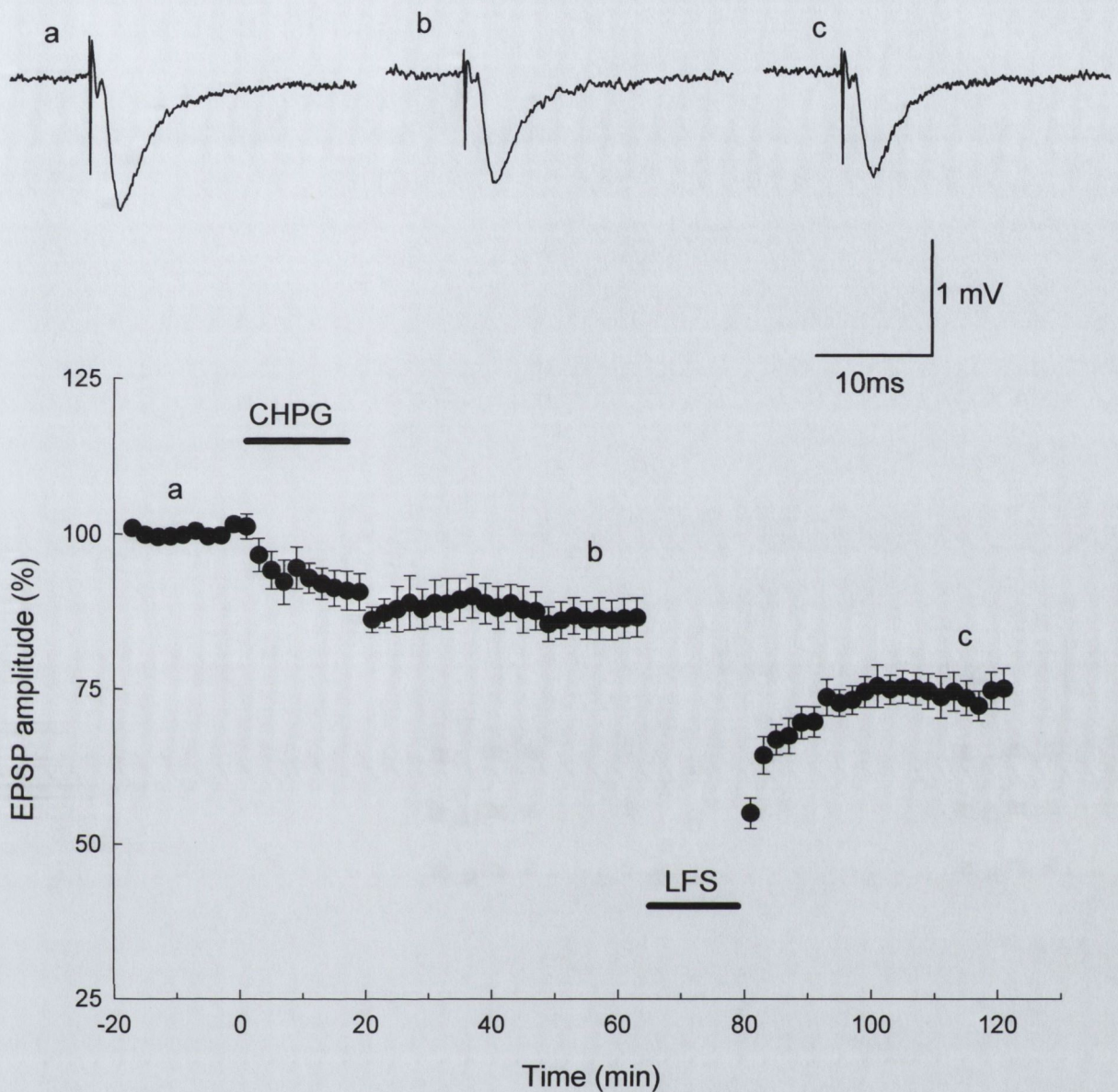


Figure 5.11 LTD induced by CHPG.

Perfusion of 1 mM CHPG for 20 min induced an LTD of $14\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 7$) at 40 min after washout, which was not significantly different from the DHPG-induced LTD (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Subsequent LFS induced a further LTD of $12\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 7$) at 40 min post-LFS. Examples of single EPSPs are shown in **a** (baseline), **b** (40 min after washout) and **c** (40 min post-LFS).

5.2.8 The effects of [CRS]-1-aminoindan-1,5-dicarboxylic acid (AIDA) on the induction of LTD.

From the experiments shown up to this point, it can be seen that activation of mGluR group I led to induction of LTD. AIDA is a selective mGluR group I antagonist (Moroni *et al.*, 1997).

A first set of experiments was carried out perfusing 20 μ M (S)-DHPG in the presence of 450 μ M AIDA.

A second set of experiments was performed to assess the effects of 450 μ M AIDA on the LFS-induced LTD.

5.2.8.1 The mGluR group I antagonist AIDA blocked the DHPG-induced LTD.

As can be seen in Fig. 5.12, when the slices were perfused with 450 μ M AIDA, a depression was induced that measured $15\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=6$) at 40 min after perfusion with the antagonist. Subsequently, 20 μ M (S)-DHPG was applied to the slices for 20 min in the presence of AIDA and then the slices were reperfused with control solution. No LTD was generated upon washout of the 20 μ M (S)-DHPG, since the depression measured only $5\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=6$) at 40 min after the start of the reperfusion with the control solution.

As can be seen in Fig. 5.13, application of 20 μ M (S)-DHPG after washout of 450 μ M AIDA induced a normal LTD.

These experiments indicated that the antagonistic action of AIDA blocked the DHPG induced LTD.

5.2.8.2 The mGluR group I antagonist AIDA partially inhibited the LFS-induced LTD.

For these experiments the slices were perfused with 450 μ M AIDA for at least 40 min prior to the start of the recordings.

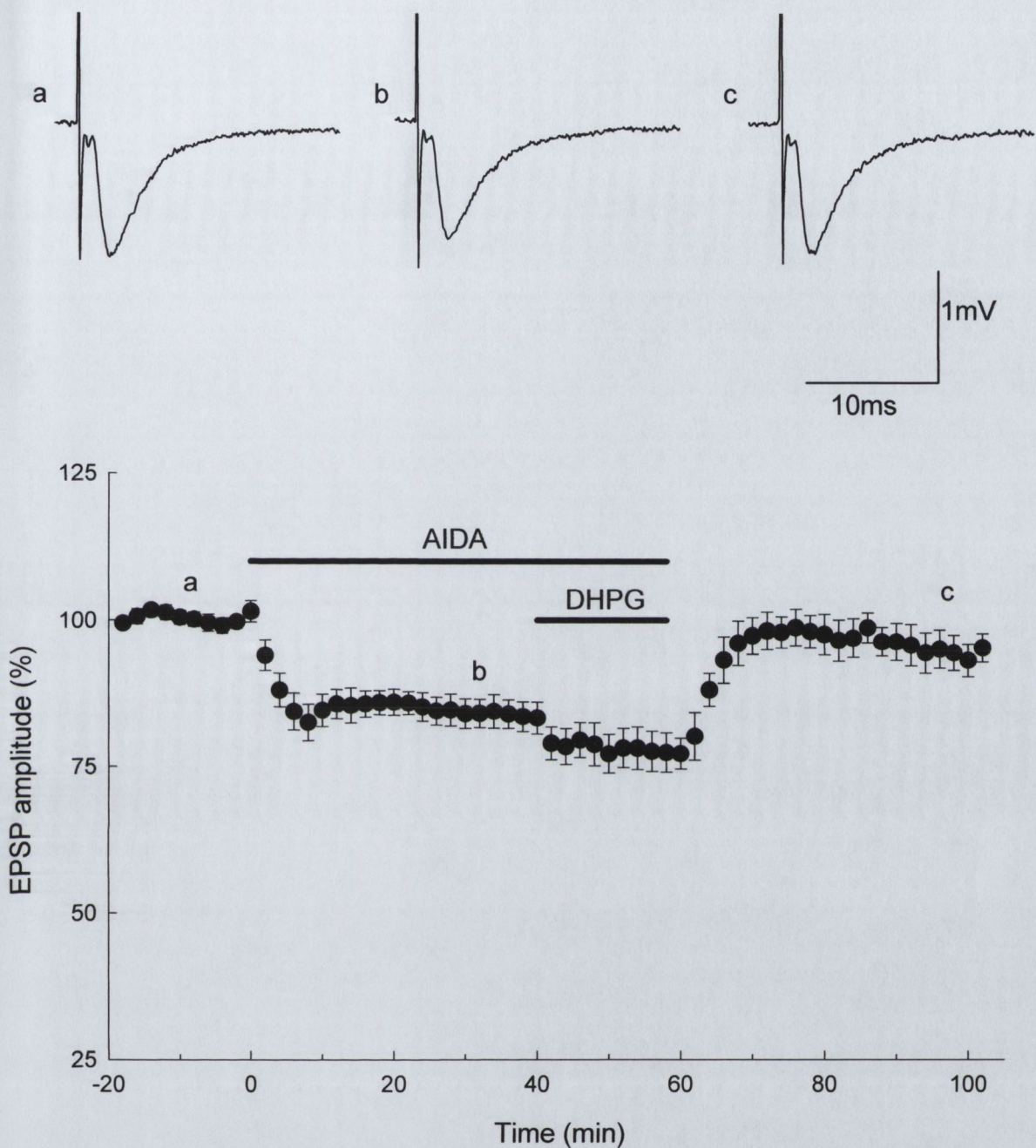


Figure 5.12 Effects of AIDA on DHPG-induced LTD.

In the presence of $450\mu\text{M}$ AIDA the EPSPs were depressed by $15\% \pm 2\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 6$) after 40 min of perfusion. Subsequent application of $20\mu\text{M}$ (S)-DHPG failed to induce any LTD (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 6$). Examples of single EPSPs are shown in a (baseline), b (40 min after perfusion with AIDA) and c (40 min after washout of AIDA and DHPG).

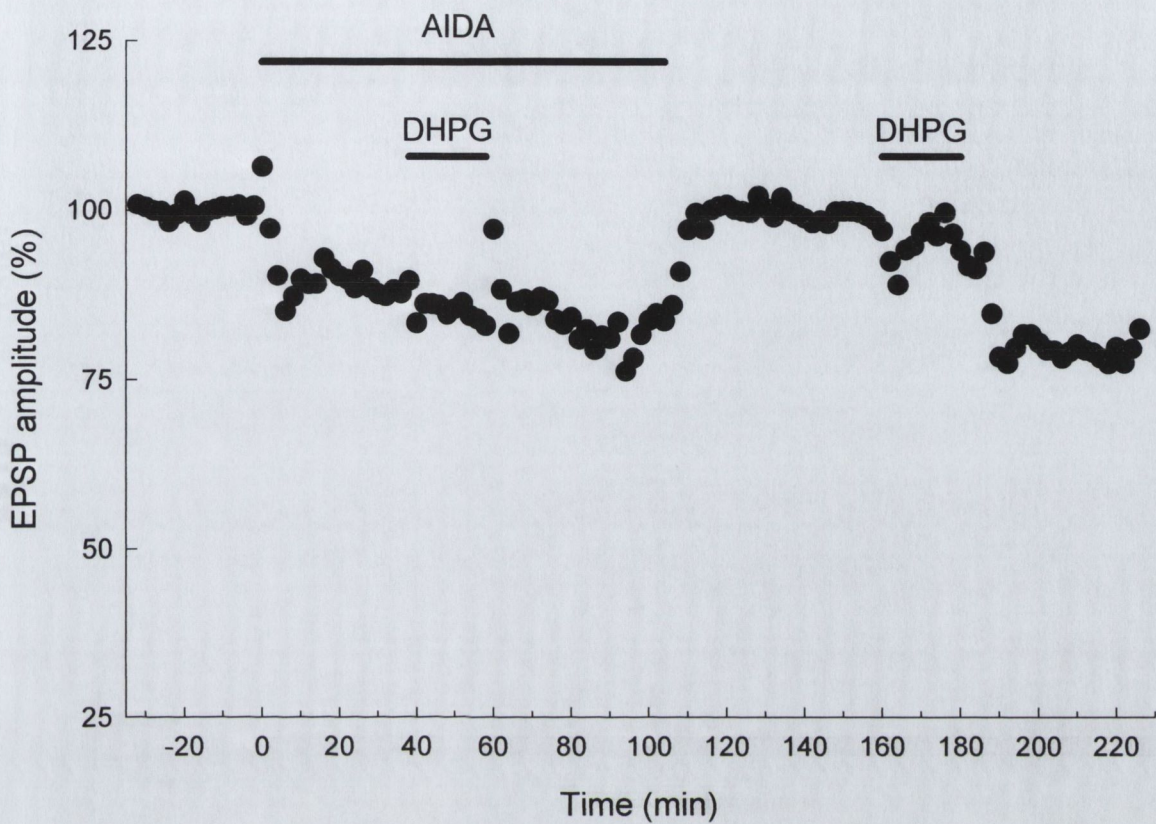


Figure 5.13 Reversible effects of AIDA.

Example of a single slice in which application of 20 μM (S)-DHPG after washout of 450 μM AIDA induced an LTD of approximately 20 % at 40 min after washout of DHPG without AIDA.

As can be seen in Fig. 5.14, when LFS was applied to slices in control solution, an LTD was induced measuring $30\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n=5$) at 30 min after the end of LFS. When LFS was applied to slices previously perfused with $450 \mu\text{M}$ AIDA, an LTD was induced that measured only $11\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n=5$) at 30 min after the end of LFS.

The LTD induced in the presence of $450 \mu\text{M}$ AIDA was significantly inhibited compared to the LFS-induced LTD obtained in control solution (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.01$).

These experiments demonstrated that the generation of LFS-induced LTD was partially inhibited in the presence of $450 \mu\text{M}$ AIDA indicating the involvement of mGluR group I in the induction of this form of LTD.

5.2.9 The induction of the DHPG-induced LTD in control mice and in mutant mice lacking the gene for mGluR5.

Because the activation of mGluR5 by CHPG induced an LTD that was similar to the DHPG-induced LTD, a study was carried out on knockout mice lacking the mGluR5 gene in order to determine more specifically the role of mGluR5 in the DHPG-induced LTD.

Two sets of experiments were carried out, one set with mice that were not genetically modified as a control and another with knockout mice lacking the mGluR5 gene.

5.2.9.1 Perfusion of $20 \mu\text{M}$ (S)-DHPG induced LTD in control mice.

In the first set of experiments shown in Fig. 5.15 the control mice were used.

When $20 \mu\text{M}$ (S)-DHPG was applied to the slices, an initial rapid and transient depression could be detected reaching a peak of $16\% \pm 1\%$ ($n=4$) in 2 min from the start of the perfusion with the agonist. However after 20 min of perfusion with $20 \mu\text{M}$ (S)-DHPG, no significant LTD could be detected, the depression measuring 1%

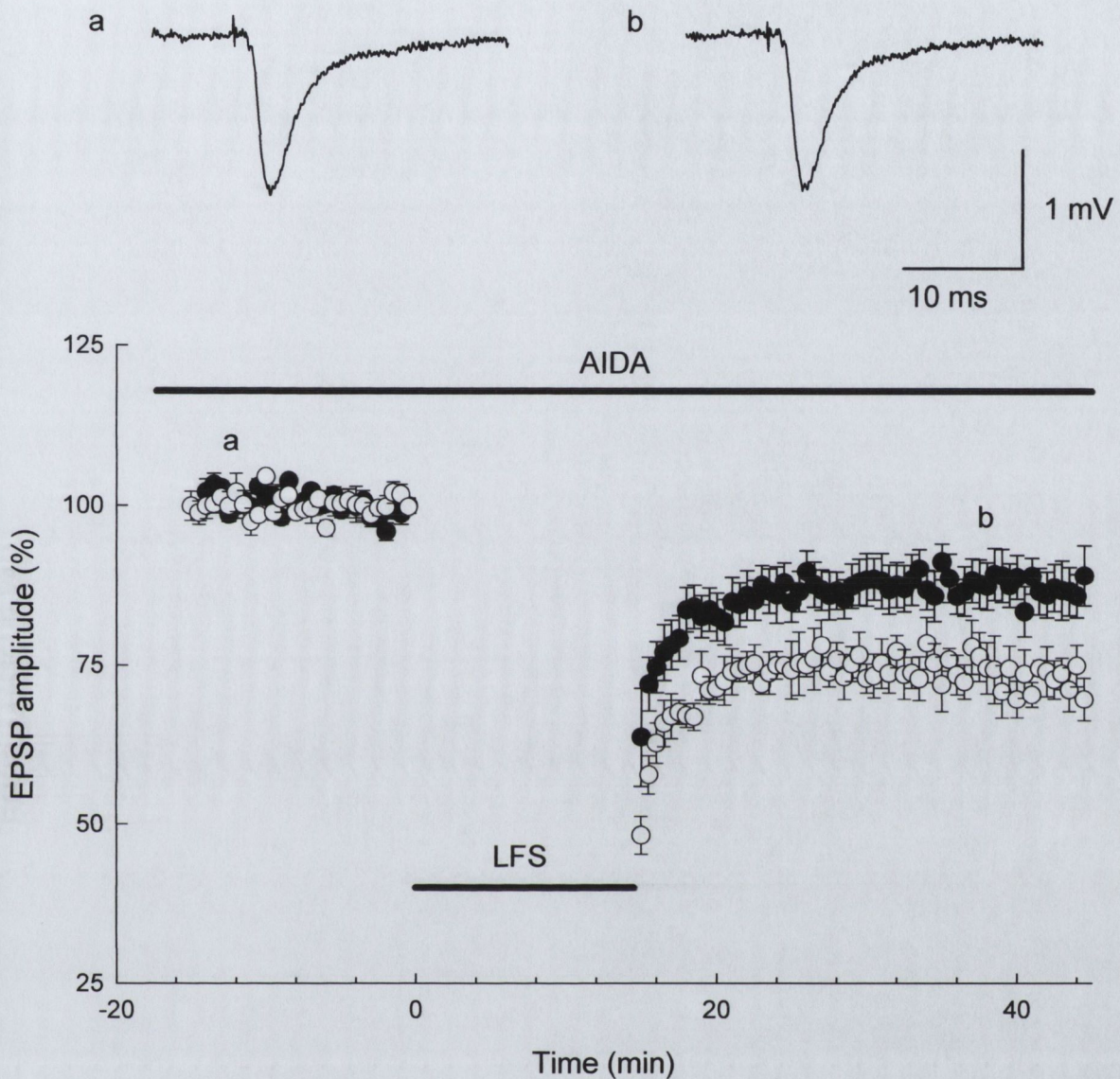


Figure 5.14 Effects of AIDA on LFS-induced LTD.

In the presence of $450 \mu\text{M}$ AIDA LFS induced an LTD of $11\% \pm 5\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n = 5$) at 30 min post-LFS. This was significantly reduced (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.01$) compared to the LFS-induced LTD obtained in control solution which measured $30\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 5$) at 30 post-LFS. Filled symbols represent the experiments in the presence of AIDA and clear symbols those in control solution. Examples of single EPSPs of an experiment in the presence of AIDA are shown in **a** (baseline), **b** (30 min post-LFS).

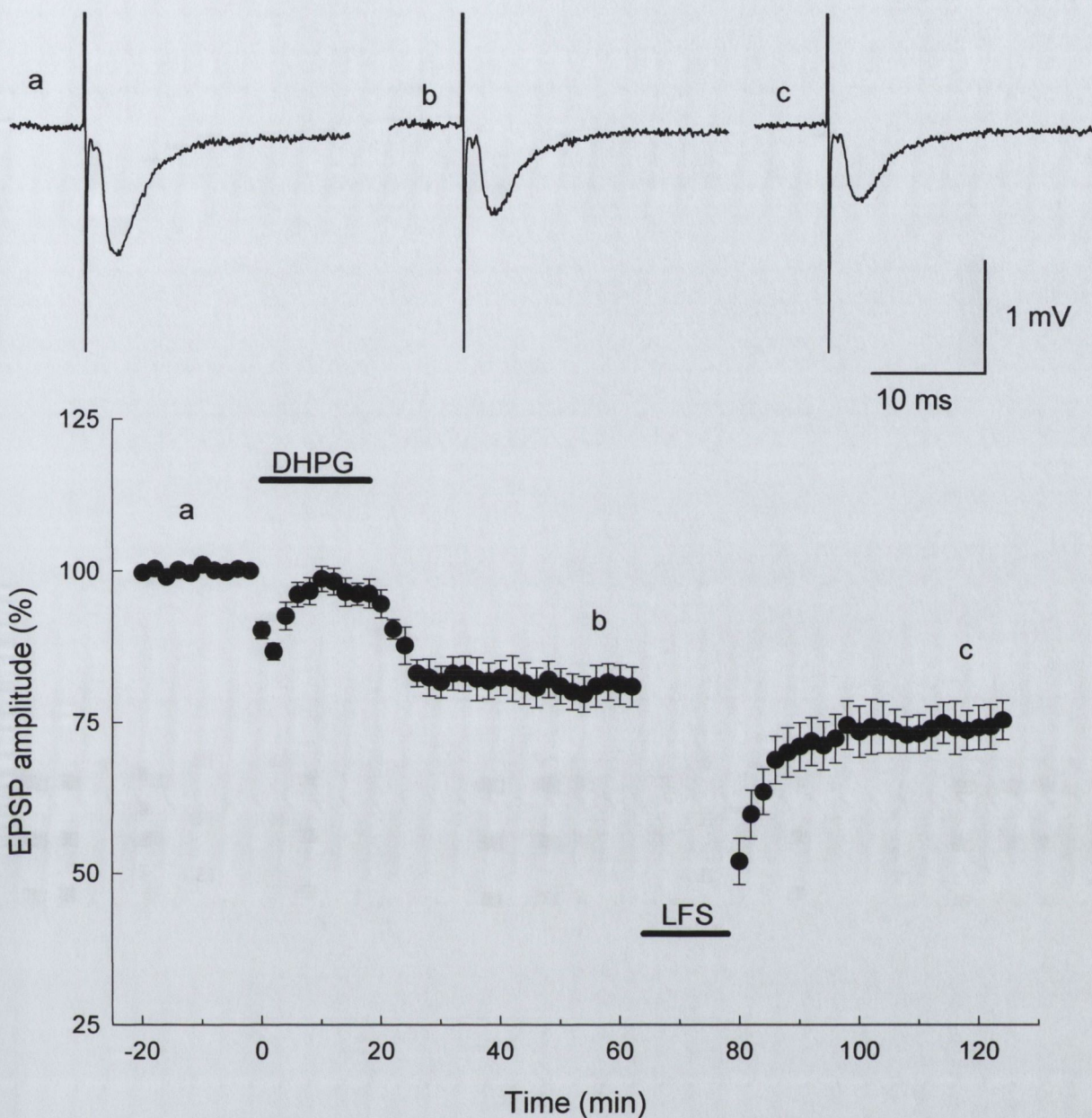


Figure 5.15 DHPG-induced LTD in control mice.

In control mice, application of 20 μ M (S)-DHPG induced an LTD of $17\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n = 4$) at 40 min after washout. Further LFS induced an LTD of $8\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 4$) at 40 min post-LFS. Examples of single EPSPs are shown in **a** (baseline), **b** (40 min after washout) and **c** (40 min post-LFS).

$\pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 4$). Upon washout with the control solution an LTD was generated that measured $17\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n = 4$) at 40 min after the reperfusion with the control solution. Subsequent application of LFS induced a further significant LTD that measured $8\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 4$) at 40 min after the end of LFS.

These experiments showed that perfusion with $20 \mu\text{M}$ (S)-DHPG induced LTD in the dentate gyrus of control mice.

5.2.9.2 DHPG-induced LTD was blocked in knockout mice lacking the gene for mGluR5.

The second set of experiments was carried out perfusing $20 \mu\text{M}$ (S)-DHPG in mutant mice which were not expressing mGluR5.

As can be seen in Fig. 5.16, when the slices were perfused with $20 \mu\text{M}$ (S)-DHPG a small but significant depression was generated that measured $4\% \pm 1\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.05$, $n = 4$) at 20 min after the start of perfusion. Upon washout with the control solution, no significant LTD was induced, the depression measuring only $3\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 4$) at 40 min after the start of the reperfusion with the control solution. Subsequent application of LFS failed to induce any LTD, the depression obtained measuring only $9\% \pm 9\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 4$) at 40 min after the end of LFS.

These experiments demonstrated that activation of mGluR5 was necessary for the induction of the DHPG-induced LTD, since in the absence of these receptors $20 \mu\text{M}$ (S)-DHPG failed to induce any LTD.

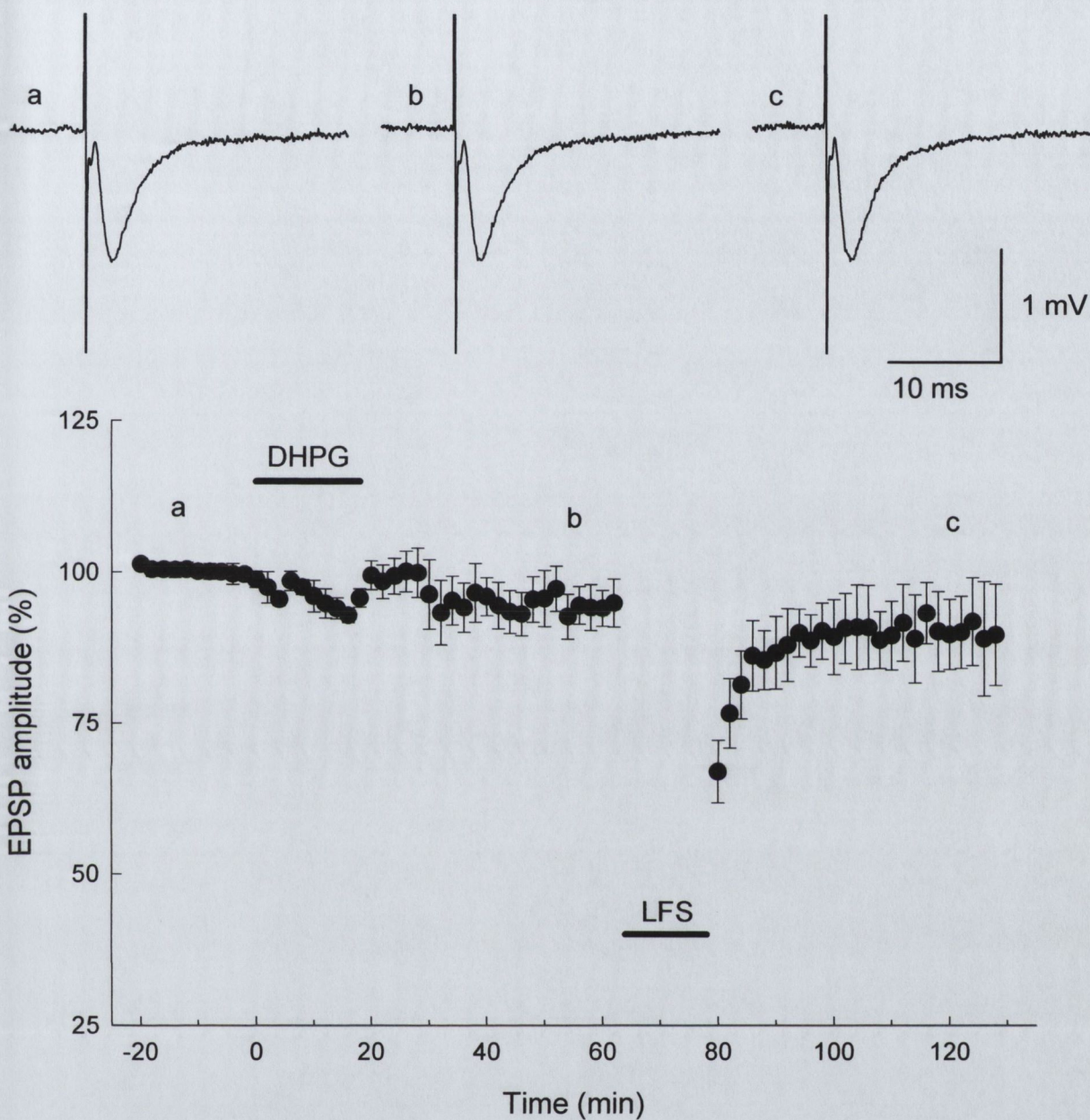


Figure 5.16 DHPG failed to induce LTD in knockout mice.

In knockout mice lacking mGluR5, application of 20 μ M (S)-DHPG failed to induce any LTD (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 4$). Subsequent LFS was also unable to generate any LTD (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 4$). Examples of single EPSPs are shown in **a** (baseline), **b** (40 min after washout) and **c** (40 min post-LFS).

5.2.10 The blocking of protein kinase C (PKC) strongly inhibited the DHPG-induced LTD.

Activation of mGluR group I results in stimulation of phospholipase C, phosphoinositide hydrolysis and stimulation of PKC through generation of diacylglycerol (Pin and Duvoisin, 1995). In order to investigate if the DHPG-induced LTD required the activation of PKC, the effects of the highly selective PKC inhibitor bisindolylmaleimide I (GF 109203X) on this form of LTD were studied. Bisindolylmaleimide I inhibits PKC with a K_i of 10 nM but affects other kinases at much higher concentrations, for example protein kinase A (PKA) at K_i of 2 μ M.

Before the start of the experiments the slices were perfused with control solution containing also 1 μ M bisindolylmaleimide I for at least an hour.

As can be seen in Fig. 5.17, after perfusion with 20 μ M (S)-DHPG in the presence of 1 μ M bisindolylmaleimide I a non significant LTD was induced that measured only $2\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 5$) at 40 min after the start of reperfusion with control solution with 1 μ M bisindolylmaleimide I. This LTD was significantly reduced compared to the DHPG-induced LTD obtained in the absence of 1 μ M bisindolylmaleimide I (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.005$). Subsequent LFS applied in the presence of 1 μ M bisindolylmaleimide I induced an LTD that measured $10\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 5$) at 40 min after the end of LFS. This LTD was significantly reduced (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$) compared to the residual LTD obtained in the absence of 1 μ M bisindolylmaleimide I after the DHPG-induced LTD.

These experiments demonstrated that activation of PKC was necessary for the induction of the DHPG-induced LTD.

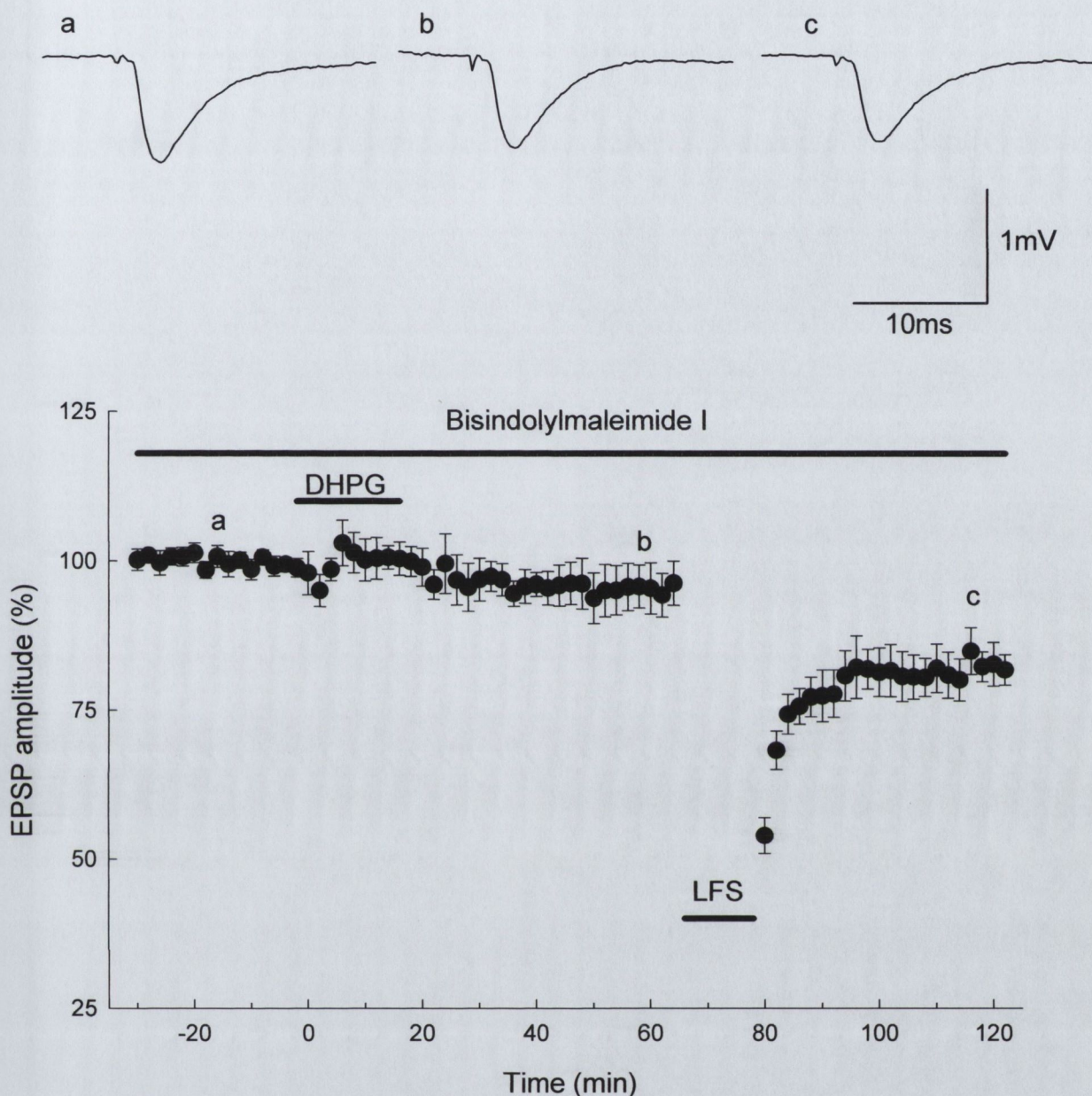


Figure 5.17 Effects of bisindolylmaleimide I on DHPG-induced LTD.

In the presence of 1 μM bisindolylmaleimide I application of 20 μM (S)-DHPG failed to induce any LTD (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 5$). Subsequent LFS induced an LTD of $10\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.001$, $n = 5$) which was significantly reduced (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$) compared to the residual LTD obtained in the absence of bisindolylmaleimide I. Examples of single EPSPs are shown in **a** (baseline), **b** (40 min after washout) and **c** (40 min post-LFS).

5.2.11 The inhibition of non-receptor protein tyrosine kinase (PTK) inhibited the DHPG-induced LTD.

It has been demonstrated that PTKs are activated by PKC and elevated intracellular free Ca^{2+} (Boxall and Lancaster, 1998 and Petryniak *et al.*, 1996).

Lavendustin A is a selective inhibitor of PTK with a K_i of 500 nM and a much lower potency for other kinases like PKA or PKC ($K_i > 200 \mu\text{M}$). In the present experiments lavendustin A was used at a concentration of 5 μM . The slices were perfused for at least 1 hour before the start of the experiments.

As can be seen in Fig. 5.18, after application of 20 μM (S)-DHPG in the presence of 5 μM lavendustin A an LTD was generated that measured $10\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n=5$) at 40 min after the start of reperfusion of control solution containing 5 μM lavendustin A. This LTD was significantly reduced (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$) compared to the DHPG-induced LTD obtained in the absence of 5 μM lavendustin A. Subsequent LFS applied in the presence of 5 μM lavendustin A failed to induce any significant LTD, the depression generated measuring $7\% \pm 6\%$ (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n=5$) at 40 min after the end of LFS.

This LTD was significantly reduced (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.01$) when compared to the LTD generated after the DHPG-induced LTD in the absence of 5 μM lavendustin A.

These experiments showed that the inhibition of PTK blocked the DHPG-induced LTD.

5.2.12 The inhibition of protein kinase A (PKA) did not affect the DHPG-induced LTD.

Because it is known that PKA can phosphorylate the IP_3 receptor in the endoplasmic reticulum, it was of interest to determine whether inhibiting PKA had any effect on the DHPG-induced LTD.

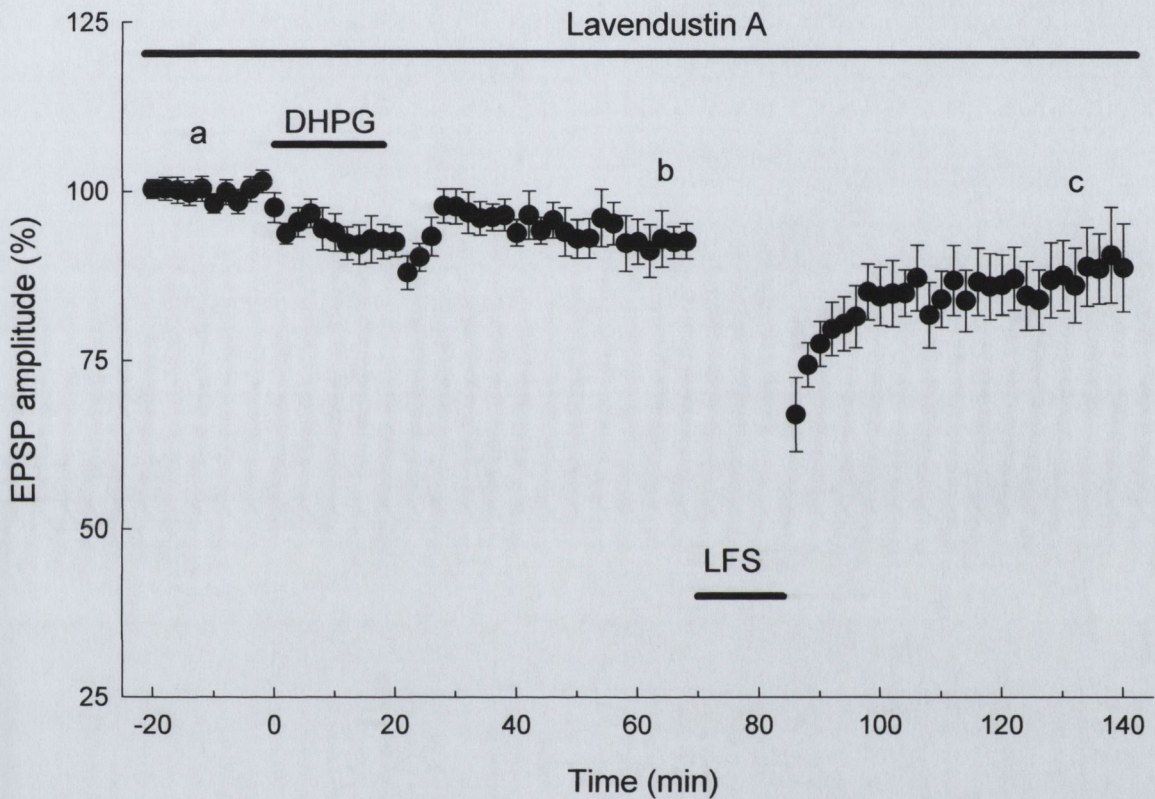
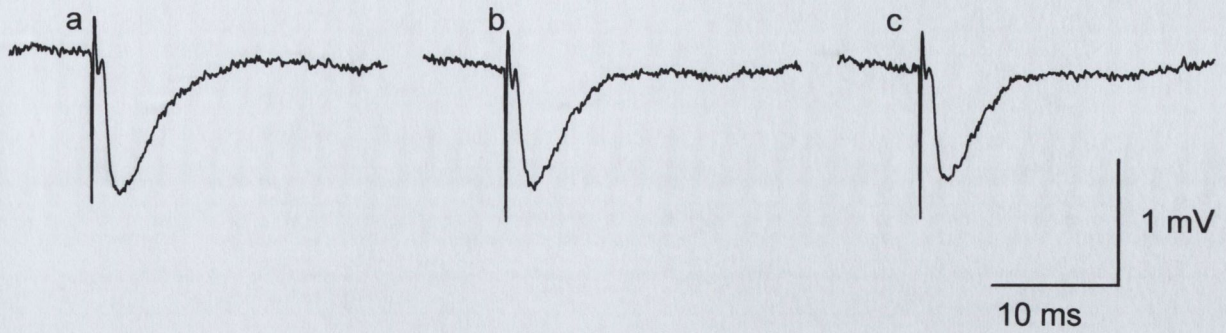


Figure 5.18 Effects of lavendustin A on DHPG-induced LTD.

In the presence of 5 μM lavendustin A application of 20 μM (S)-DHPG induced an LTD of $10\% \pm 3\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.005$, $n = 5$) at 40 min after washout. This was significantly reduced (Student's *t*-test, 2 tailed, homoscedastic, $p < 0.05$) compared to the DHPG-induced LTD in the absence of lavendustin A. Subsequent LFS failed to induce any further LTD (Student's *t*-test, 2 tailed, paired, $p > 0.05$, $n = 5$). Examples of single EPSPs are shown in **a** (baseline), **b** (40 min after washout) and **c** (40 min post-LFS).

H 89 is a potent inhibitor of the catalytic site of PKA with a K_i of 48 nM but affects other kinases at much higher concentrations, for instance CaMKII with a K_i of 30 μ M and PKC with a K_i of 32 μ M. In the present experiments H 89 was used at a concentration of 10 μ M.

Before the start of the experiments the slices were perfused with control solution containing also 10 μ M H 89 for at least 1 hour.

As seen in Fig. 5.19, after the perfusion of the slices with 20 μ M (S)-DHPG in the presence of 10 μ M H 89 an LTD was induced that measured $16\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n=5$) at 40 min from the start of reperfusion with control solution containing 10 μ M H 89. This LTD was not significantly different (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$) from the DHPG-induced LTD in the absence of 10 μ M H 89.

Subsequent application of LFS induced a further LTD measuring $14\% \pm 7\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n=5$) at 40 min after the end of LFS. This residual LTD was not significantly different (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$) from the LTD obtained after the DHPG-induced LTD in the absence of H 89.

These experiments showed that the inhibition of PKA did not affect the DHPG-induced LTD.

5.3 Discussion.

It had been shown previously that the application of DHPG in the area CA1 of the hippocampus induced LTD (Palmer *et al.*, 1997).

With the present studies it has been demonstrated that application of DHPG also induced an LTD in the medial perforant pathway of the dentate gyrus.

Two enantiomers of DHPG can be synthesised, the (S) enantiomer, which is more potent with an EC_{50} of 10.9 ± 2.13 μ M and the (R) enantiomer, the less potent one with an EC_{50} of 106 ± 2.28 μ M (Baker *et al.*, 1995). In the present experiments DHPG-induced LTD could be evoked by using the racemic mixture containing

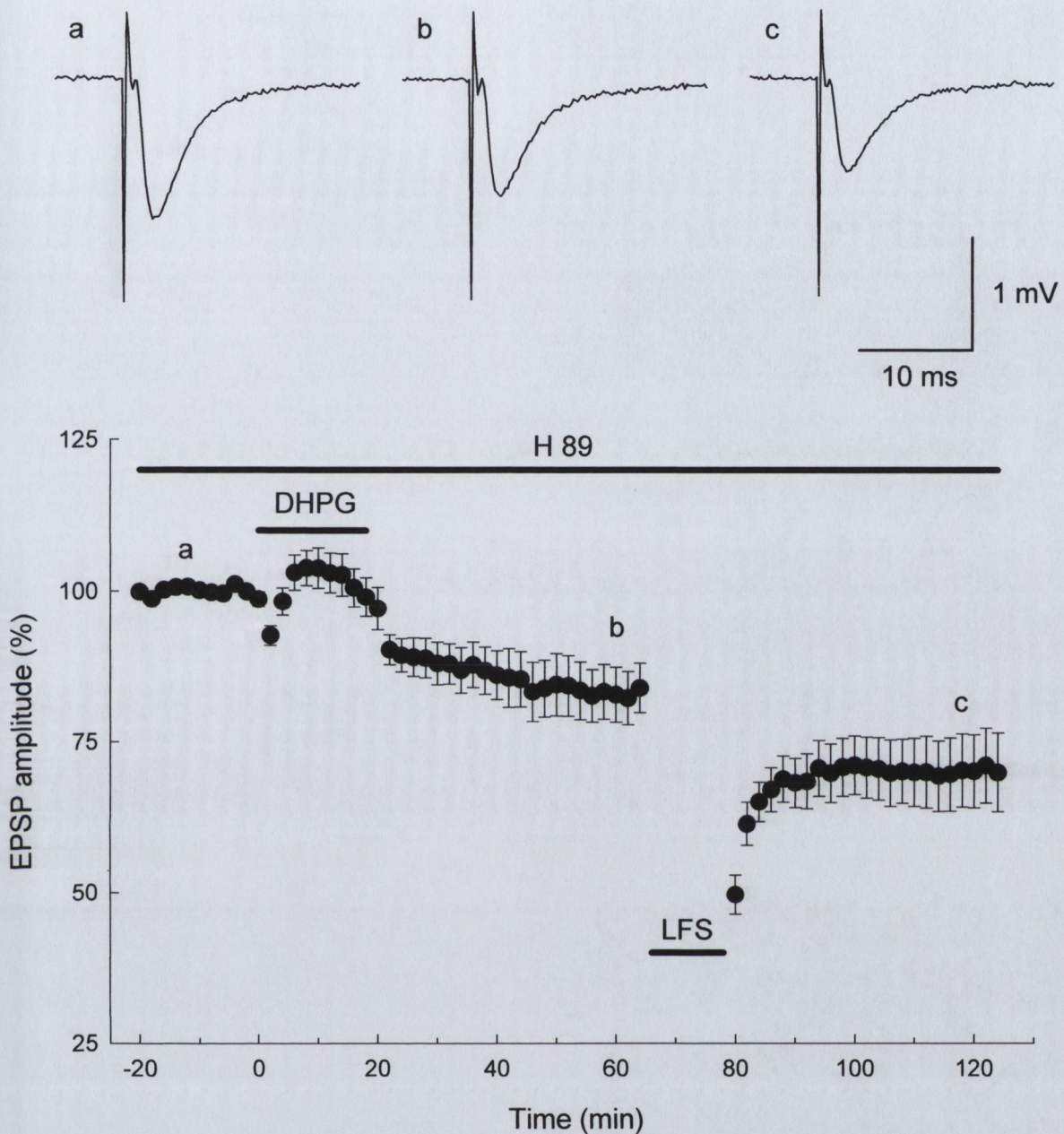


Figure 5.19 Effects of H 89 on DHPG-induced LTD.

In the presence of $10 \mu\text{M}$ H 89 application of $20 \mu\text{M}$ (S)-DHPG induced an LTD of $16\% \pm 4\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 5$) at 40 min after washout. This LTD was not significantly different from the DHPG-induced LTD in the absence of H 89 (Student's *t*-test, 2 tailed, homoscedastic, $p > 0.05$). Subsequent LFS induced an LTD of $14\% \pm 7\%$ (Student's *t*-test, 2 tailed, paired, $p < 0.01$, $n = 5$) at 40 min post-LFS. Examples of single EPSPs are shown in **a** (baseline), **b** (40 min after washout) and **c** (40 min post-LFS).

both enantiomers ((R,S)-DHPG) or by using only the more potent enantiomer ((S)-DHPG), and it was shown that there was no difference between the LTD obtained with 30 μ M (RS)-DHPG and the LTD obtained with 20 μ M (S)-DHPG. Because of these results, only the more potent enantiomer was used for all the experiments to follow and the LTD generated by the perfusion with 20 μ M (S)-DHPG was referred to as DHPG-induced LTD.

One important feature of the DHPG-induced LTD was that it was completely occluded by the LFS-induced LTD. This indicated that these two differently induced LTDs share some common induction/maintenance mechanisms. However the fact that the LFS-induced LTD was not completely occluded by the DHPG-induced LTD (since application of LFS after the induction of DHPG-induced LTD gave rise to an additional LTD) suggested that the activation of mGluR group I is one of the many pathways activated by LFS for the induction of LTD.

Neither the DHPG-induced LTD nor the LFS-induced LTD were accompanied by a change in paired-pulse depression, which indicated that both types of LTD are not accompanied by a change in the probability of transmitter release. In this way, even though a pre-synaptic involvement in the generation of LTD could not be completely excluded, the present experiments provided strong evidence that both the DHPG-induced LTD and the LFS-induced LTD were induced and/or expressed post-synaptically, in agreement with the findings by (Goda and Stevens, 1996) in cultured pyramidal neurones. In further support of this finding there is strong anatomical evidence that group I mGluRs are located post-synaptically in the hippocampus (Petralia *et al.*, 1997, Shigemoto *et al.*, 1997 and Luján *et al.*, 1996), and more specifically in the dentate gyrus both mGluR1 and mGluR5 are present post-synaptically in the peri-synaptic region (Shigemoto *et al.*, 1997, Luján *et al.*, 1996).

Fitzjohn *et al.* (1996) in the hippocampus and Ugolini *et al.* (1997) in spinal cord motoneurones have shown that application of DHPG potentiated NMDA

responses. Thiels *et al.* (1996) also demonstrated that LFS-induced LTD in CA1 was partially dependent upon activation of NMDAR.

However the DHPG-induced LTD in the present studies was not dependent on activation of NMDAR, since in the presence of 100 μ M D-AP5 20 μ M (S)-DHPG induced an LTD that was similar to the DHPG-induced LTD in control solution.

The subsequent application of LFS in the presence of D-AP5 generated a further LTD similar to that obtained in control solution when LFS was applied after the DHPG-induced LTD. O'Mara *et al.* (1995b), Wang *et al.* (1996) and Wang *et al.* (1997) have demonstrated that a substantial component of the LFS induced LTD was NMDAR independent.

Another factor to be taken into consideration is the involvement of mGluR group II in the induction of LTD in the medial perforant pathway of the dentate gyrus. Group II mGluRs are abundant in this area of the hippocampus (Shigemoto *et al.*, 1997 and Huang *et al.*, 1997) have shown that they play an important role in the induction of LTD. Therefore the LTD obtained by administration of LFS in the presence of D-AP5 after the generation of the DHPG-induced LTD could be due to the activation of mGluR group II by LFS.

Palmer *et al.* (1997) showed that in order to obtain the DHPG-induced LTD in CA1 they needed to enhance the excitability of the slices either by working in the presence of picrotoxin in order to block the GABA_A-mediated activity, or by using an experimental solution that contained no Mg²⁺.

As described in the Materials and Methods chapter, it was normal protocol to add picrotoxin to all the solutions for all the experiments carried out in the dentate gyrus. Since this altered the excitability of the slices it was decided to carry out experiments to induce LTD with DHPG in the absence of picrotoxin to determine if increased excitability was necessary for the generation of DHPG-induced LTD in the dentate gyrus. From the present studies it was found that the absence of picrotoxin affected neither the DHPG-induced LTD nor the LFS induced LTD. Therefore the DHPG-induced LTD in the medial perforant pathway of the dentate

gyrus differed from the DHPG-induced LTD in CA1 because GABA_A activity did not play a role in the induction mechanisms of this form of long-term synaptic plasticity. Because of the results obtained in the present studies it can be assumed that the inhibitory circuitry in the medial perforant pathway of the dentate gyrus does not play a role in the induction of LTD.

Group I mGluR consists of two receptors: mGluR1 and mGluR5 (for review see Conn and Pin, 1997, Pin and Duvoisin, 1995). Shigemoto *et al.* (1997) have demonstrated that both subtypes of mGluR group I are present in the medial perforant pathway of the dentate gyrus unlike in CA1 where only mGluR5 is present.

In order to determine whether one or both subtypes were responsible for the induction of LTD by activation of mGluR group I, experiments were carried out with more selective compounds. The first compound used was UPF 596. Initially this compound was described as a weak mGluR5 agonist and a very potent mGluR1 antagonist (Pellicciari *et al.*, 1996, Costantino and Pellicciari, 1996). However more recently it was observed that the agonistic properties of UPF 596 on mGluR5 were more potent than previously reported (unpublished observation by Mannaioni, G. and Attucci S.). In the present experiments the application of UPF 596 induced an LTD that was similar to the DHPG-induced LTD, indicating mGluR5 as a possible candidate for the induction of the LTD by activation of mGluR group I. Activation of mGluR5 by the selective agonist CHPG (Doherty *et al.*, 1997) generated an LTD similar to the DHPG-induced LTD, further supporting the results obtained with UPF 596. (Palmer *et al.*, 1997) demonstrated that 1 mM CHPG in CA1 was also able to induce an LTD in Mg²⁺-free conditions, although it was similar to the LTD induced by a lower concentration of DHPG than that used in their experiments.

When experiments were carried out in the presence of the specific mGluR group I antagonist AIDA (Moroni *et al.*, 1997, Chung *et al.*, 1997), the DHPG-induced LTD

was completely blocked. The small transient depression seen in the presence of AIDA could be due to activation of mGluR group II, since (Moroni *et al.*, 1997) have demonstrated that AIDA had weak agonistic properties on mGluR2 transfected cells.

However the same group claimed that AIDA displayed antagonistic activity only on mGluR1a transfected cells. This finding could be in contrast with the hypothesis that the DHPG-induced LTD in the dentate gyrus was due to activation of mGluR5. Nevertheless Oliet *et al.* (1997) have used AIDA at a similar concentration to that used in the present experiments and they blocked completely the mGluR-dependent LTD in CA1, which they demonstrated was due to activation of mGluR group I. Since the only subtype of group I mGluR expressed in CA1 is mGluR5 (Shigemoto *et al.*, 1997, Luján *et al.*, 1996) these experiments are in agreement with the findings in the present study. A possible explanation for this discrepancy in the action of AIDA could be in the fact that the preliminary studies on the potency of this drug by Moroni *et al.* (1997) and Costantino and Pellicciari (1996) were biochemical essays carried out in transfected cells expressing only one receptor at a time, while the present experiments and those reported by Oliet *et al.* (1997) were electrophysiological recordings of long-term synaptic plasticity performed on intact hippocampal slices.

The partial inhibition of LFS-induced LTD in slices perfused with AIDA gave additional evidence for the involvement of the activation of mGluR group I in the induction of LTD in the medial perforant pathway of the dentate gyrus.

In order to determine with higher accuracy whether the activation of mGluR5 was responsible for the induction of the LTD, experiments were carried out using knockout mice lacking the gene for mGluR5. In control mice the perfusion of DHPG induced LTD and subsequent LFS induced an additional small LTD. When the same experiments were carried out in mGluR5 knockout mice no LTD was generated after the perfusion with DHPG or even after administration of LFS. From these studies it can be assumed therefore that mGluR5 are essential for the

generation of the DHPG-induced LTD and play a major role in the induction of LFS induced LTD.

Activation of mGluR group I is coupled to stimulation of phospholipase C (PLC). In the phosphoinositol cascade this enzyme is responsible for the production of inositol triphosphate (IP₃) and diacylglycerol (DAG). This latter molecule activates PKC. As a result of this, activation of mGluR group I is directly linked to activation of PKC. The involvement of PKC activation in the maintenance of LTP in the dentate gyrus had already been demonstrated by Lovinger and Routtenberg (1988). It was therefore of interest to see if the inhibition of PKC could affect the DHPG induced LTD. From the present experiments in the presence of the potent PKC inhibitor bisindolylmaleimide I, it was demonstrated that inhibition of PKC strongly inhibited the DHPG-induced LTD and the additional subsequent LFS-induced LTD. The present evidence for a role of PKC in the induction of LTD supports the conclusions from previous studies in the dentate gyrus (Wang *et al.*, 1998) and CA1 (Oliet *et al.*, 1997) in which the inhibition of PKC by injection of the PKC inhibitory peptide PKC₁₉₋₃₆ in the post synaptic cell blocked LTD induction.

Boxall and Lancaster (1998) and Petryniak *et al.* (1996) have demonstrated that PTK are activated by PKC and elevated intracellular free Ca²⁺. Because of the role of PKC in the induction of DHPG-induced LTD and because the activation of mGluR group I is linked to increase in the intracellular Ca²⁺ concentration, it was thought that PTK could play a role in the generation of the DHPG-induced LTD.

As demonstrated in the present studies, the inhibition of PTK by lavendustin A inhibited both the DHPG-induced LTD and the subsequent LFS-induced LTD.

Previous studies in the cerebellum by Boxall *et al.* (1996) demonstrated that activation of PTK was essential for the induction of LTD in the cerebellum. In the hippocampus it was demonstrated that the inhibition of PTK blocked the induction of LTP in CA1 (O'Dell *et al.*, 1991, Lu *et al.*, 1998). In addition to this it was also

demonstrated that inhibition of PTK prevented the induction of LTD in the dentate gyrus *in vivo* (Abe and Saito, 1993).

The present results provided new evidence for the involvement of PTK in the induction of LTD in the medial perforant pathway of the dentate gyrus.

Since activation of group I mGluRs leads to the production of IP₃ and consequently to the activation of the IP₃-receptor and because PKA can phosphorylate this receptor, modulating its function, the involvement of PKA in the induction of LTD was investigated.

In the present studies the inhibition of PKA by the use of H 89 affected neither the DHPG-induced LTD nor the subsequent additional LFS induced LTD.

Previous studies have demonstrated that genetic mutation of certain isoforms of PKA were able to disrupt LTP of mossy fibres (Huang *et al.*, 1995) and inhibition of PKA blocked LTP at hippocampal mossy fibre synapses (Tzounopoulos *et al.*, 1998).

In addition to this, it has been shown that PKA plays a role in LTP induction in the CA1 area (Roberson and Sweatt, 1996). Another set of investigations has provided evidence for the involvement of PKA in LTD induction in CA1, since the post-synaptic injection of a PKA inhibitor produced a synaptic depression that occluded homosynaptic LTD (Kameyama *et al.*, 1998).

However from the present studies it can be assumed that activation of PKA was not necessary for the induction of LTD by activation of mGluR group I.

In conclusion the experiments here described provide new evidence for the involvement of group I mGluR in the induction of LTD in the dentate gyrus. Furthermore it has been shown that mGluR5 is the most likely candidate for the generation of the DHPG-induced LTD. It has also been demonstrated that activation of PKC and PTK is necessary for the induction of the DHPG-induced LTD.

6 Conclusions

The studies presented in this thesis describe several properties of synaptic plasticity in the medial perforant path input to the rat dentate gyrus *in vitro*. Initial experiments examined the involvement of group III mGluRs in LTP induction in this pathway. It was found that pharmacological manipulation of these mGluRs did not affect this form of synaptic plasticity. Further experiments that form the main focus of this thesis investigated the role played by calcium and the activation of group I and group III mGluRs in the induction of LTD. The findings regarding these latter investigations can be summarized as follows:

- LTD was partially inhibited by the mGluR group III antagonist MAP4.
- LTD could be induced by altering the extracellular Ca^{2+} concentration (Ca^{2+} -induced LTD).
- LTD could be induced by activation of mGluR group I with DHPG (DHPG-induced LTD).

The studies on the role played by mGluR group III in synaptic plasticity in the dentate gyrus *in vitro* showed for the first time that LTP was not affected by activity of mGluR group III while LTD was. The investigations on the Ca^{2+} -induced LTD and the DHPG-induced LTD provided evidence for novel forms of LTD expressed in the dentate gyrus *in vitro*. These new forms of LTD were induced using protocols other than LFS or weak intracellular depolarisation paired with a brief LFS. They also shared common induction and/or maintenance mechanisms with LFS-induced LTD. In this way, the studies of the properties of the Ca^{2+} -induced LTD and of the DHPG-induced LTD provided additional information regarding the properties of LTD in the dentate gyrus *in vitro*.

Although the studies reported here were quite detailed, further experiments will provide a more precise understanding of LTP and LTD. Future studies into the role played by mGluR group III in the induction of LTD and LTP will include:

1. The use of a larger number of agonists and antagonists for mGluR group III. This will reveal whether specific members of group III mGluRs are responsible for the observed effects on LTD.
2. Experiments using transgenic animals that lack the genes for specific group III mGluRs will provide a powerful tool for analysing the role of these receptors in LTD.
3. Further investigations into the nature of Ca^{2+} -induced LTD will be carried out using intracellular recordings from the postsynaptic cell. These experiments will include patch-clamp recordings in whole cell configuration or intracellular recordings using sharp electrodes. Substances that affect the release of Ca^{2+} from intracellular stores will be applied to the cell, for example, ryanodine (Meissner, 1986), heparin (Worley *et al.*, 1987), xestospongine C (Narasimhan *et al.*, 1998) and ruthenium red (Vale and Carvalho, 1973, Luthra *et al.*, 1977). This will provide more accurate information on the location (presynaptic and/or postsynaptic) and intracellular mechanisms underlying this form of LTD.
4. Experiments will be carried out using Ca^{2+} -imaging techniques to monitor Ca^{2+} movements during the induction of Ca^{2+} -induced LTD. The possible involvement of intracellular enzymes that are activated by Ca^{2+} , for example, PKC, CaMKII, and PTK, can then be investigated. Since it has been demonstrated that group I mGluRs can function as Ca^{2+} -sensing receptors (Kubo *et al.*, 1998), additional experiments will be carried out using specific antagonists for this group of mGluRs in order to determine whether they play a role in the induction of Ca^{2+} -induced LTD.
5. Activation of mGluR group I leads to the release of Ca^{2+} from the intracellular stores. Further experiments into DHPG-induced LTD will be carried out to investigate the role of intracellular Ca^{2+} stores and the possible involvement of CaMKII in the induction of this LTD. Since it has been demonstrated that DHPG-induced LTD was strongly reduced by inhibitors of PKC and PTK, the involvement of MAP kinases that are activated by PKC and PTK in DHPG-induced LTD can be tested. In addition to this, further experiments using mice

lacking mGluR 1 will characterise more fully the subtype of mGluR group I responsible for this LTD.

It is important to note that the present studies were carried out in the dentate gyrus *in vitro* and, therefore, the novel forms and properties of the LTD investigated here apply only for this preparation. Although, as explained in the introduction, LTD can be seen in the intact animal (Bramham and Srebro, 1987), it is important to understand that the *in vitro* experiments were carried out on brain slices. This preparation of brain tissue allowed easy access to single neurones or small groups of neurones and most of the properties of the cells in the specific area analysed remain intact. However, the majority of connections both within the hippocampus and between the hippocampus and other areas of the brain were disrupted in the slice. The *in vitro* technique offers the advantage of studying in very specific and detailed manner the behaviour and properties of a neurone or a small population of neurones in their environment. In this view, the studies reported here are relevant for a better understanding of the behaviour and the properties of dentate granule neurones. It would be difficult to directly link the *in vitro* studies on synaptic plasticity presented in this thesis with learning and behaviour. Nonetheless the results reported here provide important information on neurones in a specific brain area, the dentate gyrus of the hippocampus. This, in conjunction with results from *in vivo* and behavioural experiments, can help the understanding of how the electrical activity of a small group of neurones is involved in animal behaviour.

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