An Investigation of the Processes Underlying Late-phase Long-term Potentiation

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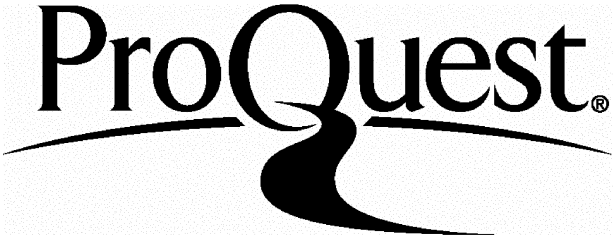

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ABSTRACT

In this thesis I have investigated the role of RNA and protein synthesis, during the late-phase of hippocampal long-term potentiation (L-LTP). Additionally, the involvement of protein kinase C (PKC) in the induction of this late phase was addressed.

Using protocols that allowed hippocampal slices to be maintained for long periods, L-LTP lasting 8 hours or more was successfully achieved. Field EPSPs were recorded from pyramidal cells in the CA1 region of the stratum radiatum. Tetanically induced L-LTP was blocked by bath application of inhibitors of transcription (actinomycin-D) and translation (emetine), whilst L-LTP in slice preparations lacking presynaptic cells bodies was unaffected. In addition, bath application of bisindolylmaleimide I, a highly selective inhibitor of PKC, was found to block both E-LTP and L-LTP induction if applied within the first 15 minutes, after the tetanus. These results are consistent with a requirement for both protein synthesis and postsynaptic RNA synthesis during L-LTP induction, coupled with a requirement for a critical period of PKC activity.

The locus of protein synthesis during L-LTP in CA1 pyramidal cells was investigated by focally applying emetine to apical dendrites, while stimulating afferents to basal and apical dendrites. This significantly reduced L-LTP in the projection to apical dendrites, whilst leaving L-LTP unaffected in the projection to basal dendrites. Focal application of emetine to the cell bodies had no significant effect on L-LTP in either the apical or basal dendrites. Furthermore, preliminary data suggest that L-LTP in the basal dendrites is unaffected by focal emetine application. These experiments provide evidence that pyramidal cells in the CA1 region of the hippocampus can support two different forms of L-LTP: type I, in the apical dendrites (stratum radiatum) that depends on protein synthesis, and a component of which relies on local dendritic protein synthesis and type II, in the basal dendrites (stratum oriens), that remains protein synthesis-independent for at least 8 hours.

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LIST OF ABBREVIATIONS

3'-UTR:	3'-untranslated region
5-HT:	Serotonin
AA:	Arachidonic acid
AC:	Adenylate cyclase
ACSF:	Artificial cerebro-spinal fluid
Act-D:	Actinomycin-D
AMPAR:	α-amino-3-hydroxy-5-methyl-4-isoxaline propionic acid receptor
AP3:	2-amino-3-phosphonopropionate
APP:	Amyloid precursor protein
AP5:	D-2-amino-5-phosphonovalerate
Arc:	Activity regulated cytoskeletal-associated protein (also termed Arg3.1)
ATP:	Adenosine 5'-triphosphate
BDM I:	Bisindolylmaleimide I .
BDNF:	Brain derived neurotrophic factor
BLA:	Basolateral amygdaliod complex
CaM:	Calmodulin
cAMP:	Cyclic adenosine monophosphate
CaMKII:	Calcium/calmodulin-dependent protein kinase II
CEA:	Central nucleus of the amygdala
cGMP:	Cyclic guanosine monophosphate
CNQX:	6-cyano-7-nitroquinoxaline-2,3-dione
CNS:	Central nervous system
CO:	Carbon monoxide
CPE:	Cytoplasmic polyadenylation element
CPEB:	Cytoplasmic polyadenylation element binding protein
CRE:	cAMP responsive element
CREB:	cAMP-response element-binding protein
CS:	Conditioned stimulus
CV:	Coefficient of variation
DG:	Dentate gyrus
DHPG:	(*RS*)-3,5-dihydroxyphenylglycine

DNA:	Deoxyribonucleic acid
DRB:	5,6-dichloro-1-β-D-ribofuranosyl benzimidazole
eEF2:	Eukaryotic elongation factor 2
EGTA:	Ethyleneglycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
eIF2β:	Eukaryotic initiation factor 2β
E-LTP:	Early-phase long-term potentiation
EPSC:	Excitatory postsynaptic current
EPSCaT:	Evoked postsynaptic Ca^{2+} transients
EPSP:	Excitatory postsynaptic potential
ER:	Endoplasmic reticulum
ERK:	Extracellular signal-regulated kinase
FMRP:	Fragile X mental retardation protein
GA:	Golgi apparatus
GABA:	γ-amino-n-butyric acid
GEF:	Guanine-nucleotide-exchange factor
GFP:	Green fluorescent protein
G-protein:	GTP binding protein
gsk-3β:	Glycogen synthase kinase 3β
GTP:	Guanine trisphosphate
HEK-293:	Human embryonic kidney-293
HF:	Hippocampal formation
HFS:	High frequency stimulation
hnRNP:	Heterogenous nuclear ribonucleoproteins
HO:	Haem oxygenase
I1:	Inhibitor-1 protein
IEG:	Immediate early gene
IP_3:	Inositol 1,4,5 trisphosphate
ITI:	Inter-train interval
LFS:	Low-frequency stimulation
LTD:	Long-term depression
LTF:	Long-term facilitation
LTM:	Long-term memory
LTP:	Long-term potentiation

L-LTP:	Late-phase long-term potentiation
MAP2:	Microtubule-associated protein 2
MAPK:	Mitogen-activated protein kinase
MEK:	Mitogen-activated protein kinase kinase
mEPSC:	Miniature excitatory postsynaptic current
mGluR:	Metabotropic glutamate receptor
MGN:	Medial geniculate nucleus
MLC:	Myosin light chain kinase
MPP:	Medial perforant path
MRI:	Magnetic resonance imaging
mRNA:	Messenger ribonucleic acid
MTL:	Medial temporal lobe
NMDAR:	N-methyl-D-aspartate receptor
NO:	Nitric oxide
NOS:	Nitric oxide synthase
NT-3:	Neurotrophin-3
PAF:	Platelet-activating factor
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PDZ:	PSD95, Drosophila tumour suppresser dlgA, ZO-1
PET:	Positron emission tomography
Pim-1:	Provirus integration site for moloney murine leukemia virus
PKA:	cAMP dependent protein kinase
PKC:	Phospholipid/calcium dependent protein kinase (Protein kinase C)
PKG:	cGMP-dependent protein kinase
PP1:	Protein phosphatase-1
PPF:	Paired-pulse facilitation
PSD:	Postsynaptic density
PTP:	Post-tetanic potentiation
Ras-GRF:	Ras-guanine nucleotide releasing factor
Raf:	Replication defective, acute transforming retrovirus
rCBF:	Regional cerebral blood flow
RNA:	Ribonucleic acid

rRNA:	Ribosomal ribonucleic acid
RTK:	Receptor tyrosine kinase
Src:	Sarcoma virus transforming protein
STG:	Stomatogastric ganglion
STM:	Short-term memory
STP:	Short-term potentiation
tPA:	Tissue-plasminogen activator
TrkB:	BDNF receptor (an RTK)
tRNA:	Transfer ribonucleic acid
US:	Unconditioned stimulus
VSCC:	Voltage sensitive calcium channels
ZBP:	Zipcode binding protein

INTRODUCTION

1.1 LEARNING AND MEMORY

Memory is the ability of organisms to retain and use information or knowledge that has been acquired. The acquisition of memory always involves learning.

1.1.1 Short-term and Long-term Memory

One of the most widely accepted ideas concerning memory is that two forms of memory storage exist: short-term memory (STM) and long-term memory (LTM). STM has a time course measured in minutes and can be distinguished from LTM, whose time course is hours, days or years. This distinction has been demonstrated by observation of amnesic patients that have intact STM, despite severely impaired LTM (Milner, 1971). Amnesic patients with damage to the hippocampus have been found to perform entirely normally in a number of different STM tests, suggesting that STM is independent of LTM and independent of the structures and connections damaged in amnesia (Cave and Squire, 1992). Information is thought to initially enter short-term memory and subsequently become incorporated into a more stable long-term memory.

1.1.2 Multiple Forms of Long-term Memory

One idea that has become the subject of widespread interest since the early 1980's is that long-term memory is not a single entity but is composed of several different components, which are mediated by separate brain systems. In 1980, Cohen and Squire performed a series of experiments on amnesic patients showing that they could learn the task of mirror reading (i.e. reading the mirror images of print) at a rate equivalent to that of normal patients, and that this ability could be maintained for at least 3 months. This provided evidence for an idea long favoured by the cognitive psychologists that the brain supported two different memory systems. Cohen and

Squire termed these 2 types *declarative* (or explicit or relational) memory and *non-declarative* (or procedural or implicit) memory. Declarative memory is the ability to consciously recall facts and events. Declarative memory includes the memory for faces and spatial layouts. It relies on the medial temporal lobe, which includes the hippocampus. Non-declarative memory underlies changes in skilled behaviour and the ability to respond to stimuli through practice. Many forms of learning fall into this non-declarative group such as habituation, sensitisation and classical conditioning. (reviewed by Squire and Knowlton, 1994).

1.1.3 Hippocampal Involvement in Learning and Memory

The ability to form and retain new long-term declarative memories requires the medial temporal lobe. This comprises the hippocampus, entorhinal, perirhinal and parahippocampal cortices. The evidence for the medial temporal lobe's importance comes from a number of different observations; the most famous of which is the case of an American male known as HM. In 1953, in an attempt to cure his severe epilepsy he underwent bilateral resection of the medial temporal lobe, including the hippocampus (Scoville and Milner, 1957). Whilst improving his epilepsy, the operation left him with severe memory impairments, the most dramatic of which was the inability to form new memories. He had, however, retained the ability to learn new physical tasks and improve at them without being aware he had ever performed them. Another case exhibiting deficits similar to H.M, but less extreme, was that of R.B who, following an ischemic infarc during surgery incurred a bilateral lesion to the CA1 region of the hippocampus (Zola-Morgan *et al.*, 1986). The resulting memory impairment revealed that even partial damage to the hippocampus could give rise to severe memory defects. That H.M's impairments were greater than R.B's presumably reflects the importance of brain regions adjacent to the hippocampus, as well as the hippocampus itself in memory function. Lesions similar to those experienced by H.M and R.B have also been shown to impair declarative memory function in monkeys, rats and rabbits (reviewed by Squire, 1992; Nadel and Moscovitch, 1997).

Further support for the role of the hippocampus in declarative memory formation comes from magnetic resonance imaging (MRI). Patients with severe medial temporal

lobe amnesia displayed markedly shrunken and atrophic (57% of normal size) hippocampal regions, whilst the remainder of the temporal lobe appeared normal (Squire *et al.*, 1990). Other imaging methods such as Positron Emission Tomography (PET) have been used to assess the involvement of different brain areas in certain behavioural tasks. Maguire *et al.* (1997) used PET to image the brains of London taxi drivers as they recalled information about routes around the city. They observed greater activation of the right hippocampus during recall of route information than during the recall of famous landmarks, movie plot lines or movie scenes. This suggested a strong role for the hippocampus in spatial information processing. However, there have been numerous PET studies with differing suggestions as to the nature of the role of the hippocampus and the medial temporal lobe (MTL) in human memory. These range from the hippocampus playing a role in memory of past events (Mattioli *et al.*, 1996), through to it being involved in the detection and assessment of novelty of incoming information (Tulving *et al.*, 1994). In a recent review, Tulving *et al.* (1999) warn that these findings need to be interpreted cautiously and that findings from individual studies should not be over interpreted.

1.1.4 Cellular Models of Learning and Memory

Almost a century has passed since the publication of Ramon y Cajal's description of the nervous networks within the brain. These led Sherrington (1906) to state that, neurons communicate via a 'surface of separation', which he termed the 'synapse'. This led to the idea that the brain used long lasting modifications in synaptic strength in order to retain information. In 1949, Donald Hebb in his book, *The Organization of Behavior*, developed this idea and formulated a 'coincidence detection rule'. This stated that when an axon of cell A excites cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's efficacy as one of the cells firing B is increased.

In 1973 Bliss and Lømo, demonstrated that the synapses within the hippocampus were able to undergo increases in efficacy. Whilst stimulating the perforant path of the hippocampus in anaesthetised rabbits, they found that short high-frequency trains of stimuli could augment the response of the postsynaptic cell populations of that

pathway. This increase in synaptic efficacy is called long-term potentiation (LTP). In the last 20 years LTP has become the dominant model of activity-dependant synaptic plasticity in the mammalian brain (Bliss and Collingridge, 1993) and will be discussed in greater detail in the following sections.

1.2 LTP: A NEURAL CORRELATE OF LEARNING AND MEMORY?

Even though LTP seems to have a number of properties that make it attractive as a candidate memory mechanism (see later sections), it still remains unclear whether it is involved in the storage of declarative memories (e.g. spatial memory), in the hippocampus.

1.2.1 LTP and Spatial Memory

The first evidence that LTP may play a role in spatial learning was provided by Morris *et al.* (1986) who found that rats that had been given the drug AP5, an NMDA receptor antagonist (and selective blocker of LTP) showed impaired spatial memory in the water-maze. However, more recently, it has been shown that rats can perform spatial learning tasks following AP5 infusion if they had already received training in the task (Bannerman *et al.*, 1995). Similar results have been seen with rats injected with NPC17742, a potent NMDA receptor antagonist (Saucier and Cain, 1995). Thus spatial learning may be NMDA independent, but there may be some non-spatial components of the water-maze task (i.e. learning that the platform is a refuge) which are NMDA dependent (Morris and Frey, 1997).

Moser *et al.* (1998) used a different approach to examine the involvement of LTP in spatial memory. They attempted to saturate LTP in the hippocampus using a multielectrode stimulating array and then tested to see if spatial learning was disrupted. Spatial learning was found to be impaired in rats that had <10% residual LTP. Interestingly, rats that had been pre-trained in the water-maze and then saturated with LTP, exhibited no spatial impairments when trained in a second water-maze (Otnaess *et al.*, 1999). These results are consistent with those seen using NMDA

receptor antagonists and call into question the role of LTP in this type of spatial learning.

Within the last 10 years, much of the data suggesting a role for LTP in spatial memory has come from studies using transgenic animals. Mutant mice that do not express α-calcium calmodulin-dependent kinase II (αCaMKII) were found to be deficient in their ability to produce LTP in the Schaffer collateral CA1 pathway of the hippocampus (Silva *et al.*, 1992b). These mice were also found to exhibit specific spatial learning impairments (Silva *et al.*, 1992a). Similar spatial memory deficits have been seen in *fyn* (a non-receptor tyrosine kinase gene) mutant mice (Grant *et al.*, 1992). However, experiments of this type are open to criticism. The manipulations are not specific to particular pathways, but occur throughout the brain. Also, the deficits are present throughout the development of the animal and so abnormalities in the connectivity of the neurons cannot be ruled out. Later studies have attempted to address these problems. Tsien *et al.* (1996a) produced a mouse strain that lacked expression of the NMDAR1 gene, only in the CA1 pyramidal cells of the hippocampus. They found that this produced a specific disruption in LTP in the Schaffer collateral/CA1 pathway whilst having no effect on LTP in the dentate gyrus (Tsien *et al.*, 1996b). The specific LTP disruption in the CA1 region was accompanied by a marked impairment of the spatial memory of these mice when tested in the Morris water maze (Tsien *et al.*, 1996b). In addition, Mayford *et al.* (1996a) eliminated the problems of developmental consequences of knocking out specific genes. They produced a genetically modified mouse in which the expression of the transgene of interest (in this case a calcium-independent form of CaMKII) could be turned on and off. Expression of the activated CaMKII resulted in a loss of LTP in response to low frequency stimulation (10Hz), accompanied by a deficit in spatial memory. These effects were reversed when the transgene expression was suppressed.

In certain cases however, genetic abolition of LTP is not always associated with an impairment in spatial memory. Mice lacking the AMPA receptor subunit GluR-A showed no LTP in the CA1 region *in vitro*; however, spatial learning in the water-maze was not affected (Zamanillo *et al.*, 1999). A similar disparity between

LTP and spatial learning has also been seen in mutant mice lacking the neuronal glycoprotein Thy-1. LTP, recorded *in vivo*, from anaesthetised Thy-1 deficient mice, although normal in the CA1 region, was found to be absent in the dentate gyrus (Nosten-Bertrand *et al.*, 1996). Spatial learning of these Thy-1 mice in the Morris water-maze was however, unaffected (Nosten-Bertrand *et al.*, 1996). Taken together these results suggest that LTP in either the CA1 region or the dentate gyrus may not be required for spatial learning. It is important to note however that the electrophysiology and water-maze training were not performed in the same animals in these experiments, a situation which makes it difficult to directly establish a meaningful correlation between LTP and behaviour (Errington *et al.*, 1997).

Finally, spatial exploration of rats in a novel environment has been found to reverse the potentiation produced by high-frequency stimulation in the CA1 region of the hippocampus (Xu *et al.*, 1998). This strongly suggests a link between LTP and spatial exploration in these animals. However, due to conflicting results, obtained using a number of different techniques, it seems that we are still a long way from a definitive answer surrounding the relationship between LTP and spatial memory.

1.2.2 LTP and Place Cells

In 1971 O'Keefe and Dostrovsky showed that individual hippocampal pyramidal cells could fire in response to a rat entering highly restricted regions within its environment. Each cell has its own region of elevated firing, termed a 'place field' (or firing field). It is thought that spatial location is encoded by the pattern of firing of these individual cells. Place fields are extremely stable for a given environment (up to 6 months) changing only when the rat is placed in a new environment or the old environment is changed dramatically so that it appears novel (reviewed by Muller, 1996). It is thought that the hippocampus forms a cognitive map of the environment and that this map is made up of place fields. Is LTP involved in the formation or maintenance of place fields?

This question was addressed by McHugh *et al.* (1996) and Rotenberg *et al.* (1996) using mutant mice that lacked expression of the NMDAR1 gene (Tsien *et al.*, 1996b),

or mice that expressed a calcium-independent form of CaMKII (Mayford *et al.*, 1996a). Despite previous reports of LTP and spatial memory deficits occurring in these mice, (Mayford *et al.*, 1996a; Tsien *et al.*, 1996b), both genotypes displayed place fields, although their characteristics had changed (McHugh *et al.*, 1996; Rotenberg *et al.*, 1996). NMDAR1 mutant mice had lost correlations between overlapping place cells that were present in normal mice (McHugh *et al.*, 1996), whilst the CaMKII mice showed a decrease in total firing (Rotenberg *et al.*, 1996). Furthermore, the place fields from CaMKII mice were found to show significant instability, whilst those in the NMDAR1 mice appeared normal (McHugh *et al.*, 1996; Rotenberg *et al.*, 1996). Similarly αCaMKII (lacking the ability to autophosphorylate) and CREB (cAMP-response-element-binding protein; see later sections) mutant mice have impaired LTP and spatial learning (Bourtchuladze *et al.*, 1994; Kogan *et al.*, 1997; Giese *et al.*, 1998) and show a decrease in place cell spatial selectivity and stability (Cho *et al.*, 1998). More recently, pharmacological blockade of NMDA receptors has shown that, although they were essential for the long-term stabilisation of place fields, they were not involved in formation or short-term stability of these fields (Kentros *et al.*, 1998). LTP may therefore have a role, not in the formation of place cells but in their fine-tuning and stability over time.

1.2.3 LTP and Fear Conditioning in the Amygdala

LTP is not thought to be exclusively involved in the storage of declarative memories. It has also been implicated in emotionally charged non-declarative memories such as Pavlovian fear conditioning. Fear conditioning is the ability of the animal to associate an innocuous stimulus such as a loud tone (the conditioned stimulus or CS), with an aversive stimulus such as a foot shock (the unconditioned stimulus or US), when they are presented so that the tone (CS) precedes the foot shock (US). After a few trials the animals exhibit a fear response (i.e. freezing, elevated blood pressure) in response to the tone alone. It is thought that the amygdala is involved in this type of emotional memory. The amygdala consists of two separate but linked subsystems: the basolateral amygdaliod complex or BLA (consisting of the lateral, basolateral and basomedial nuclei), and the central nucleus of the amygdala (CEA). The BLA responds to stimuli from auditory, visual, somatic, spatial and olfactory regions within

the brain. This information is then integrated, before being sent to the CEA, which in turn sends projections to brain areas involved in fear responses (reviewed by Swanson and Petrovich, 1998).

Tetanic stimulation of axonal projections in the hippocampal formation (HF) of anaesthetised rats has been shown to induce LTP in the basolateral amygdala (Maren and Fanselow, 1995). LTP induction in the BLA is dependent on NMDA receptor activation (Maren & Fanselow, 1995). Moreover, infusion of the NMDA receptor antagonist AP5, into the BLA in rats, prevents new fear learning such as freezing (Lee and Kim, 1998) and fear-potentiated startle (Miserendino *et al.*, 1990). These results suggest that LTP may underlie the formation of conditioned fear responses in behaving rats. This question was further explored by Rogan *et al.* (1997), who recorded from the BLA of freely moving rats. The authors found that animals that underwent fear conditioning, exhibited persistent increases in auditory-evoked field potentials, similar to increases seen during LTP induction. These increases were enduring, reflected the acquisition of CS–elicted fear behaviour and were absent in rats that had not undergone pairing of the CS and US. Around the same time McKernan *et al.* (1997) found that fear conditioning in rats was accompanied by increases in AMPA receptor mediated transmission in the medial geniculate nucleus (MGN)-lateral nucleus pathway. These increases were measured *in vitro*, via whole cell recordings from brain slices prepared from rats that had undergone this training. Taken together the results from Rogan *et al.* (1997) and McKernan *et al.* (1997) suggest that fear conditioning induces LTP in the amygdala, and provide some of the best evidence to date, linking LTP to learning and memory.

A number of mutant mice studies have provided indirect data to link LTP and fear conditioning in the amygdala. Mice over expressing a calcium-independent form of CaMKII are not only impaired in the ability to support hippocampal LTP, but are also deficient in both conditioned and cued fear conditioning (Mayford *et al.*, 1996a). Similarly, mutant mice with targeted disruption of the α and δ isoforms of CREB, do not support long lasting LTP in the hippocampus and are dramatically compromised in their ability to retain cued and contextual fear conditioning (Bourtchuladze *et al.*,

1994). Interestingly, CREB activated gene expression is elevated in the basolateral amygdala of fear conditioned mice (Impey *et al.*, 1998b).

More-direct genetic evidence for the involvement of amygdala LTP in fear conditioning was provided by Brambilla *et al.* (1997), studying mutant mice lacking the neuronal-specific guanine-nucleotide-exchange factor (Ras-GRF), a component of the mitogen activated protein kinase (MAPK) pathway. These mice showed a rapidly decaying LTP in the amygdala (recorded in brain slices), coupled with a reduction in contextual and cued fear conditioning after 24 hours. This disruption was specific to the amygdala; hippocampal LTP and spatial memory in these mice remained unaffected, suggesting different signal transduction pathways for LTP in these two brain structures. Inhibition of the MAPK pathway has also been shown to block fear conditioning in rats (Atkins *et al.*, 1998). It therefore appears that in addition to its proposed role in the establishment of declarative memories in the hippocampus, LTP may also be involved in the storage of non-declarative, emotional memories occurring in the amygdala.

1.3 INDUCTION OF LTP

1.3.1 Properties of LTP

There are a number of properties of LTP that make it an attractive candidate as a cellular memory storage mechanism. LTP can be induced rapidly via brief stimulation (usually lasting tens of milliseconds at a frequency of around 100-400Hz)(Brown *et al.*, 1988). Once induced, LTP is persistent, outlasting other forms of synaptic enhancement such as post-tetanic potentiation. LTP can last for hours in the anaesthetised animal and in slice preparations (Bliss & Lomo, 1973; Schwartzkroin and Wester, 1975; Alger and Teyler, 1976) and up to days or even weeks in awake freely moving animals (Bliss and Gardner-Medwin, 1973). LTP has 3 important cellular properties, cooperativity, associativity and input specificity. These characteristics are defined as follows. Cooperativity - multiple inputs are required to reach a threshold for LTP induction that cannot be reached by a single input (McNaughton *et al.*, 1978). Associativity - a weak input can only be potentiated if its

activity is paired with that of a strong input (McNaughton *et al.*, 1978; Levy and Steward, 1979). Input specificity - only those inputs that are activated at the time of the tetanus show potentiation (Andersen *et al.*, 1977; Lynch *et al.*, 1977). It should be noted that observations in which LTP can be induced in a single cell in response to pairing of postsynaptic depolarisation with presynaptic stimulation (see following paragraph), has called the role of cooperativity during LTP induction into question. Indeed, cooperativity as characterised by McNaughton *et al.* (1978), in which LTP induction in the dentate gyrus required a threshold stimulus intensity during high frequency stimulation (corresponding to the coactivity of a considerable number of afferent fibres), can also be explained in the context of associativity. For example, increasing stimulus strength and thus increasing afferent fibre recruitment could lead to LTP induction via associative interactions with convergent projections from different brain structures that may use different transmitter systems. Recently, reinforcement of a protein synthesis independent 'early' LTP into a protein synthesis dependent 'late' LTP (see later sections) occurring in the dentate gyrus of freely moving rats, was shown to involve a heterosynaptic associative interaction with the basolateral amygdala via the muscarinergic and β-adrenergic receptors (Frey *et al.*, 2001). Therefore the mechanisms underlying cooperativity and associativity may be similar and it may be more appropriate to refer to cooperativity only within its historical context.

These three properties suggest that LTP occurs at a specific synapse, only if activity coincides with sufficient depolarisation in the postsynaptic cell. In 1986 this hypothesis was tested directly in the hippocampus by a number of groups (Kelso *et al.*, 1986; Malinow and Miller, 1986; Sastry *et al.*, 1986; Wigstrom *et al.*, 1986); each reached similar conclusions. For example, Kelso *et al.* (1986) found that applying postsynaptic depolarisation alone (using a current step to force action potential firing), or presynaptic stimulation whilst the postsynaptic cell soma is voltaged clamped, did not produce LTP. However, if presynaptic stimulation was then paired with postsynaptic depolarisation, LTP was induced. More recently, studies have show that this postsynaptic depolarisation may take the form of backpropagating action potentials. Studying pyramidal cells from the neocortex of rats, Markram *et al.* (1997) showed that EPSP amplitude was increased when a postsynaptic neuron was

depolarised to produce a burst of action potentials (APs) that coincided with a high frequency train of EPSPs. An increase in EPSP amplitude was not seen however, following pairing of high frequency EPSPs with a postsynaptic depolarisation sub-threshold for AP initiation, or when APs and EPSPs did not coincide within a specific time window (<100ms) (Markram *et al.*, 1997). Similar results have also been seen in CA1 neurons of the hippocampus (Magee and Johnston, 1997). Taken together, these findings show the spatiotemporal characteristics of LTP as being similar to those postulated by Donald Hebb (1949) in his model of a synaptic learning mechanism.

1.3.2 Calcium and the NMDA Receptor

In 1983, Lynch *et al.* found that injection of the postsynaptic cell with the Ca^{2+} chelator EGTA, blocked the induction of LTP. This result suggested that LTP is dependent on postsynaptic modifications, which are at least in part brought about by an increase in postsynaptic Ca^{2+} levels. However, Gustafsson *et al.* (1987) found that depolarising the postsynaptic cell for 2 minutes at –40 mV, thereby inducing continuous Ca^{2+} spiking due to Ca^{2+} entry via dendritic voltage dependent Ca^{2+} channels (the obvious candidate for this Ca^{2+} influx), failed to modify synaptic transmission. Therefore the rise in postsynaptic Ca^{2+} was thought to come from some other source. The favoured candidate was an ionotropic glutamate receptor called the N-methyl-D-aspartate receptor (NMDAR). Not only is this receptor permeable to Ca^{2+} (MacDermott *et al.*, 1986; Ascher and Nowak, 1988); addition of the NMDAR antagonist 2-amino-5-phosphonovalerate (AP5) blocks LTP at the Schaffer collateral/CA1 synapses of the hippocampus (Collingridge *et al.*, 1983). The NMDAR has specific characteristics related to its role in LTP induction. The most important of these is that the NMDA receptor channel has a voltage dependent Mg^{2+} block (Nowak *et al.*, 1984; Ascher & Nowak, 1988). Under resting membrane conditions the channel pore is blocked by Mg^{2+}. Only when the membrane becomes depolarised is this block relieved, and ion flux proceeds.

During basal synaptic transmission (at the Schaffer collateral/CA1 synapse), glutamate is released from the presynaptic terminal and binds to both NMDA and

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxalinepropionate) receptors localised on the dendritic spines of the postsynaptic cell. However, basal activation does not provide sufficient depolarisation to adequately reduce the Mg^{2+} channel block of the NMDA receptor. At the resting membrane potential (–70mV) the current generating the synaptic response flows via the AMPA receptor channel (Collingridge *et al.*, 1983) and can be blocked pharmacologically by addition of the AMPAR antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) (Andreasen *et al.*, 1989). It is worth noting that during low frequency transmission a small proportion of NMDA receptors do see a relief of the Mg^{2+} block. However, due to the slow activation kinetics of these receptors (Lester *et al.*, 1990), by the time a significant amount of channels are open, the membrane is already in a hyperpolarised state (due to concurrent inhibition from GABAergic interneurons), which serves only to increase the Mg^{2+} block on these receptors (Collingridge *et al.*, 1988b). As a result, NMDAR contribution to the synaptic response is small and has no effect on the efficiency of synaptic transmission (Bliss & Collingridge, 1993).

Under conditions, where the postsynaptic membrane becomes depolarised, such as repetitive synaptic activation, the Mg^{2+} block is relieved, allowing an influx of Ca^{2+} and Na^{+} through the NMDA receptors. In this situation, depression of the GABA mediated inhibition occurs via $GABA_B$ autoreceptors (Davies *et al.*, 1991), which serve to increase the depolarisation (via summation of NMDA receptor-mediated synaptic components) to a level sufficient to induce LTP. The NMDA receptor therefore acts as a molecular coincidence detector that permits Ca^{2+} influx only when afferent activity occurs in conjunction with depolarisation in the target dendrite. Importantly, this quality of the NMDA receptor provides an explanation of the cooperativity, associativity and selectivity, characteristics of LTP.

Although LTP is dependent on the NMDAR at some synapses, others have shown LTP without NMDAR involvement. Harris and Cotman (1986) found that AP5 did not block LTP at guinea pig mossy fiber/CA3 synapses. Also, under certain conditions in the CA1 region involving high frequency stimulation (200Hz), a non-NMDAR dependent LTP can be induced, that is suppressed by nifedipine, a blocker of L-type voltage dependent calcium channels (Grover and Teyler, 1990).

Other results implicate metabotropic glutamate receptors (mGluRs) in LTP. The mGluR inhibitor MCPG is found to block induction of both NMDAR-dependent LTP in the CA1 region, as well as NMDAR-independent LTP at mossy fibre/CA3 synapses (Bashir *et al.*, 1993).

More recently, imaging studies of calcium transients in single dendritic spines of hippocampal pyramidal cells have shown unequivocally that NMDARs are activated during basal transmission (Yuste and Denk, 1995; Emptage *et al.*, 1999). Furthermore, Emptage *et al.* (1999) demonstrated that during basal synaptic transmission the NMDA receptor evokes substantial calcium release from internal stores without inducing LTP. These results differ from those that suggest the NMDARs are predominately blocked during basal transmission (Collingridge *et al.*, 1988a).

In light of such findings, are there other coincidence detection mechanisms present in these cells that can bring about the induction of LTP? Yuste and Denk (1995) provide some evidence for this hypothesis. They report a supralinear rise in Ca^{2+} in the postsynaptic cell when an action potential is paired with synaptic stimulation. Indeed, LTP can be induced by a pairing protocol that generates a supralinear calcium signal (Magee & Johnston, 1997). Following this discovery, Nakamura *et al.* (1999) showed (in the presence of ionotropic receptor agonists) that this Ca^{2+} rise resulted from mGluR activation, and was mediated by IP_3 dependent Ca^{2+} release. They also found that pairing individual action potentials with presynaptic activity produced significant calcium release from intracellular stores. Whether or not the IP_3 receptor acts as a coincidence detector of Ca^{2+} (via back propagating action potentials) and IP_3 (via the mGluR) to induce LTP has yet to be shown.

1.4 EXPRESSION OF LTP

1.4.1 Signal Transduction Pathways Involved in E-LTP

Although the importance of the activation of the NMDA receptor and its subsequent Ca^{2+} influx has been demonstrated and is widely accepted, the downstream signalling

cascades triggered by this Ca^{2+} influx and the subsequent expression mechanisms of LTP are poorly understood. A feature of LTP expression that has added to its complexity is the demonstration that LTP consists of a number of mechanistically distinct temporal components. These can be divided as follows: short-term potentiation (STP) lasting for 1-3 hours, early-LTP (E-LTP) lasting 3-6 hours, and late-LTP (L-LTP) lasting at least 8 hours. E-LTP is dependent predominately on short-term kinase activity whilst L-LTP exhibits a requirement for protein synthesis. The mechanisms responsible for STP however, remain unclear.

The most striking feature of STP is that it appears to occur independently of protein kinase activity. In the CA1 region of hippocampal slices, application of a number of broad range protein kinase inhibitors during tetanisation, results in a decremental LTP that lasts between 1-3 hours and is insensitive to protein kinase inhibition (Lovinger *et al.*, 1987; Malinow *et al.*, 1988; Reymann *et al.*, 1988a; Malinow *et al.*, 1989; Colley *et al.*, 1990; Denny *et al.*, 1990; Muller *et al.*, 1990; Reymann *et al.*, 1990; Matthies *et al.*, 1991; Wang and Feng, 1992)(see chapter 7 for more detail). Although the mechanisms responsible for the expression of STP have been difficult to elucidate, a candidate molecule involved in STP may have been identified. This molecule is nitric oxide synthase (NOS)(see section 1.4.5). The NOS synthase inhibitor *N*-nitro-L-arginine has been shown to block all stages of LTP, including STP, when applied during the tetanus (O'Dell *et al.*, 1991; Schuman and Madison, 1991; Haley *et al.*, 1993; Williams *et al.*, 1993). Furthermore, STP is also blocked during inhibition of either guanylate cyclase or ADP-ribosyltransferase, both of which are thought to be components of the NOS signalling pathway (Schuman *et al.*, 1994; Zhuo *et al.*, 1994). However, the exact role of NOS in STP (if any) remains unclear.

The remainder of this section will concentrate on those pathways involved in the early phase of LTP; mechanisms implicated in L-LTP will be dealt with in later sections. The list of candidate molecules reported to have some involvement in the conversion of the initial Ca^{2+} trigger signal, into a long lasting modification is large. In a recent article, Sanes and Lichtman (1999) list over a hundred such molecules, but draw attention to the fact that a satisfactory molecular explanation for LTP has yet to be provided. Molecules implicated in the early stages of LTP, include glutamate

receptors, intercellular messengers, ion channels, vesicle and synapse associated proteins, adhesion molecules, proteases, phosphatases, phospholipases and kinases (for a complete list see Sanes & Lichtman, 1999). It is important to recognise that some of these molecules and maybe many of them, are in fact modulators of LTP, that is, they can alter the process but are not essential for its occurrence. Alternatively, they may have an affect on brain development or intermediary metabolism that could indirectly effect LTP without directly being involved in mediating it. There are very few molecules for which the data provides clear evidence for a role in E-LTP expression. These include protein kinases, in particular Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC).

In 1985, Lisman proposed a mechanism whereby a kinase could become activated by autophosphorylation, with activation continuing even after the triggering event was over. Subsequently, it was discovered that CaMKII possessed such properties. Miller and Kennedy (1986) found that CaMKII became active following a rise in the intracellular Ca^{2+} concentration and that this activity was sustained beyond the duration of the elevated calcium signal. CaMKII is appropriately localised in the cell for such a role. CaMKII is found in high concentrations in the postsynaptic density (PSD) (Kennedy *et al.*, 1983), a tiny structure located beneath the postsynaptic membranes of dendritic spines, that is thought to be involved in the clustering of NMDARs and AMPARs and may be a scaffold for Ca^{2+} dependent signal transduction mechanisms (Kennedy, 1997).

CaMKII involvement in LTP has also been shown. Intracellular postsynaptic injection of $CaMKII_{(273\text{-}302)}$, a specific inhibitor of CaMKII, caused potentiated synaptic responses to return to baseline after about 1 hour (Malinow *et al.*, 1989). Using similar techniques, potent calmodulin antagonists (that inhibit CaMKII auto- and substrate phosphorylation) have also been shown to block LTP (Malenka *et al.*, 1989). Additionally, Silva *et al.* (1992b) found that mutant mice that did not express αCaMKII were unable to support LTP. Further evidence for a role of CaMKII in LTP generation came from experiments on hippocampal CA1 neurons expressing a constitutively active form of CaMKII. These neurons showed increased levels of CaMKII accompanied by enhanced synaptic transmission and occlusion of LTP

(Pettit *et al.*, 1994). A correlation between CaMKII autophosphorylation and LTP has also been shown. High frequency stimulation resulted in CaMKII autophosphorylation and this was prevented by the addition of the NMDAR antagonist AP5 (Fukunaga *et al.*, 1995). Furthermore, a requirement for CaMKII autophosphorylation in LTP and learning has been demonstrated. Mice were produced that contained a point mutation (at Thr^{286}) in the αCaMKII gene, thereby blocking autophosphorylation of the kinase, whilst having no effect on its CaM-dependent activity. These mice were found to be deficient in their ability to produce LTP as well as showing impaired spatial learning (Giese *et al.*, 1998).

Lastly, CaMKII has been shown to interact directly with AMPARs. CaMKII can phosphorylate AMPARs *in vitro*, increasing kainate–induced ion currents by three to four fold (McGlade-McCulloh *et al.*, 1993). Barria *et al.* (1997) have shown an increase in [^{32}P] labelling of AMPARs in response to LTP induction. The AMPAR phosphorylation was correlated with CaMKII autophosphorylation and blocked by KN-62, an inhibitor of CaMKII. More recently, electrophysiologically tagged AMPARs have been shown to move into synapses in response to increased CaMKII activity or LTP. This movement was found to be dependent on the interaction of the GluR1 subunit of the AMPARs with a PDZ domain protein (thought to be involved in membrane protein subcellular localisation) (Hayashi *et al.*, 2000).

A large proportion of the data implicating PKC in E-LTP is from pharmacological experiments. However, these results are often difficult to interpret, largely because the inhibitors used block other kinases in addition to PKC. In 1988, Malinow *et al.*, demonstrated that bath application of the protein kinase inhibitor H7, could block both induction and expression of E-LTP. However, in the same paper, the inhibitor sphingosine, a competitor of second messenger activators of PKC, blocked E-LTP induction but not expression. This suggested that kinase activity sustaining E-LTP was autonomous. More direct evidence for the involvement of PKC has come from studies using selective inhibitor peptides, however these peptides are membrane impermeable and must therefore be injected into the cell. Postsynaptic injection of the PKC inhibitor peptide $PKC_{19\text{-}31}$ has been shown to block E-LTP when present in the cell at the time of the tetanus (Malinow *et al.*, 1989; Wang & Feng, 1992) whilst

injection of PKC_{19-31} and polymyxin B (a PKC as well as CaMK inhibitor) up to 3 hrs after the tetanus caused LTP to return to baseline (Wang & Feng, 1992). This result suggests that the continued activity of PKC is required for E-LTP expression.

In addition to inhibitor studies, activator studies have also suggested a role for PKC in E-LTP. Application of phorbol esters, potent activators of PKC, induce potentiation of synaptic transmission in the CA1 region of the hippocampus and occlude LTP (Malenka *et al.*, 1986). Postsynaptic intracellular injection of PKC (but not inactivated PKC) has also been shown to enhance synaptic transmission (Hu *et al.*, 1987). In 1993 two groups using direct *in vitro* methods reported NMDAR dependent increases in PKC activation within minutes of tetanic LTP induction (Klann *et al.*, 1993; Sacktor *et al.*, 1993). Furthermore Klann *et al.* (1993) showed that this PKC activation lasted well into the maintenance phase of E-LTP (30-180 minutes) and appeared to be dependent on phosphorylation. Data from experiments with knockout mice has proven controversial. Mutant mice lacking the γ subtype of PKC show reduced LTP, however, this LTP can be recovered by preceding the tetanus with low frequency stimulation (Abeliovich *et al.*, 1993). This led the authors to propose that PKC may be involved more in a modulatory role, rather than as a direct mediator of LTP.

One final line of evidence relates to the function that PKC may have in the expression of E-LTP. Wang *et al.* (1994) using hippocampal neuronal cultures, showed that intracellular injection of the catalytic fragment of PKC (PKCM) caused AMPAR mediated responses to become potentiated, whilst coapplication of the inhibitory subunit $PKCI_{19-36}$ prevented this. This PKCM injection caused increases in mean miniature EPSC (mEPSC) amplitude and/or time constant of decay. Similarly activation of PKC by phorbol esters causes an increase in mEPSC frequency as well as amplitude (Carroll *et al.*, 1998). Taken together, these results implicate PKC as having a role in the control of synaptic strength via AMPAR modulation, (similar to the role proposed for CaMKII). Consistent with this view, Roche *et al.* (1996) provide evidence that PKC specifically phosphorylates the GluR1 subunit of the AMPAR at Ser^{831} in HEK-293 cells and cultured neurons. However, direct evidence showing AMPAR phosphorylation via PKC during LTP has yet to be shown. PKC is also

thought to play a role in NMDAR modulation. PKC activation, via phorbol ester addition to spinal cord slice preparations, has been shown to enhance the depolarising responses of dorsal horn neurons to NMDA. This potentiation of the NMDA response was blocked by AP5 (Gerber *et al.*, 1989). Chen *et al.* (1992) showed that PKC potentiated the NMDA response by reducing the voltage dependent Mg^{2+} block and increasing the probability of channel opening. Additionally, PKC has been shown to phosphorylate NMDA receptor subunits, NR1, NR2A and NR2B (Leonard and Hell, 1997). However, the relationship between PKC-induced phosphorylation of the NMDAR subunits to PKC-induced NMDAR potentiation or LTP is unclear.

A number of other kinases are proposed to be involved in E-LTP, although the evidence for each is less convincing than that for CaMKII or PKC. The cAMP dependent protein kinase (PKA) is thought to have a role in gating CaMKII activity during E-LTP. Studies have shown that LTP can increase CaMKII activity in a cAMP dependent manner via the phosphorylation (and activation) of inhibitor-1 protein (I1), a specific protein phosphatase-1 (PP1) inhibitor (Blitzer *et al.*, 1998; Makhinson *et al.*, 1999). Therefore, PKA may be indirectly involved in AMPAR modulation via removal of inhibitory phosphatase activity thereby prolonging CaMKII activation. Indeed PKA has been seen to affect AMPA receptor function. Bath application of activators of PKA, or intracellular injection of the catalytic subunit of PKA, induce potentiation of AMPAR mediated currents in hippocampal neuronal cultures. Furthermore, addition of the competitive PKA inhibitor Rp-cAMPS, causes depression of these currents (Wang *et al.*, 1991). Greengard *et al.* (1991) showed an increase in opening frequency and mean opening time of AMPAR single channel recordings in response to exposure to PKA. Additionally, evidence for the specific phosphorylation of the AMPAR GluR1 subunit at Ser^{845} has been published (Roche *et al.*, 1996). In addition to its involvement in E-LTP, PKA has also been implicated in the modulation of gene expression leading to L-LTP (discussed in section 1.5.2).

Tyrosine kinases, particularly the tyrosine kinase Src, are also involved in E-LTP. There is some evidence that the target protein for these kinases is the NMDA receptor subunit NR2B. Application of the tyrosine kinase inhibitor genisten to spinal dorsal horn neurons causes a reduction in the NMDAR mediated currents, as well as a

reduction in the amplitude of Ca^{2+} responses. Furthermore, these currents can be potentiated by the intracellular injection of the tyrosine kinase $pp60^{c\text{-}src}$ (Wang and Salter, 1994). Zheng *et al.* (1998) showed that this potentiation of NMDAR mediated current was due the relief of zinc inhibition by Src phosphorylation of C-terminal tyrosine residues. Increases in tyrosine phosphorylation of the NR2B subunit have been observed 3 hours after LTP induction. These increases were absent when LTP was blocked by addition of the NMDAR antagonist MK801 (Rostas *et al.*, 1996). Similarly, Rosenblum *et al.* (1996) report an NMDAR dependent tyrosine phosphorylation of the NR2B subunit that is correlated with LTP in the dentate gyrus. LTP induction produces a rapid increase in activation of the tyrosine kinase Src, whilst Src inhibitors block LTP (Lu *et al.*, 1998). It is hypothesised that during LTP induction, tyrosine kinases enhance NMDAR mediated currents, thus enhancing the Ca^{2+} trigger required by other signal transduction pathways to potentiate the AMPAR mediated current (Soderling and Derkach, 2000). Indeed, postsynaptic Src injection induces a potentiation in the AMPAR EPSCs that is dependent on both increased intracellular Ca^{2+} and NMDAR activation (Lu *et al.*, 1998). Whether or not tyrosine kinase phosphorylation of the NMDAR is rapid enough to make this feasible is yet to be demonstrated.

Finally, recent evidence suggests that the mitogen activated protein kinase (MAPK) pathway is involved in E-LTP. The MAP kinase, ERK (extracellular regulated kinase), is activated in hippocampal slices in response to LTP-inducing high frequency stimulation (HFS) and is dependent on NMDA receptor activation (English and Sweatt, 1996). Furthermore, inhibitors of the MAPK pathway block this HFS induced ERK activation, in addition to significantly attenuating LTP over 60-90 minutes (English and Sweatt, 1997; Atkins *et al.*, 1998). LTP induced in the insular cortex *in vivo*, in response to stimulation in the basolateral amygdala is also associated with an increase in ERK2 activation (Jones *et al.*, 1999). Also, micro injection of PD98059 into the insular cortex 30 minutes prior to tetanisation blocks LTP, causing potentials to return to baseline after 40 minutes (Jones *et al.*, 1999). Behavioural experiments have also shown a role for ERK in learning and memory. ERK activation increases in rats, one hour after contextual and cued fear conditioning. Conversely, intraperitoneal injection of the MAPK pathway inhibitor SL327, 1 hour

before training blocks both contextual and cued fear conditioning measured 24 hours later (Atkins *et al.*, 1998). The role of MAPK in LTP may be two fold; in addition to a role in E-LTP it is also thought to mediate L-LTP via the modulation of gene expression (see section 1.5.2).

1.4.2 The Locus of Expression for LTP

The locus of LTP expression has been the subject of intense debate over the last 20 years. However, due to the technical difficulties associated with answering this question, a definitive explanation has yet to be presented. Evidence in support of either postsynaptic or presynaptic mechanisms is available, although LTP expression may actually involve both. Candidate postsynaptic modifications include increases in the number of receptors or a change in their functional characteristics (i.e. changes in affinity for glutamate, probability of channel opening, or channel conductance). Presynaptic modifications include enhanced glutamate release, due to changes in quantal size (the amount of transmitter per vesicle), the number of vesicles released or changes in the probability of release of a vesicle.

1.4.3 Postsynaptic Expression Mechanisms

Evidence for a postsynaptic expression of LTP comes from studies on miniature EPSCs (mEPSCs). These are postsynaptic currents evoked by the spontaneous release of individual synaptic vesicles. These studies rely on the assumption that synaptic vesicles are of constant size and contain a fixed amount of neurotransmitter. Therefore, a change in the size of these mEPSCs would reflect a postsynaptic change in the response to the neurotransmitter. In 1992 Manabe *et al.* showed that brief NMDA application caused a large increase in mEPSCs amplitude whilst LTP induction led to a lasting (at least 50 minutes) increase in the mEPSC amplitude. Furthermore, an increase in mEPSC size, seen in response to LTP induction, could be completely reversed by depotentiating stimuli (Oliet *et al.*, 1996). These increases in mEPSCs are thought to reflect changes in the function or number of AMPA receptors. Davies *et al.* (1989) showed that the depolarisation in CA1 neurons, induced via

AMPA application, developed in magnitude after the induction of LTP, reaching a maximum response around 2 hours post tetanus. Prevention of LTP induction, by the addition of the NMDAR agonist AP5 blocked this response.

A number of studies have examined the mechanism by which a change in the AMPAR response may occur. The main candidate is AMPAR phosphorylation. CaMKII, PKC and PKA have all been shown to directly phosphorylate the AMPAR GluR1 subunit (McGlade-McCulloh *et al.*, 1993; Roche *et al.*, 1996; Barria *et al.*, 1997)(see also section 1.4.1). Increases in AMPAR phosphorylation during LTP have been correlated with CaMKII autophosphorylation and blocked by inhibition of CaMKII (Barria *et al.*, 1997). Additionally, AMPAR currents can be enhanced in response to CaMKII phosphorylation (McGlade-McCulloh *et al.*, 1993) or exposure to the catalytic subunit of PKA or PKC (Greengard *et al.*, 1991; Wang *et al.*, 1991; Wang *et al.*, 1994)(see section 1.4.1). Derkach *et al.* (1999) showed an increase in GluR1 single channel conductance in HEK-293 cells, in response to coexpression with activated CaMKII. Consistent with these results, the AMPAR single channel conductance increases in response to LTP induction in CA1 pyramidal cells of hippocampal slices (Benke *et al.*, 1998). Finally, it has been shown that LTP is absent in the CA1 region in mutant mice lacking the AMPAR GluR1 subunit (Zamanillo *et al.*, 1999).

Although the role of the NMDA receptor in LTP induction is certainly important, it seems that the NMDAR mediated current contributes less to the maintenance of LTP than that of the AMPAR mediated current. However, as already discussed, some enhancement of NMDAR currents does appear to occur following LTP induction. What is the mechanism behind such changes? Much of the evidence points to the phosphorylation of the NMDAR by tyrosine kinases (particularly Src) and PKC. Src has been shown to phosphorylate the NMDA receptor subunit NR2B (Rosenblum *et al.*, 1996; Rostas *et al.*, 1996), whilst PKA and PKC phosphorylate subunits, NR1 NR2A and NR2B (Leonard & Hell, 1997). Application of both Src and PKC has also been shown to potentiate NMDAR mediated currents (Chen & Huang, 1992; Wang & Salter, 1994; Zheng *et al.*, 1998)(for more detail see section 1.4.1). Furthermore, NMDA dependent increases in tyrosine phosphorylation of the NR2B subunit have

been observed following LTP induction (Rosenblum *et al.*, 1996; Lu *et al.*, 1998) some of which can be seen for up to 3 hours (Rostas *et al.*, 1996)(see section 1.4.1). These results suggest a role for tyrosine phosphorylation of NMDA receptors in LTP maintenance.

1.4.4 Evidence against a Presynaptic Locus of Expression

Many of the early studies used electrophysiological methods to examine whether an increase in transmitter release occurred following LTP induction. The close proximity of AMPA and NMDA receptors on the postsynaptic membrane (Bekkers and Stevens, 1989) would make one predict that they should show a similar increase in synaptic current in response to an increase in glutamate release. In conditions in which glutamate release was artificially elevated (e.g. by the addition of phorbol esters, $GABA_A$ agonists, adenosine antagonists, or changes in stimulation frequency) this is indeed the case. (Asztely *et al.*, 1992; Perkel and Nicoll, 1993). However, after LTP induction this is not seen. Instead a differential increase occurs, with NMDAR mediated response being only moderately affected in relation to the AMPAR mediated response (Kauer *et al.*, 1988; Muller *et al.*, 1988; Asztely *et al.*, 1992). Asztely *et al.* (1992) report that the NMDA component shows an increase of about one third of that of the AMPA component. These findings argue against a role for increased glutamate release in LTP expression. It is worth noting that, Clark and Collingridge (1995) have reported a similar magnitude of AMPA and NMDA receptor potentiation after LTP.

Paired pulse facilitation (PPF), an increase in the amplitude of a second EPSP of a pair of temporally linked EPSPs, is a form of short-lasting plasticity. It is well documented that PPF is presynaptic in origin (reviewed by Zucker, 1999). However, changes in PPF have been shown not to occur following LTP induction (Manabe *et al.*, 1993; Pananceau *et al.*, 1998; Selig *et al.*, 1999). Manabe *et al.* (1993) found that LTP was associated with no change in PPF, but presynaptic manipulations such as GABA agonists or adenosine antagonists, that produced potentiation similar in magnitude to that induced by LTP, significantly reduced PPF. Once again conflicting

data exist. Schulz *et al.* (1994) have results that show LTP induction can cause changes in PPF.

Attempts have been made to directly measure glutamate release by applying use-dependent antagonists for both the NMDAR and the AMPAR. The rate of decline of the NMDAR mediated current in the presence of the irreversible NMDA open channel blocker MK801 can be used to assess the probability of release of transmitter. However, Manabe *et al.* (1994) found that the rate of decline of the response was similar at synapses expressing LTP or not expressing LTP. In contrast to these results Kullman *et al.* (1996) found that the rate of block of NMDAR mediated currents by MK801 was greater after LTP, this suggests an increase in the probability of release of glutamate during LTP. Similar experiments have been performed using the use-dependent AMPAR antagonist HPP-SP. This drug is selective for AMPARs lacking the GluR2 subunit. In mutant mice lacking GluR2 the rate of AMPAR block by HPP-SP was unchanged after LTP, again indicating that transmitter release does not increase after LTP (Mainen *et al.*, 1998). Glial cells have also been used as a measure of glutamate release at synapses. Electrogenic glutamate transporters present in glial cell membranes can detect glutamate released from the synapse. By recording currents evoked by these transporters, changes in presynaptic glutamate release can be detected. Glial cell transporter currents are sensitive to manipulations that alter the release of glutamate, but are unaffected by the induction of LTP (Diamond *et al.*, 1998; Luscher *et al.*, 1998) once again suggesting that increased glutamate release is not involved in LTP.

1.4.5 Presynaptic Expression Mechanisms

The earliest evidence for a presynaptic mechanism of expression in LTP, was seen in experiments in which neurotransmitter release was measured following LTP induction. Pre-accumulated [^{3}H]-D-aspartate release from Schaffer collateral fibres was significantly increased in response to bursts of electrical stimulation (Skrede and Malthe-Sorenssen, 1981). Additionally, Dolphin *et al.* (1982) used a push–pull canulla technique to measure [^{3}H] glutamate release from the synaptic region of the rat dentate gyrus *in vivo*. Following LTP induction, glutamate levels were seen to

increase and remain elevated for at least 1 hour. However, others have seen that LTP in the CA1 or dentate gyrus regions of the anaesthetised rat is not accompanied by a significant, long-lasting increase in the release of either glutamate or aspartate (Aniksztejn *et al.*, 1989). Therefore, the involvement of increased transmitter release in LTP is still open to debate.

The majority of evidence implicating a presynaptic locus for LTP expression has come from quantal analysis studies. In such experiments the amplitude of either evoked EPSCs or spontaneous miniature EPSCs is plotted against their frequency of occurrence. These plots reveal peaks thought to correspond to release of single or multiple quanta. Assuming that the quantal size (the amount of neurotransmitter per vesicle) is consistent, the distance between the peaks corresponds to the quantal amplitude (i.e. the postsynaptic response to a single quantum of neurotransmitter). Changes in the amplitude, frequency or shape of these peaks can provide insights into changes occurring during LTP. A number of studies have attempted to determine whether quantal size, the number of quanta released or the probability of quantal release, changes after LTP. The majority of papers suggest some presynaptic component is involved in the expression of LTP, but they report different levels of involvement. Bekkers and Stevens (1990) suggest that LTP is a purely presynaptic mechanism, depending on the probability of transmitter release (P_r). Others report a decrease in the number of failures following LTP, suggesting either an increase in the number of vesicles available for release and/or increases in the probability of release (Malinow and Tsien, 1990), however, postsynaptic modifications are not ruled out. Subsequent studies point to both pre and postsynaptic expression mechanisms (Kullmann and Nicoll, 1992), the relative importance of each depending on the initial setting of the presynaptic release mechanism (Larkman *et al.*, 1992).

Several studies have used quantal analysis to look at the postsynaptic responses produced by 'minimal stimulation', in which only one or a few, presynaptic fibres are stimulated. These methods attempt to look at changes in individual quanta following LTP induction. This approach has shown that LTP induction is associated with a decrease in the number of failures (Malinow, 1991; Stevens and Wang, 1994; Stricker *et al.*, 1996), suggesting a presynaptic involvement in LTP. Additionally, Bolshakov

and Sieglebaum (1995) recording from pairs of pyramidal neurons in hippocampal slices, showed that only a single quantum of transmitter was released from a CA3 presynaptic bouton in response to an action potential. However, the probability of release of the quantum was unique for a particular cell pair. In response to PPF, 2-3 week old rats with low initial P_r synapses (P_r <0.5) showed a decrease in the number of failures to the second pulse, however this decrease did not occur in young animals (4-8 days) that contained high P_r synapses (P_r= 0.9). Furthermore, LTP was correlated with an increase in P_r in older animals whereas LTP was occluded in young animals, perhaps due to the initial high P_r of the synapses.

Recently, Ca^{2+} imaging studies have been employed to address the same question. This approach offers the advantage that only single synapses are studied. Emptage *et al.* (2000) imaged calcium transients in single dendritic spines of hippocampal pyramidal cells before and after LTP induction. The probability of evoking a postsynaptic Ca^{2+} transient (EPSCaTs) in response to a single synaptic stimulus increased following LTP. Assuming that EPSCaTs reflect the release of transmitter at a synapse, these results show a change in P_r following LTP induction. Increases in EPSCaT amplitude were also seen following LTP, perhaps suggesting that LTP is expressed at individual synapses via both pre and postsynaptic mechanisms.

Although the majority of studies in this area support the view that an increase in the probability of neurotransmitter release is a likely presynaptic mechanism in LTP expression, there are other possibilities. In theory, transmitter release could also be influenced by the quantal size or the number of quanta released. However, for either of these mechanisms to be effective it is essential that the postsynaptic receptors are not saturated by neurotransmitter under conditions of basal transmission. Early evidence from quantal analysis studies revealed only modest variation in quantal amplitude which suggested postsynaptic receptor saturation (Larkman *et al.*, 1991; Liao *et al.*, 1992). Additionally, studies looking at mEPSCs in cultured hippocampal neurons found that the peak amplitudes of closely timed pairs of mEPSCs (thought to originate from a common release site) were extremely similar, also suggesting receptor saturation (Tang *et al.*, 1994). However, there are results that contradict these findings. Forti *et al.* (1997) examined individual synapses by capturing an isolated

presynaptic bouton inside a loose patch pipette and recording from a lose seal on the associated postsynaptic membrane. They found that the variability of the conductance of any single synapse was larger than could be accounted for by the random opening of receptor channels, suggesting that the postsynaptic receptors were not saturated in response to a quantum of neurotransmitter. In line with these findings, NMDAR-mediated synaptic calcium transients in single dendritic spines of CA1 pyramidal cells, have been shown to summate in response to two closely paired shocks (occurring <10ms apart). This suggests that a single release event does not saturate spine NMDARs. (Mainen *et al.*, 1999). Therefore, one cannot rule out the possibility that a synapse could increase its efficacy by increasing the neurotransmitter content of its vesicles or the number of vesicles released.

1.4.6 Retrograde Messengers

It is widely accepted that LTP *induction* is postsynaptic, following the demonstration by Lynch *et al.* (1983) that postsynaptic injection of the Ca^{2+} chelator EGTA blocks the induction of LTP. Therefore, the idea that a presynaptic mechanism may contribute to the expression of LTP raises a problem. How is the postsynaptic induction of LTP communicated to the presynaptic cell? One suggestion is that a *retrograde messenger* is released from the postsynaptic cell that diffuses back across the synaptic cleft and modifies transmitter release from the presynaptic cell (Bliss *et al.*, 1986). The properties that a candidate retrograde messenger should possess are as follows: 1) the retrograde messenger should be released from the postsynaptic cell in response to NMDA dependent LTP induction; 2) Inhibitors of the production or action of this messenger should block LTP; 3) application of this messenger in the presence of afferent stimulation should cause a synaptic enhancement that is NMDAR independent and will occlude LTP. Four candidate retrograde messengers are arachidonic acid (AA), nitric oxide (NO), carbon monoxide (CO), and platelet-activating factor (PAF). To date, none of these molecules have adequately met the criteria required of a retrograde messenger.

The fatty acid, arachidonic acid (AA) was the first candidate molecule to be described. In 1989, Williams *et al.* (1989) showed that AA application, coupled with

weak activation of the perforant path of rats *in vivo* caused a slow onset potentiation that occluded further LTP. Furthermore, AA induced potentiation *in vitro* was accompanied by an increase of glutamate levels in the perfusing solution (Williams *et al.*, 1989). It has also been shown that inhibition of phospholipase A_2, an enzyme that liberates AA from phospholipids blocks LTP (Williams and Bliss, 1989). AA is released from cultured striatal neurons in response to NMDAR activation by glutamate and NMDA (Dumuis *et al.*, 1988) and can potentiate NMDA receptor currents in cerebellar granule cells via an increase in channel open probability (Miller *et al.*, 1992). Thus AA released by activation of NMDARs could potentiate the NMDAR current, thereby amplifying the rise in intracellular calcium during LTP induction. AA has also been found to inhibit the uptake of glutamate by glial cells, which may contribute to increases in the synaptic response that occur during LTP (Barbour *et al.*, 1989). However, there are problems with AA as a candidate retrograde messenger. LTP produced by AA has a delayed onset (1-2 hours) (Williams *et al.*, 1989), whilst observed increases in neurotransmitter release occur immediately after LTP induction (Dolphin *et al.*, 1982). Furthermore, since retrograde messenger production is presumed to be downstream of NMDAR activation, AA application would be expected to enhance synaptic strength during the blockade of NMDARs, however this is not the case. O'Dell *et al.* (1991), found that AA induced potentiation is blocked by the NMDAR antagonist AP5.

The possibility that nitric oxide (NO) is the retrograde messenger has received a great deal of support. NO is a diffusible gas formed from L-arginine by a calcium calmodulin-dependent enzyme, NO synthase. NO is released from cultured cerebellar cells in response to NMDAR activation via application of glutamate or NMDA (Garthwaite *et al.*, 1988). In addition, E-LTP is blocked by the bath application or postsynaptic injection of NO synthase inhibitors (O'Dell *et al.*, 1991; Schuman & Madison, 1991). More recently, NO synthase inhibitors have also been shown to block protein synthesis dependent late-phase LTP (Lu *et al.*, 1999). However, NO synthase inhibitors applied 30 minutes after LTP induction, have no effect on established LTP, suggesting a role for NO during the induction of LTP (O'Dell *et al.*, 1991).

In dissociated cell cultures, Arrancio *et al.* (1996), found that pre or postsynaptic injection of the membrane impermeable NO scavenger oxymyoglobin blocked LTP. However, LTP was only blocked by postsynaptic injection of NO synthase inhibitors. Furthermore, bath application of the membrane impermeable molecule haemoglobin, known to bind NO, abolished E-LTP (O'Dell *et al.*, 1991; Schuman & Madison, 1991). Taken together, these results suggest that LTP may be dependent on NO that is produced postsynaptically and liberated into the extracellular space where it acts presynaptically.

Application of NO has also been shown to cause a rapid enhancement of mEPSC frequency (O'Dell *et al.*, 1991). In addition, NO paired with weak tetanic stimulation, produces a rapid, long-lasting synaptic potentiation in guinea pig hippocampal slices (Zhuo *et al.*, 1993). This enhancement occluded LTP and was not blocked by the NMDA antagonist AP5, suggesting NO acts downstream of the NMDA receptor (Zhuo *et al.*, 1993). Therefore, NO displays many of the characteristics of a retrograde messenger.

In contrast with the pharmacological data, early studies on NO mutant mice were inconclusive. Mice expressing a disrupted neuronal-specific NO synthase (nNOS) gene were found to support relatively normal LTP compared to wild types (O'Dell *et al.*, 1994). This led to the suggestion that the endothelial isoform of NO synthase (eNOS) (expressed in CA1 neurons) maybe important for LTP. This was confirmed *in vitro* by Kantor *et al.* (1996) who injected a truncated eNOS into CA1 pyramidal cells. The mutant eNOS bound to native eNOS thus inhibiting its function and blocking the induction of E-LTP. However, *in vivo*, mice with a targeted mutation in either the nNOS or eNOS isoforms show normal LTP. Only when the double mutation ($nNOS^-/eNOS^-$) was present, did the animals show a reduction in LTP (Son *et al.*, 1996).

Although evidence in support of NO as a retrograde messenger is largely compelling some concerns have arisen. LTP induced by strong stimulation does not seem to require NO (Wilson *et al.*, 1997; Wilson *et al.*, 1999). Wilson *et al.* (1999) showed that in hippocampal slices taken from mice expressing a mutation in the eNOS gene,

E-LTP is absent under weak stimulation conditions. However, in response to strong stimulation these mice exhibit LTP that is insensitive to NO synthase inhibitors. Similar results have been seen *in vivo*. The NO synthase inhibitor, L-NAME (N_{ω}-nitro-L-arginine methyl ester) did not effect strong tetanus induced LTP in the rat dentate gyrus, whilst an apparent LTP block by NO inhibition under weak tetanisation conditions could be explained by a reduction in baseline responses brought about by the drug (Bannerman *et al.*, 1994). Finally, NO synthase inhibitors block LTP induction in hippocampal slices prepared from young rats and subsequently incubated at room temperature (23-25°C). However, these inhibitors are ineffective at blocking LTP induced in slices from adult rats, or LTP induced in slices incubated at 29-30°C, that are prepared from these young rats (Williams *et al.*, 1993).

Limited evidence exists in support of a role for either carbon monoxide (CO) or platelet activating factor (PAF) as retrograde messengers. CO is a diffusible membrane permeable gas produced by the action of haem oxygenase (HO). Application of CO when paired with either low frequency stimulation or weak tetanic stimulation induces a rapid long lasting EPSP potentiation. This potentiation occluded further LTP induced by strong tetanisation and was not blocked by the NMDR antagonist AP5 (Zhuo *et al.*, 1993). Application of the haem oxygenase inhibitor ZnPP (zinc protoporphyrin IX), has been shown to block LTP induction (Stevens and Wang, 1993; Zhuo *et al.*, 1993). Furthermore, pre-established LTP is eliminated by addition of ZnPP up to 45 minutes post-tetanus (Stevens & Wang, 1993), suggesting that the maintenance of LTP may depend on the constant production of CO.

Platelet activating factor (PAF) is a membrane-derived lipid second messenger and is generated by Ca^{2+}-dependent phospholipase A_2, the enzyme that also produces arachidonic acid. PAF is released into the extracellular space from cultured cerebellar granule cells (Yue *et al.*, 1990). Application of the PAF analogue carbamyl-PAF to cultured rat hippocampal neurons causes an increase in the frequency of mEPSCs, whilst having no effect on amplitude, suggesting a presynaptic site of action (Clark *et al.*, 1992). Similar results have been observed in hippocampal slices (Kato *et al.*, 1994). The PAF-receptor antagonist BN-52021 has been shown to block CA1 LTP in rats, which can be 'rescued' by the presence of PAF analogues (Kato *et al.*, 1994).

Application of PAF or PAF analogues induce a stable increase in EPSP amplitude which can be blocked by PAF-receptor antagonists and is occluded by tetanically induced LTP (Wieraszko *et al.*, 1993; Kato *et al.*, 1994). Kato *et al.* (1994) also showed that postsynaptic injection of PAF analogues, coupled with low frequency stimulation in the presence of the NMDAR antagonist AP5, is sufficient to induce a stable EPSC potentiation that would not have occurred in the presence of either PAF analogues or low frequency stimulation alone. However, the apparent NMDA independent effect of PAF-induced potentiation is disputed (Wieraszko *et al.*, 1993). More recently, the role of PAF and its associated receptor in LTP has been questioned. Hippocampal slices taken from PAF receptor deficient mice exhibit normal LTP (Kobayashi *et al.*, 1999). Furthermore, application of PAF receptor antagonists to LTP induced in wild type mice did not block LTP; whilst PAF application produced no EPSP potentiation (Kobayashi *et al.*, 1999).

1.4.7 Silent Synapses

As already discussed, research into the locus of expression of LTP has produced compelling evidence for both presynaptic and postsynaptic expression. How can data suggesting a postsynaptic receptor modification be reconciled with quantal analysis studies indicating increased presynaptic release of glutamate? One hypothesis that could explain these conflicting results in favour of a postsynaptic locus is the 'silent synapse' model. This model states that a subset of synapses exist that only contain functional NMDA receptors. During normal synaptic transmission, synapses would remain 'silent', due to the voltage dependent block on the NMDA receptors and the absence of an AMPAR current. However, during LTP induction, a process could occur in these synapses that would cause them to express functional AMPA receptors, thus reducing failure rate at the synapse with no change in glutamate release. Kullmann (1994) provided indirect evidence in support of this model. Recording from the CA1 region of hippocampal slices, Kullman showed that the coefficient of variation (CV) was larger in the AMPA receptor mediated current than in the NMDA receptor mediated current. Furthermore, LTP induction caused a decrease in the CV of the AMPAR current whilst having no effect on either the CV or average amplitude of the NMDAR current. The CV is inversely proportional to the number of activated

synapses, which led the author to conclude that these results could be explained if AMPA receptors were absent, or non functional in a proportion of synapses, and that LTP induction caused AMPAR insertion or activation in the postsynaptic membrane.

Following these results several groups have shown that a large proportion of synapses in the CA1 region transmit using only functional NMDA receptors (Isaac *et al.*, 1995; Liao *et al.*, 1995; Durand *et al.*, 1996). Under depolarising conditions (+30 to +60mV) it is possible to record responses from these synapses that can be completely blocked by the NMDAR antagonist AP5 (Isaac *et al.*, 1995; Liao *et al.*, 1995; Durand *et al.*, 1996). Additionally, silent/NMDAR only synapses can be converted (via an NMDAR dependent mechanism) into synapses expressing functional AMPARs, following LTP induction (Isaac *et al.*, 1995; Liao *et al.*, 1995; Durand *et al.*, 1996). Immunocytological evidence from cultures and intact tissue supports this difference between AMPA and NMDA receptor clustering in the hippocampus. The majority of synapses are seen to posses NMDARs whilst the number of synapses expressing detectable AMPA receptors can vary dramatically, increasing during neuronal maturation (Liao *et al.*, 1999; Petralia *et al.*, 1999), AMPAR blockade (Liao *et al.*, 1999); and increasing PSD diameter (Takumi *et al.*, 1999). Two recent studies have provided evidence of possible mechanisms that may be involved in the unmasking of AMPARs at silent synapses following LTP. Shi *et al.* (1999) transiently expressed the AMPAR subunit GluR1 tagged to green fluorescent protein (GFP) in hippocampal CA1 neurons. Tetanic stimulation caused an NMDAR dependent targeting of this GluR-GFP protein into dendritic spines. Similarly Hayashi *et al.* (2000) have shown that following LTP induction, electrophysiologically tagged AMPARs move into synapses via a process dependent on the interaction of the GluR1 subunit with a PDZ domain protein.

More recently, a second hypothesis has been proposed that is also consistent with the data used to support the postsynaptically silent synapse model. This is the glutamate spillover hypothesis (for a review see Kullmann and Asztely, 1998). It states that glutamate can diffuse from the synaptic cleft at which it is released, into adjacent synapses, in sufficient concentrations to activate NMDARs but not AMPARs. Support for this model first came from Kullmann *et al.* (1996) who found that tetanic

stimulation caused potentiation of NMDAR mediated currents, recorded from a single cell, in which the AMPAR mediated LTP had been blocked. These results suggested that the NMDARs on this individual cell could respond to increases in glutamate release from neighbouring synapses (on other cells) at which LTP had been induced. However, Malenka and Nicoll (1997) state, that to explain the sudden appearance of AMPA receptor-mediated currents following LTP induction (Isaac *et al.*, 1995; Liao *et al.*, 1995; Durand *et al.*, 1996), the spillover hypothesis would still require the conversion of silent synapses into functional synapses. The locus of this conversion would however be presynaptic. Following LTP induction, presynaptic boutons that previously released little or no glutamate, would increase their neurotransmitter release, thus recruiting additional AMPARs from neighbouring synapses.

1.5 LATE-PHASE LTP

LTP, like behavioural memory, consists of mechanistically distinct temporal components that can be experimentally dissected into: post-tetanic potentiation (PTP), short-term potentiation (STP), early LTP (E-LTP) lasting 3-6 hours, and late LTP (L-LTP) lasting 8 hours or more. This section will examine L-LTP, a mechanism thought perhaps to underlie the storage of long-term memories.

1.5.1 L-LTP is Dependent on Transcription and Translation

The idea that memory is dependent on *de novo* protein synthesis is not a new one. In 1963, Flexner *et al.* showed that intracerebral injection with the protein synthesis inhibitor puromycin caused memory loss in mice. Since then, a large body of literature has been generated examining the behavioural effects of cerebral application of protein synthesis inhibitors. The early literature is reviewed by Davis and Squire (1984). The authors summarise the findings of these studies as follows. Protein synthesis during, or shortly after, training is required for the formation of long-term memory (days to weeks); however the initial acquisition and retention of short-term memory, lasting over a period of many minutes, is protein synthesis independent. Additionally, the relative effectiveness of protein synthesis inhibition on learning

based performance decreases, as the delay between training and inhibitor application increases. This suggests that long-term memory is dependent on a critical time period of protein synthesis occurring after training. Finally, the inhibition of protein synthesis in these studies is thought to interfere with memory storage, rather than with retrieval.

A frequent criticism levelled at these experiments was that the amnesic effects induced by protein synthesis inhibitors could be due to a run down of a constitutive brain protein, rather than a block of new protein synthesis. This scenario seems unlikely. Intracerebral injection of the protein synthesis inhibitor acetooxycycloheximide, 18 hours before training (resulting in a high level of protein synthesis inhibition for many hours before training, but dropping below the effective dose for block at training), had no effect on long-term memory (Barondes and Cohen, 1967). However this experiment did not rule out proteins with very short half-lives (i.e. a few minutes). Squire and Barondes (1976) addressed this issue positing a hypothetical protein to which they had assigned a half-life of 10 minutes. Mice trained 118 minutes after cycloheximide injection (a time point at which protein levels would be low, but drug concentration would be below the effective dose, therefore allowing protein synthesis at the time of training) were unaffected, whilst those trained 1 min after drug injection (at which time protein levels were high but new protein synthesis was blocked) exhibited significantly impaired memory retention. These experiments suggest that long-term memory is dependent on new protein synthesis occurring around the time of learning.

Following the description of LTP by Bliss and Lømo (1973), it was possible to carry out similar experiments in the context of a candidate memory storage mechanism. In 1984 Krug *et al.* recorded LTP in the dentate gyrus of freely moving rats. They found that intraventricular injection of the protein synthesis inhibitor anisomycin 15 minutes prior to tetanisation, whilst having no effect on the induction of LTP, did cause EPSP slope and population spike amplitude to return to pre-tetanus levels after 5-6 hours. LTP in control animals, however, remained elevated for 7 days. These results were the first to suggest that LTP consists of at least two phases, a protein synthesis independent early phase (lasting about 3-6 hours), and a late phase requiring protein

synthesis. Similar results have been seen in anaesthetised rats (Otani and Abraham, 1989; Otani *et al.*, 1989). Injection of several different types of protein synthesis inhibitors (anisomycin, emetine, cycloheximide and puromycin) 1 hour before tetanisation caused EPSP slope to decrease to baseline levels quicker than controls (Otani *et al.*, 1989). Anisomycin showed the most dramatic effect, an 88% decay over 3 hours (Otani *et al.*, 1989). However, it should be noted that population spike amplitude was not affected by injection of any of these drugs (Otani *et al.*, 1989). Additionally, anisomycin injection, 15 minutes post-tetanus, caused no significant block of EPSP LTP when compared to controls. This suggests that *in vivo*, the late phase of LTP (L-LTP) is dependent on protein synthesis initiated within the first 15 minutes following tetanisation. Furthermore, anisomycin applied 4 hours prior to the tetanus had no greater effect on LTP than when applied 1 hour before tetanus (Otani *et al.*, 1989). This suggests that L-LTP is blocked by the inhibition of new proteins rather than the depletion of existing proteins.

Early studies of protein synthesis inhibition on hippocampal slices *in vitro* were not in agreement with the *in vivo* data. Deadwyler *et al.* (1987) found that induction of population spike LTP in the CA1 region was completely blocked following a 30 minute pre-incubation with cycloheximide. Therefore protein synthesis inhibition appeared to have an 'all or none' effect on LTP. This may have resulted from the high doses of drug used (1mM). Similarly, the frequency of population spike LTP induction, was found to decrease in a dose-dependent manner, following bath application of emetine, cycloheximide or puromycin, 30 minutes prior to the tetanus (Stanton and Sarvey, 1984). Curiously, anisomycin was the only protein synthesis inhibitor that failed to block CA1 LTP (Stanton & Sarvey, 1984). However, LTP was only followed for 30 minutes post-tetanus, providing little insight about effects on LTP for longer time periods.

The late phase of LTP (>3 hours) has been studied less extensively *in vitro* than the early phase. This is largely due to the technical difficulties encountered in maintaining adequate hippocampal slice health for long periods of time. However, in the mid 1980's a German group pioneered methods for prolonging the lifespan of the *in vitro* hippocampal slice preparation, enabling recording from, and the subsequent study of

the maintenance phase of LTP for up to 10 hours (Reymann *et al.*, 1985; Thiemann *et al.*, 1986). Using these techniques, Frey *et al.* (1988) showed that bath application of anisomycin for a period of 3 hours directly following the tetanus, caused CA1 population spike LTP in rat hippocampal slices to return to baseline levels over 8 hours. Control slices that had not received anisomycin maintained a stable LTP over the same time period (Frey *et al.*, 1988). Similar results have also been seen in mice, where synaptic potentiation was blocked over a period of 3 hours, following a 1 hour incubation with anisomycin, added 30 minutes prior to the tetanus (Nguyen and Kandel, 1997). The protein synthesis inhibitor emetine has also been found to block the induction of L-LTP in the CA1 region *in vitro* (Frey and Morris, 1997). In agreement with the *in vivo* data, CA1 LTP *in vitro* has been shown to depend on a 'critical period' of protein synthesis. Application of anisomycin, 35 minutes after the tetanus did not block L-LTP (Frey & Morris, 1997). It is not only LTP in the CA1 region of hippocampal slices that has been shown to have a protein synthesis dependent component. Mossy fibre EPSP potentiation, that remains stable for at least 3 hours *in vitro*, returns to baseline over this period following a 1 hour application of anisomycin, beginning immediately before the tetanus (Huang *et al.*, 1994). Additionally, a 40 minute application of emetine, overlapping the tetanus, has been shown to block L-LTP in the medial perforant pathway of the dentate gyrus in hippocampal slices (Nguyen and Kandel, 1996). Recently, L-LTP in the lateral amygdala *in vitro* has also been shown to be dependent on protein synthesis (Huang *et al.*, 2000).

The involvement of protein synthesis in LTP has also been demonstrated using a different approach. Early radiolabelling studies on hippocampal slices showed increased levels of labelled protein following the induction of LTP, at either CA1 or dentate gyrus regions (Duffy *et al.*, 1981). More recently, Fazeli *et al.* (1993) used two-dimensional gel electrophoresis to look at changes in [^{35}S] methionine radiolabelled proteins, following LTP induction in rat dentate gyrus *in vivo*. Density changes (reductions as well as increases) were seen for 11 proteins, 3 hours after LTP induction, suggesting complex changes in protein levels on a time scale comparable to the onset of L-LTP. Furthermore, there is evidence to suggest that dendritic protein synthesis can occur during LTP (see section 1.7).

In addition to a role for translation, early experiments also examined the role of gene transcription in long-term memory. In 1971, Daniels showed that short-term memory (<3 hours) in rats that had undergone hippocampal injection with the transcription inhibitor actinomycin-D (Act-D), 4 hours prior to training, was unaffected. However, long-term memory (i.e. >4 hours) in these animals was completely abolished. This result was not however in agreement with a number of previous studies which could find no evidence for a role of transcription in learning and memory over the same time periods, despite extensive inhibition of brain RNA synthesis (Barondes and Jarvik, 1964; Cohen and Barondes, 1966; Squire and Barondes, 1970).

Initial studies examining the effects of actinomycin-D on LTP, were also in agreement with the majority of the behavioural data. Otani *et al.* (1989) found that intraventricular injection of Act-D in anaesthetised rats, 1 hour prior to tetanisation, had no effect on LTP for either EPSP slope or population spike amplitude, over 3 hours in the dentate gyrus. In further experiments, focal injection of Act-D into the dorsal dentate gyrus, 1 hour prior to tetanisation, was also found to produce no significant effect on LTP over 6 hours (Otani & Abraham, 1989). This led the authors to state that proteins necessary for the maintenance of LTP in the dentate gyrus over at least 6 hours, are synthesised from pre-existing mRNA. However, similar experiments using freely moving rats found that intrahippocampal or intraventricular injection of Act-D, 2 hours before tetanisation blocked population spike L-LTP in the dentate gyrus (Frey *et al.*, 1996b). The reason for the difference in results between these two groups however, remains unclear. Interestingly, recent *in vivo* experiments investigating the mechanisms involved in long-term depression (LTD)(a persistent reduction in synaptic strength) have produced similar results to that seen in the *in vivo* LTP data. LTD persisting for >8 hours in the CA1 region of freely moving rats, has been shown to return to pre-tetanus levels over ~4.5 hours in response to protein synthesis inhibition. However, LTD expression was unaffected by application of actinomycin-D. These results suggest that LTD within the CA1 region *in vivo* is dependent on the translation of pre-existing mRNA but not on *de novo* mRNA transcription (Manahan-Vaughan *et al.*, 2000).

In vitro studies examining the dependence of L-LTP on transcription are also far from conclusive. In 1994, Nguyen *et al.* (1994) were the first to show a requirement for transcription in L-LTP in the CA1 region of hippocampal slices. The transcription inhibitors, Act-D and DRB (5,6-dichloro-1-β-D-ribofuranosyl benzimidazole) when applied for 2 hours immediately after the tetanus, blocked L-LTP and caused EPSPs to return to pre-tetanus levels over 3-4 hours. LTP in control experiments remained stable over the same time period. Delayed application of Act-D, 2 hours after the tetanus, did not block L-LTP. These results suggest a critical period of transcription, i.e. within 2 hours of induction, that is essential for the formation of L-LTP (Nguyen *et al.*, 1994). A critical period of transcription for L-LTP has also been shown to occur in mice, although the time period is shorter. One hour application of Act-D, produced a block of L-LTP when added 30 minutes prior to the tetanus, but had no effect on L-LTP if the application was delayed until 30 minutes post-tetanus (Nguyen & Kandel, 1997). However, conflicting results have been published. Using a similar experimental configuration and following recordings from hippocampal slices for up to 8 hours post tetanus, Frey *et al.* (1996b) examined the effects of Act-D on L-LTP. The authors found that Act-D blocked L-LTP of the population spike in the CA1 region over 8 hours, whilst causing only a slight attenuation of EPSP L-LTP over the same time period. These effects may be explained by the fact that Act-D was applied for only 30 minutes and washed off before the tetanus. Despite this, Frey *et al.* (1996a) did observe that a 30 minute application of Act-D, 20 minutes after tetanisation, did not block the previously sensitive population spike L-LTP, again suggesting a critical time window of transcription. In line with the findings that L-LTP in the CA1 region is dependent on gene transcription, L-LTP of both mossy fibre and medial perforant path EPSPs has also been shown to be blocked by the addition of Act-D and Act-D or DRB, respectively (Huang *et al.*, 1994; Nguyen & Kandel, 1996). Therefore, although the findings between groups are inconsistent and the data is far from conclusive, the data does suggest in some cases that new RNA synthesis is required for the formation of robust L-LTP.

In addition to the pharmacological dissection of E-LTP and L-LTP, reports have been published showing that variations in tetanisation procedures can produce a dissociation. In the majority of the literature, hippocampal L-LTP in the Schaffer

collateral or mossy fibre pathways *in vitro* has been shown to be induced by 3-4 trains consisting of 100Hz stimulation (1 second duration) with an inter-train interval (ITI) of 5-10 minutes (Frey *et al.*, 1988; Frey *et al.*, 1993; Huang and Kandel, 1994; Huang *et al.*, 1994; Nguyen *et al.*, 1994; Huang and Kandel, 1996; Impey *et al.*, 1996; Abel *et al.*, 1997; Frey & Morris, 1997; Baranes *et al.*, 1998; Impey *et al.*, 1998a; Winder *et al.*, 1998; Lu *et al.*, 1999). In areas where greater synaptic inhibition is present, such as in the medial perforant pathway, stronger stimulation is required, consisting of 10 trains @ 100Hz for 1 second, with an ITI of 1 minute, applied in the presence of low magnesium (Nguyen & Kandel, 1996). In addition, a number of groups have reported that weaker stimulation paradigms, typically consisting of 1 train @ 100Hz for 1 second, can induce an E-LTP that decays back to pre-tetanus levels over a time frame similar to that shown by LTP in the presence of either transcription or translation inhibitors (Huang & Kandel, 1994; Huang *et al.*, 1994; Huang & Kandel, 1996; Baranes *et al.*, 1998; Winder *et al.*, 1998). Once again, the medial perforant pathway required slightly stronger stimulation (3 trains @ 100Hz for 1 sec, ITI 1 min, in lowered magnesium) (Nguyen & Kandel, 1996).

It is important to note however, that despite claims to the contrary, L-LTP is not exclusively induced by these widely spaced multiple trains of 100Hz for 1 second. L-LTP in the Schaffer collateral pathway in rats has been induced using 4 trains of 20 pulses @ 100Hz with a 6 second ITI (Wong *et al.*, 1999) or by a single train @ 100Hz for 1 second (U. Frey, personal communication.). In addition, 15 trains of 4 pulses @100Hz ITI 200msec(Nguyen & Kandel, 1997) or 3 trains of 30 pulses @100Hz 5s ITI (Korte *et al.*, 1998) can induce Schaffer collateral L-LTP in mice. Similarly, Schaffer collateral E-LTP is not exclusively dependent on a 1 sec 100Hz train and can be induced from as little as 21 pulses @ 100Hz (Frey & Morris, 1997; Frey and Morris, 1998b). Although this distinction is important to make, it remains the case that, irrespective of the stimulation protocols used, early and late LTP can be dissected electrophysiologically, as well as pharmacologically. These findings further highlight the mechanistic differences between the two phases and have proved useful in a number of experiments concerned with the nature of the mechanism by which E-LTP is converted into L-LTP (see section 1.6.2).

1.5.2 Signal Transduction Pathways Involved in L-LTP Induction

To date, a large amount of evidence has suggested that a cyclic adenosine monophosphate (cAMP) regulated signalling pathway plays a key role in the processes underlying the development of L-LTP. Behavioural screening of the fruit fly *Drosophila*, for genetic mutations that effect learning and memory, has identified four different genotypes. Three of these mutations effect molecules involved in the cAMP signalling pathway (Dubnau and Tully, 1998). In addition, cAMP has been shown to be important in synaptic plasticity occurring in the marine snail *Aplysia californica*. *Aplysia* has been studied for the last 20 years as a simple model system in which to investigate associative and non-associative conditioning. Weak tactile stimulation of the siphon or mantle shelf of *Aplysia* (the conditioned stimulus or CS), paired repeatedly with strong electrical shocks to the animal's tail (the unconditioned stimulus or US) results in prolongation of the animal's withdrawal reflex in response to the subsequent presentation of the CS. The pairing of CS and US during conditioning results in an activity dependent presynaptic facilitation in the sensorimotor connections that mediate the response. In 1988 Schacher *et al.* showed that prolonged application of cAMP to neurons involved in the gill withdrawal reflex of *Aplysia*, produced a long-lasting synaptic facilitation lasting 24 hours, that is dependent on protein synthesis (Schacher *et al.*, 1988).

The first evidence that cAMP could be involved in L-LTP in mammals, came from Frey *et al.* (1993). The authors showed that L-LTP in the CA1 region of rat hippocampal slices was blocked by the application of Rp-cAMPS (an inhibitor of the regulatory subunit of cAMP dependent protein kinase (PKA)) when applied during the tetanus. The time course of LTP block was similar to that produced by inhibitors of protein synthesis. Similar findings have been reported by Huang *et al.* (1994) using the drug KT5720 (an inhibitor of the PKA catalytic subunit), although application, 30 minutes after the start of tetanisation, produced no effect on LTP. Rp-cAMPS and KT5720 have also been shown to block L-LTP in mice when applied during the tetanus, although both drugs are ineffective when applied 30 minutes following the tetanus (Nguyen & Kandel, 1997). An involvement of the cAMP pathway in L-LTP induction, has also been reported in both the mossy fibres and in the medial perforant

path, although these will not be discussed here (Huang *et al.*, 1994; Weisskopf *et al.*, 1994; Nguyen & Kandel, 1996).

In the CA1 region, application of the cAMP analogue Sp-cAMPS, known to cause PKA activation (via binding to its regulatory subunits), induces a potentiation that lasts for at least 3 hours, occludes tetanically induced L-LTP and is dependent on protein synthesis (Frey *et al.*, 1993; Bolshakov *et al.*, 1997; Nguyen & Kandel, 1997). Increases in cAMP levels have also been seen one minute following LTP, in response to tetanisation consisting of three trains @ 100Hz or via bath application of NMDA or glutamate (Chetkovitch *et al.*, 1991; Frey *et al.*, 1993). These transient increases (lasting for less than 10 minutes), did not occur in response to a single 100Hz train and were blocked by application of the NMDA receptor blocker AP5, the Ca^{2+} chelator EGTA, or the dopamine D1 receptor agonist SCH 23390 (Chetkovich *et al.*, 1991; Frey *et al.*, 1993). LTP-induced PKA activation has been shown to follow a similar time course to that of cAMP. PKA was transiently activated 2 and 10 minutes after either high frequency stimulation, or NMDA application, and was blocked by the addition of AP5 (Roberson and Sweatt, 1996). This Ca^{2+} and NMDA receptor dependent increase in cAMP has been linked to the enzyme adenylate cyclase (responsible for the conversion of intracellular ATP to cAMP), via calcium/calmodulin (Ca^{2+}/CaM). Chetkovich and Sweatt (1993), using membranes prepared from the CA1 region of hippocampal slices found that adenylate cyclase activity was stimulated by Ca^{2+}/CaM, and that this was blocked by the Ca^{2+}/CaM antagonists W7 and TFP. Furthermore, application of both drugs blocked cAMP increases in the CA1 region of intact hippocampal slices, in response to high frequency stimulation or NMDA application. In agreement with these results, increasing cAMP levels, via application of the drug forskolin, an agonist of adenylate cyclase, has been shown to produce a form of synaptic potentiation (Chavez-Noriega and Stevens, 1992; Huang & Kandel, 1994; Impey *et al.*, 1996; Wong *et al.*, 1999) that occludes the induction of L-LTP (Huang & Kandel, 1994).

Dopamine receptors coupled to adenylate cyclase form part of a modulatory mesolimbic dopaminergic pathway that is known to innervate area CA1. These receptors have also been shown to be important in L-LTP induction. The broad range

dopamine receptor blocker domperidone has been shown to block the induction of L-LTP in the CA1 region of rat hippocampal slices. Both EPSP slope and population spike potentiation decayed to baseline levels over 8 hours (Frey *et al.*, 1990). Subsequent experiments using the selective D1 receptor blocker SCH 23390 also showed a block of L-LTP induction (Frey *et al.*, 1991; Huang and Kandel, 1995). As previously mentioned, SCH 23390 can also block the transient increases in cAMP seen after LTP induction (Frey *et al.*, 1993). In addition to inhibitor studies, application of agonists of the D1/D5 receptors to hippocampal slices induces a long-lasting EPSP potentiation (>6 hours). This potentiation is partially dependent on NMDA receptor activation, occluded by tetanic stimulation and PKA activation, and dependent on protein synthesis (Huang & Kandel, 1995). D1/D5 receptor activation has also been shown to increase the magnitude of E-LTP and to inhibit depotentiation (Otmakhova and Lisman, 1996; Otmakhova and Lisman, 1998). This mechanism has been suggested to occur via adenylate cyclase and cAMP (Otmakhova & Lisman, 1996; Otmakhova & Lisman, 1998).

Evidence from behavioural experiments with rats also points to some involvement of the dopamine/cAMP signalling pathway in spatial memory and L-LTP. Bach *et al.* (1999) showed that old mice (18 months) displayed defects in hippocampal dependent spatial learning tasks and a reduction in the levels of L-LTP (measured *in vitro*) when compared to young animals (3 months). However, these deficits could be 'rescued' by the application of either D1/D5 receptor agonists or a cAMP phosphodiesterase inhibitor, both of which would increase the levels of cAMP (Bach *et al.*, 1999). Similarly, the late memory consolidation phase of rats trained in an inhibitory avoidance task is blocked by the hippocampal infusion of the D1/D5 antagonist SCH 23390, and the PKA catalytic subunit inhibitor KT5720, 3 or 6 hours post-training (Bernabeu *et al.*, 1997). Memory consolidation can also be enhanced, in response to infusion at 3 and 6 hours post-training with either the D1/D5 agonist (SKF 38393), the cAMP analogue 8Br-cAMP or the adenylate cyclase activator, forskolin (Bernabeu *et al.*, 1997).

More recently, genetic evidence in the form of mutant mice studies has provided further support for the involvement of the cAMP signalling pathway in the induction

of L-LTP. Mice expressing an inactivating mutation in the $C\beta_1$ isoform of the catalytic subunit of PKA are significantly impaired in their ability to express L-LTP, but not E-LTP *in vitro*, when compared to wild types (Qi *et al.*, 1996). Similarly, hippocampal slices taken from transgenic mice expressing R(AB), an inhibitory form of the PKA regulatory subunit, show a block of L-LTP induction, with potentiated EPSPs returning to pre-tetanus levels over 3 hours (Abel *et al.*, 1997). In addition, these mice exhibit impaired spatial memory and deficits in long-term (24 hours) but not short-term (1 hour) contextual fear conditioning, thereby mimicking the effects produced by protein synthesis inhibition (Abel *et al.*, 1997). Eight isoforms of adenylate cyclase exist, two of which (AC type I and AC type VIII) are calcium/calmodulin dependent and are present in the CA1 pyramidal cells of the hippocampus. Initial studies on mutant mice in which hippocampal Ca^{2+} stimulated adenylate cyclase (AC) activity was reduced by 50%, due to inactivation of AC type I, found that these animals exhibited normal L-LTP (*in vitro*) and spatial memory (Wu *et al.*, 1995). Similar results were seen in AC type VIII mutant mice in which Ca^{2+} stimulated AC activity was reduced by 30% with no effect on L-LTP induction or long-term memory (Wong *et al.*, 1999). However, mice in which both AC type I and AC type VIII were disrupted, showed a complete block of *in vitro* L-LTP induction, paralleled by a disruption of long-term memory for passive avoidance learning and contextual fear conditioning (Wong *et al.*, 1999). Additionally, infusion of the AC activator forskolin, into the CA1 region of these mutant mice, 15 minute prior to passive avoidance training, restored long-term memory (Wong *et al.*, 1999). Early genetic evidence for dopamine receptor involvement in L-LTP seems to be in agreement with the previous pharmacological data. Hippocampal slices taken from mutant mice deficient in the D1 dopamine receptor do not support L-LTP, with EPSP potentiation returning to pre-tetanus levels over 8 hours (Matthies *et al.*, 1997a).

Recently, a growing body of evidence suggests that the cAMP/PKA pathway is involved in both structural and functional changes occurring during L-LTP. Bolshakov *et al.* (1997) studying synaptic transmission between connected pairs of single CA3 and CA1 neurons in hippocampal slices showed that E-LTP (induced by a pairing protocol) was accompanied by an increase in the probability of release. However, cAMP-induced L-LTP in the same system, involved an increase in the

number of quanta released, possibly due to recruitment of new sites of synaptic transmission (Bolshakov *et al.*, 1997). In line with these results, application of Sp-cAMP has been shown to increase the number of active presynaptic boutons in cultured CA3 and CA1 neurons via a mechanism dependent on PKA, protein synthesis and NMDA and AMPA receptor activation (Ma *et al.*, 1999). Finally, evidence implicating PKA in a postsynaptic mechanism maintaining L-LTP has also been proposed. L-LTP can occur in CA1 mini-slices which lack the CA3 region and therefore the presynaptic cell bodies (Nayak *et al.*, 1998). In addition, increased AMPA receptor synthesis (GluR 1 and GluR2/3) has been shown to occur 3 hours following the induction of LTP in the mini-slice. In these preparations, both L-LTP and AMPAR synthesis are blocked by inhibitors of PKA (Nayak *et al.*, 1998).

As previously outlined, both pharmacological and genetic evidence points to an involvement of the cAMP signalling pathway in the generation of L-LTP. Inhibiting the pathway at a variety if points (cAMP, PKA, D1/D5, AC), produces LTP that returns to pre-tetanus levels over a time course comparable to that seen in the presence of transcription and translation inhibitors. In addition, potentiation induced by artificially increased levels of cAMP, is dependent on transcription and translation, whilst AMPAR synthesis following L-LTP induction is dependent on PKA. These findings have been suggested by some, to be an indication that cAMP inducible gene expression may underlie L-LTP in the CA1 region of the hippocampus. A possible mechanism by which this may occur, involves a family of transcription factors called the cAMP-response-element-binding proteins (CREB). In response to phosphorylation on Ser-133 by either PKA or CaM kinases I and II (activated by increased levels of cAMP and Ca^{2+} respectively) (Gonzalez and Montminy, 1989; Sheng *et al.*, 1991), CREB binds to, and promotes the transcription of genes containing cAMP response elements (CREs) in their promotor sequences. Early evidence that CREB-mediated gene expression may be important in learning and memory came from studies on invertebrates. Dash *et al.* (1990) studying *Aplysia*, inhibited CREB function via injection of a CRE oligonucleotide into the nucleus of the presynaptic cell. This resulted in a block of long-term synaptic facilitation (dependent on transcription and translation) at this synapse whilst leaving short-term facilitation unaffected. Studies on the fruit fly *Drosophila*, show that associative

long-term memory in the behaving animal requires CREB function (Yin *et al.*, 1994). Heat shock induced expression of a dominant negative isoform of the fly CREB gene transgene completely blocked a translation dependent form of long-term memory whilst having no effect on a translation insensitive short-term memory. In a similar study, transgenic flies expressing an activator isoform of CREB showed enhanced long-term memory formation when compared to wild type animals. Furthermore, this enhancement was dependent on the phosphorylation of Ser-231 (equivalent to Ser-133 on mammalian CREB) (Yin *et al.*, 1995).

The first evidence that CREB may play a role in LTP and long-term memory in mammals came from Bourtchuladze *et al.* (1994) who produced a mutant mouse expressing a targeted disruption in the α and δ isoforms of CREB. Hippocampal slices taken from these mice, tetanised with a single 1 second train @ 100Hz, produced a decremental LTP that returned to pre-tetanus levels over 2 hours. Wild type mice exhibited a 25% EPSP slope potentiation in response to this stimulus, over the same time period. In parallel with these results, $CREB^{\alpha\delta-}$ mice were deficient in long-term memory for cued and contextual conditioning (measured 24 hours after training), whilst initial memory (measured 30 minutes after training) remained unaffected. Spatial memory in these mice was also affected, although these deficits could be partially rescued by intensive training (Bourtchuladze *et al.*, 1994). In agreement with the previous paper, others have demonstrated a rescue of the long-term memory deficits observed in these $CREB^{\alpha\delta-}$ mice, in response to changes in the training protocol. Deficits seen in contextual fear conditioning, spatial learning and social transmission of food preferences in $CREB^{\alpha\delta-}$ mice, were overcome by lengthening the inter-trial interval during training, from 1 to 60 minutes (Kogan *et al.*, 1997). More recently, contradictory reports have been published stating that $CREB^{\alpha\delta-}$ mice show no significant impairment in spatial memory or social transmission of food preference, whilst LTP in both the CA1 and dentate gyrus of hippocampal slices taken from these animals was unaffected over 2 hours post tetanus (Gass *et al.*, 1998). The CREB mutant mice results are confusing and although varying genetic backgrounds of the mice may account for some of the differences, a clear role for CREB in L-LTP or memory in mammals has yet to be shown.

Circumstantial evidence for CREB involvement in L-LTP does exist. CREB phosphorylation (measured using a phospho-specific anti CREB antibody, or pCREB) occurs in response to L-LTP (>24 hours), but not E-LTP (<6 hours) induction in the dentate gyrus of rats *in vivo* (Schulz *et al.*, 1999). This CREB phosphorylation is NMDA dependent and biphasic, with the first peak at 30 minutes post-tetanus and a second peak beginning 2 hours post-tetanus and lasting for at least 24 hours (Schulz *et al.*, 1999). A rapid and transient increase in CREB phosphorylation, peaking at 30 minutes and returning to control levels at 1 hour, has also been seen in the CA1 region of hippocampal slices in response to high frequency stimulation (Matthies *et al.*, 1997b). Increases in pCREB immunoreactivity have been observed in the CA1 region of the hippocampus, immediately, 3 and 6 hours after avoidance task training in rats (Bernabeu *et al.*, 1997). Although CREB has previously been shown to be phosphorylated by PKA (Gonzalez & Montminy, 1989), whether the CREB phosphorylation occurring during LTP and/or learning, is mediated by the cAMP/PKA pathway remains to be seen. Some evidence suggests that it is not. Deisseroth *et al.* (1996) show that pCREB immunoreactivity is increased in cultured CA1/CA3 hippocampal neurons in response to, but not exclusively by LTP inducing stimuli and blocked by a combination of AP5 and CNQX, or AP5 and the L-type Ca^{2+} channel blocker, nimodipine. In addition, application of the calmodulin inhibitor calmidazolium or the CaMKII inhibitor KN-62 blocked pCREB in response to LTP inducing stimuli, whist the PKA inhibitors KT5720 and Rp-cAMPS had no significant effect (Deisseroth *et al.*, 1996). These results suggest that CREB phosphorylation, in this system at least, occurs via CaM kinase activity in response to increases in Ca^{2+}/CaM brought about by synaptic activity, rather than via the cAMP pathway.

Results published around the same time as those of Deisseroth *et al.*, suggest that the cAMP/PKA pathway does indeed play a role in CRE mediated gene expression associated with L-LTP. Impey *et al.* (1996) produced mutant mice that contained six copies of the cAMP response element (CRE) linked to a LacZ reporter gene (CRE-LacZ). This provided an assay by which CRE induced gene expression could be monitored in response to L-LTP induction. Increased CRE-regulated gene expression was seen in cultured hippocampal neurons in response to application of the adenylate

cyclase activator forskolin and the D1/D5 receptor agonist SKF-38393, and in hippocampal slices in response to forskolin. In addition, stimulation paradigms that induced L-LTP but not those that induced E-LTP, produced increased levels of CRE-mediated gene expression in the CA1 region of hippocampal slices, when measured 4 hours after tetanisation (Impey *et al.*, 1996). Inhibition of L-LTP by the application of PKA or L-type Ca^{2+} channel inhibitors, resulted in an associated inhibition of this CRE-mediated gene expression (Impey *et al.*, 1996). These CRE-LacZ transgenic mice have also been used to look at the levels of CRE-mediated gene expression during hippocampal dependent learning. Both contextual conditioning and passive avoidance learning have been shown to increase CRE-mediated gene expression, as well as CREB phosphorylation in the hippocampal CA1 region, when measured 8 hours, and 30 minutes after training, respectively (Impey *et al.*, 1998b).

Although much of the early interest in L-LTP induction has involved the cAMP/PKA pathway and its modulation of CREB and CRE, a number of other pathways may also play a role in L-LTP induction. One of these is the Ras-MAPK (mitogen activated protein kinase) pathway. In addition to an involvement in E-LTP (see section 1.4.1) MAPK may modulate the induction of L-LTP via stimulation of gene expression. This signalling pathway is vast and complex and is reviewed elsewhere (Orban *et al.*, 1999), although it is briefly summarised below. In response to GEF (guanine-nucleotide exchange factors) activation, members of the Ras family of GTP binding proteins activate the Raf family of proteins which relocate to the cell membrane and phosphorylate MEKs (mitogen activated protein kinase kinases). The MEKs in turn, activate MAPKs (mitogen activated kinases). Activation of GEFs (and thus Ras) can occur via ligand-activation of receptor tyrosine kinases (RTKs) such as the TrkB receptor, sensitive to the neurotrophin, BDNF (brain derived neurotrophic factor). Interestingly, application of antisera or fusion proteins that block TrkB function (either before or up to 30 minutes after tetanisation), block L-LTP, giving rise to an E-LTP that is protein synthesis independent and returns to pre-tetanus levels over 3 hours (Kang *et al.*, 1997; Korte *et al.*, 1998). Similarly, mutant mice that lack BDNF do not express L-LTP (Korte *et al.*, 1998; for a recent review of BDNF in LTP see Lu and Chow, 1999). Therefore, BDNF may lead to L-LTP induction via MAPK activation, mediated by its affect on the TrkB receptor.

Pharmacological experiments have also shown a role for the MAP kinase, ERK (extracellular regulated kinase), in L-LTP induction. Bath application of the MEK inhibitor PD98059, when applied during the tetanus caused EPSP slope potentiation to return to baseline levels over 3 hours in hippocampal slices (Impey *et al.*, 1998a). Additionally, L-LTP induced chemically in hippocampal slices, via the application of forskolin, is attenuated by either PD98059 addition, or in slices taken from rats that had undergone injection with MAPK antisense oligonucleotide (Wu *et al.*, 1999).

MAPKs phosphorylate a large range of substrates within the cell, including several transcription factors. NMDA-dependent Ca^{2+} influx in cultured hippocampal neurons has been shown to activate expression of the immediate early gene *c-fos* via a mechanism dependent on the phosphorylation of the transcription factor ElK-1 by ERK (Xia *et al.*, 1996). Similarly, phosphorylation of another transcription factor, *c-jun*, is also positively regulated by MAPKs (Pulverer *et al.*, 1991). In addition, Impey *et al.* (1998a) using hippocampal cell cultures, showed that both ERK and PKA activity were required for Ca^{2+} activated CRE-mediated gene transcription. Increases in intracellular Ca^{2+}, were found to cause the translocation of ERK to the nucleus and the subsequent phosphorylation and activation of CREB via Rsk2 (a Ca^{2+} stimulated CREB kinase). Furthermore, this ERK translocation to the nucleus was dependent on PKA activation. The authors suggest that the dependence of CREB-mediated gene expression on PKA may be due to its involvement in the nuclear translocation of ERK rather than in the direct phosphorylation of CREB (Impey *et al.*, 1998a). Recently, similar results to those of Impey *et al.* have been seen *in vivo*. LTP induction in the dentate gyrus of rats was shown to give rise to rapid phosphorylation and nuclear translocation of ERK, accompanied by an increase in phosphorylation of the transcription factors CREB and Elk-1 and the upregulation of the IEG *zif268* (Davis *et al.*, 2000). In addition, LTP and these associated changes were blocked by the injection of the MEK inhibitor SL327, 1 hour before LTP induction (Davis *et al.*, 2000).

A role for MAPKs in behaviour has recently been demonstrated in rats. A transient increase in activated ERK and Elk-1 levels (peaking at 30 minutes and subsiding to basal levels at 60 minutes) was seen in the insular cortex of rats in response to

experiencing a novel taste but not a familiar taste (Berman *et al.*, 1998). In addition, long-term taste aversion (in which rats avoided a novel taste that had been previously paired with a malaise-inducing agent), was attenuated by injection of the MEK inhibitor PD98059, whilst short-term memory was unaffected (Berman *et al.*, 1998). Finally, rats injected with inhibitors of protein synthesis, PKA or MAPK show similar deficits in long-term (24 hours) but not short-term (1 hour) memory for contextual and cued fear conditioning (Schafe *et al.*, 1999).

In addition to a role for protein kinases in L-LTP, protein phosphatases such as Ca^{2+}-sensitive calcineurin (protein phosphatase 2B) have also been implicated. Calcineurin can dephosphorylate inhibitor 1 (I-1), which, when phosphorylated inhibits the function of protein phosphatase 1 (PP1). Therefore dephosphorylation of I-1 by calcineurin activates PP1 and leads to the dephosphorylation of a large set of proteins. In addition, calcineurin acts in opposition to the cAMP cascade by dephosphorylating the site on I-1 phosphorylated by PKA. Recent experiments suggest that the cAMP/PKA pathway may suppress the actions of PP1 thereby gating LTP. (Blitzer *et al.*, 1998; Makhinson *et al.*, 1999). Winder *et al.* (1998) found that mutant mice, overexpressing calcineurin do not show L-LTP. Furthermore, the authors define an intermediate LTP or I-LTP, that was induced by multiple trains and dependent on PKA but not protein synthesis. I-LTP in the calcineurin overexpressing mice was found to be reduced when compared to LTP in wild type animals, but could be rescued by the protein phosphatase inhibitor, calyculin A. The authors suggest that calcineurin places an inhibitory constraint on I-LTP that can be relieved by PKA, and that this mechanism could gate the induction of L-LTP (Winder *et al.*, 1998). A possible mechanism by which this may occur is via PKA and calcineurin acting to regulate CREB activation. The role of PKA in CREB activation has already been discussed, whilst inhibitors of PP1 have been shown to block the dephosphorylation (and thus inactivation) of CREB in cultured hippocampal neurons (Bito *et al.*, 1996). Further support implicating calcineurin in memory formation comes from behavioural studies. Calcineurin overexpressing mice, show deficits in long-term but not short-term, spatial and non-spatial memory. However, long-term spatial memory deficits could be rescued by increasing the number of training trails per day, from 1 to 4 (Mansuy *et al.*, 1998).

1.5.3 Immediate Early Genes and L-LTP

Much of the pharmacological and genetic evidence highlighted thus far, points to an involvement of gene expression in L-LTP. Following extracellular stimulation such as that provided by growth factors or neurotransmitters, a small subset of genes undergo a rapid and transient increase in expression that is protein synthesis independent. These are called immediate early genes (IEGs). What are the candidate IEGs expressed in response to L-LTP induction, and what role might they play (if any) in the consolidation of long term memory? In 1987, Morgan *et al.* reported that following the injection of a seizure inducing drug, mRNA levels of the transcription factor *c-fos* were increased in mouse brain, within 60 minutes, declining to baseline levels over 3 hours. Similarly, a transient increase in *c-fos* protein immunoreactivity was seen in the dentate gyrus of rats within 30 minutes of an electrically-induced seizure produced by kindling (Dragunow and Robertson, 1987). Following these studies, a number of mRNAs from genes encoding for the transcription factors *zif268*, *c-jun*, and *jun-B*, in addition to *c-fos* were found to be induced in hippocampal neurons as well as other brain areas in the rat, in response to drug induced seizure (Saffen *et al.*, 1988). The mRNA increases were rapid and transient, occurring within 1 hour and returning to baseline within 2 hours. Furthermore, *zif268* (also known as *Krox-24*, *EGR-1*, or *NGFI-A*) mRNA levels in the dentate gyrus of anaesthetised rats, were found to increase in response to high frequency (but not low frequency) stimulation that could induce LTP (Cole *et al.*, 1989). The increases in *zif268* expression, only occurred in the ipsilateral hippocampus and were blocked by NMDA receptor inhibitors (Cole *et al.*, 1989). Initially, the levels of *zif268* (and to a lesser extent *c-jun*, *jun-B*, *jun-D* and *fos* related mRNAs) induction in the dentate gyrus of awake rats following LTP had been shown to be highly correlated with the duration of the LTP rather than with the magnitude of the initial LTP. (Richardson *et al.*, 1992; Abraham *et al.*, 1993). However, other studies have shown that the link between LTP and the induction of these transcription factor IEGs is not quite so straightforward. Wisden *et al.* (1990), reported that LTP in the dentate gyrus of anaesthetised rats did not result in the induction of mRNA for *c-fos*, *c-jun*, *jun-D* and a number of other IEGs. Although *zif268*, and to a lesser extent, *jun-B* induction was seen following NMDA dependent LTP, non-specific induction of all the IEGs examined occurred in

response to forms of stimulation that activated granule cells but did not induce LTP. In addition, no changes in IEG induction were seen in animals trained for spatial learning in the water-maze (Wisden *et al.*, 1990). Consistent with these results, *c-fos* induction in unanaesthetised rats did not correlate with LTP induction (Dragunow *et al.*, 1989).

Despite the ambiguity of the early findings, recent evidence does suggest that *zif268* is necessary for both L-LTP and long-term memory (Jones *et al.*, 2001). *In vivo* recordings from awake mutant mice with a targeted disruption of *zif268*, showed that although LTP was unaffected for the first hour after induction, it was absent when measured 24 and 48 hours post-tetanus. Furthermore, these mice displayed deficits in long-term but not short-term memory for a number of spatial and non-spatial learning tasks (Jones *et al.*, 2001). The finding that both *zif268* and *c-fos* contain cAMP response elements (CREs) in their promotor sequences (Sassone-Corsi *et al.*, 1988; Sakamoto *et al.*, 1991), suggests a possible role for these transcription factor IEGs in the CREB mediated regulation of expression of, yet unknown, late effector genes, that could govern the duration of L-LTP. However, although it appears that *zif268* is necessary for the induction of L-LTP it has still not been determined whether this gene is produced constitutively, or is specifically induced during L-LTP induction.

In addition to the transcription factor IEGs, there is also a small set of IEGs that have the potential to directly modulate changes involved in LTP. One of the best characterised of these IEGs is tissue-plasminogen activator (tPA), an extracellular serine protease that converts plasminogen into plasmin. *In situ* hybridisation showed that tPA is rapidly induced (within 1 hour) in the whole brain in response to injection with seizure inducing drugs, or increased throughout the hippocampus in response to kindling stimulation in the perforant path (Qian *et al.*, 1993). Furthermore, tPA expression was found to rapidly increase (within 1 hour), specifically in the ipsilateral dentate granule neurons, in response to an LTP inducing tetanus delivered to the perforant path. The tPA increase was transient (decreasing at 6 hours and returning to baseline levels at 24 hours) and blocked by the NMDA receptor inhibitor MK801 (Qian *et al.*, 1993).

Inhibitors of tPA have been shown to block L-LTP induced by either tetanic stimulation or forskolin application in both the CA1 and CA3 regions (Baranes *et al.*, 1998). Furthermore, application of tPA paired with a stimulus that would normally induce E-LTP can produce an L-LTP in both these regions (Baranes *et al.*, 1998). A possible mechanism involving the production of L-LTP by tPA mediated synaptic growth is provided by Baranes *et al.* (1998). The authors showed that cultured dentate granule cells secrete tPA and undergo axonal elongation and formation of presynaptic varicosities in response to forskolin application. These changes were blocked by inhibitors of tPA and could be induced by the application of tPA alone. Furthermore, mutant mice lacking the tPA gene are impaired in their ability to exhibit L-LTP (Huang *et al.*, 1996; Calabresi *et al.*, 2000), and show reduced potentiation in response to application of cAMP analogs and D1/D5 agonists (Huang *et al.*, 1996). However, not all studies agree. Frey *et al.* (1996c) found that L-LTP in tPA mutant mice was unaffected, although L-LTP in these mice, was blocked by inhibition of GABAergic transmission. In addition, conflicting results seen from behavioural studies, have made it difficult to determine whether tPA is involved in hippocampal-dependent learning (Huang *et al.*, 1996; Calabresi *et al.*, 2000).

Another IEG that may have a role in the consolidation of L-LTP is the activity regulated cytoskeletal-associated protein, *Arc* (also termed *Arg 3.1*). *Arc* mRNAs are enriched in brain and hippocampus after drug or electrically-induced seizures. In addition, LTP-inducing, high-frequency stimulation, applied to the perforant path resulted in a robust induction of *Arc* in the granule cells of the ipsilateral hippocampus (Link *et al.*, 1995; Lyford *et al.*, 1995). Interestingly, both *Arc* mRNA and protein are localised in the soma as well as in the dendrites of the granule cells (Link *et al.*, 1995; Lyford *et al.*, 1995; Wallace *et al.*, 1998). It is thought that *Arc* may play a role in the activity-dependent response of synapses by modifying the function of existing proteins. Indeed *Arc* mRNA and protein have been shown to selectively localise to regions of recent synaptic activity in granule cell dendrites (Steward *et al.*, 1998). More recently, using *in situ* hybridisation and confocal microscopy, *Arc* mRNA has been visualised in the nucleus within minutes of the exposure of a rat to a specific environment. Furthermore, within 30 minutes this *Arc* mRNA had accumulated in the cytoplasm (Guzowski *et al.*, 1999). The ratio of the

nuclear to cytoplasmic *Arc* mRNA could be used to infer the recent environmental exposure of the animals (Guzowski *et al.*, 1999). These results provide evidence that *Arc* may be induced in response to behaviour in the intact animal. Finally, inhibition of *Arc* protein expression, via hippocampal infusion of antisense oligodeoxynucleotides has been shown to block L-LTP and long-term but not short-term spatial memory in rats (Guzowski *et al.*, 2000).

In addition to *Arc* and tPA a number of other IEGs seem to be correlated to LTP induction. The IEG *Homer* is induced in the dentate gyrus of freely moving rats in response to LTP inducing stimulation. Homer protein appears to target excitatory synapses and dendritic spines. Homer contains a PDZ-like binding domain, and can bind to type1 and type 5 metabotropic glutamate receptors (mGluRs) (Brakeman *et al.*, 1997). Expression of the IEG form of *Homer* can modulate mGluR-induced intracellular Ca^{2+} release (decreasing amplitude and increasing latency), suggesting a mechanism by which it could modulate synaptic function during L-LTP (Tu *et al.*, 1998). Recently, a serine/threonine kinase, *Pim-1* (provirus integration site for Moloney murine leukemia virus), has been shown to be consistently, and strongly induced in dentate granule cells in response to LTP inducing high frequency stimulation, showing both nuclear and dendritic localisation (Konietzko *et al.*, 1999). Mutant mice deficient for *Pim-1* show no L-LTP in the CA1 region, whilst E-LTP is relatively unaffected (Konietzko *et al.*, 1999). A number of other IEGs exist that encode proteins with the potential to directly modify synaptic plasticity, such as *BAD2* (a MAPK phosphatase)(Qian *et al.*, 1994), *Cox-2* (an inducible form of cyclooxygenase), *Rheb* (a Ras homologue), and *Narp* (a calcium-dependent lectin), however less evidence for their involvement in LTP exists (if any), and these are discussed elsewhere (for a recent review see Lanahan and Worley, 1998). In summary, the evidence for the involvement of a small number of IEGs in L-LTP and long-term memory is promising. However, it remains to be seen whether or not the specific induction of these genes during potentiation is necessary for the maintenance of L-LTP.

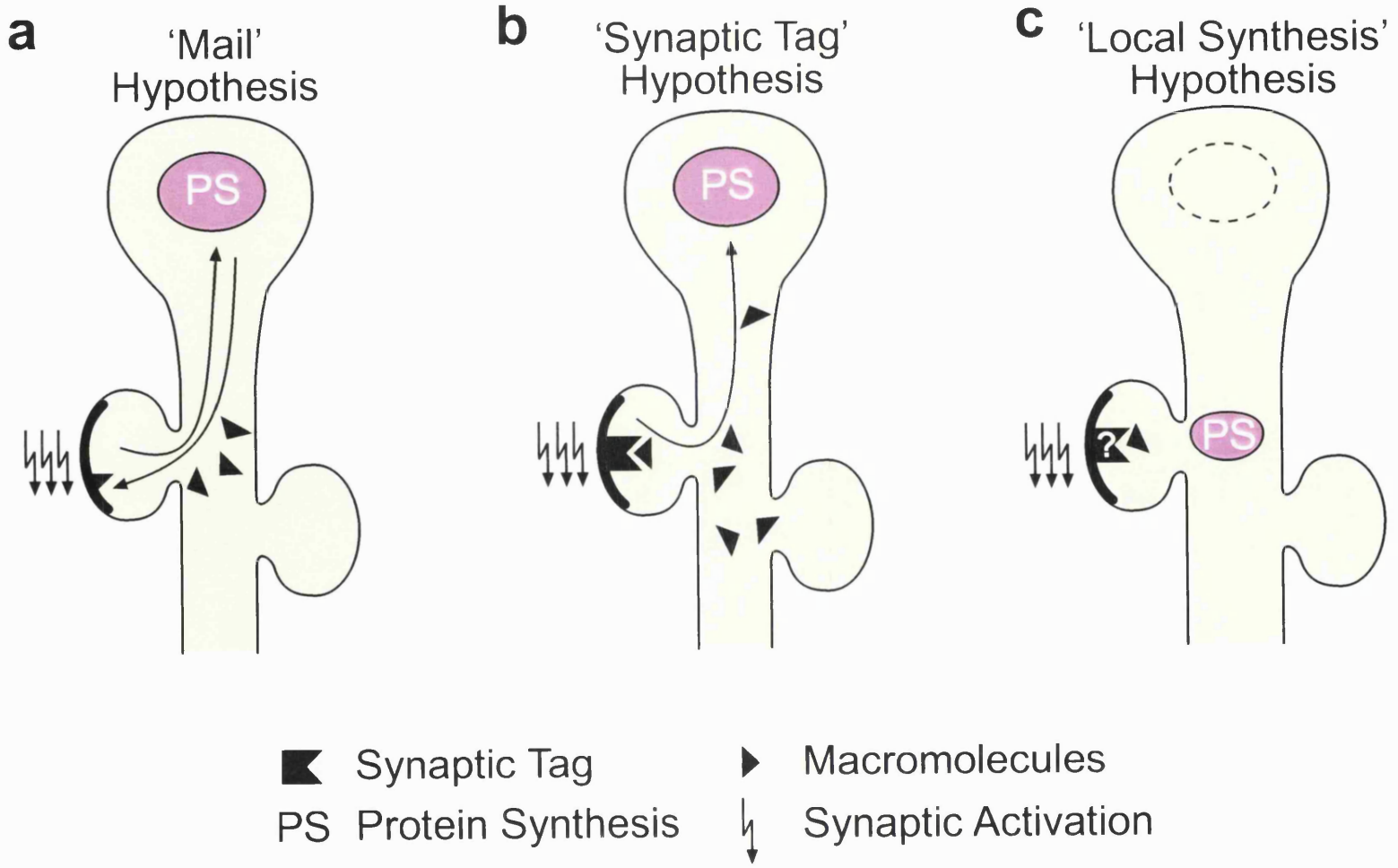
1.6 THE LOCUS OF PROTEIN SYNTHESIS DURING L-LTP

As we have seen in the previous sections, L-LTP exhibits a requirement for both transcription and translation. Transcription, and to a lesser extent translation are somatic events, taking place in the cell bodies of neurons, some distance away from the site of potentiation in the dendrites. Since a key feature of LTP is input specificity, separate synapses or groups of synapses must be potentiated on a single neuron, whilst others remain unchanged. How, therefore can a dependence on somatic mRNA and protein synthesis be reconciled with the synapse specificity required for L-LTP? In other words, how do mRNAs or proteins reach potentiated synapses, whilst unpotentiated synapses remain unaffected by their synthesis. Three basic hypotheses have been proposed outlining how synapse specificity in L-LTP could be achieved (reviewed by Schuman, 1997; Frey and Morris, 1998c): the 'mail' hypothesis, the 'synaptic tag' hypothesis and the 'local synthesis' hypothesis (see Fig. 1.1). Each of these will be discussed in turn below.

1.6.1 The 'Mail' Hypothesis

In the 'mail' hypothesis, L-LTP induced at the synapses, generates a signal that can travel back to the soma inducing transcription and translation (Fig 1.1a). Newly synthesised proteins are then specifically targeted via a mechanism activated at the time of their synthesis, to the potentiated synapses that require them. This hypothesis seems overly complex, especially when you consider that an individual CA1 pyramidal cell may have more than 10,000 dendritic spines corresponding to over 10,000 individual synapses. To target one or a small group of these synapses, from instructions laid down in the nucleus seems unlikely. To date there is no direct evidence that this process actually occurs within neurons during synaptic plasticity. Recently, Steward *et al.* (1998) found that repeated high-frequency stimulation of the medial perforant path (MPP) caused newly synthesised protein for the IEG *Arc*, to localise to the middle molecular layer of the dentate granule cell dendrites; the area that is directly innervated by the MPP. Therefore, newly synthesised protein can localise at sites of recent synaptic activity. Using the same technique, area specific localisation in response to synaptic activity has also been seen for CaMKII and MAP2

Figure 1.1 Three hypotheses for synapse specificity in L-LTP. a, The 'mail' hypothesis involves elaborate intracellular trafficking of proteins back to individual synapses (indicated by curved line).**b**, The 'synaptic tag' hypothesis involves setting a tag at activated synapses that sequesters plasticity related proteins (synthesised in the soma) that are distributed throughout the dendritic tree. **c**, The 'local synthesis' hypothesis requires that the relevant protein synthetic machinery is present at the level of the dendrites or single spines. Therefore plasticity related proteins are synthesised from mRNA present in the dendrites (modified from Frey and Morris 1998).


a
'Mail' Hypothesis
PS
b
'Synaptic Tag' Hypothesis
PS
c
'Local Synthesis' Hypothesis
?
PS
Synaptic Tag
PS Protein Synthesis
Macromolecules
Synaptic Activation

protein (Steward and Halpain, 1999). It is important to note however, that this does not indicate a specific trafficking mechanism for these proteins and could equally be due to activity dependent sequestering of protein distributed randomly throughout the dendrites (see the 'synaptic tag' hypothesis), or local translation of protein at the dendrites (see 'local synthesis' hypothesis).

1.6.2 The 'Synaptic Tag' Hypothesis

An alternative mechanism by which L-LTP at specific synapses could be achieved is via a synaptic tag (Fig. 1.1b). This hypothesis states that L-LTP induction at a single or small group of synapses, sets a tag whose purpose is to sequester proteins. Again a message travels back to the soma, activating transcription and translation. However, instead of being specifically trafficked, these proteins are distributed randomly throughout the dendritic processes and are only captured at those synapses expressing the tag. This hypothesis was proposed by Frey and Morris (1997). To explore this idea they set up a 'two pathway' experiment in the CA1 region of hippocampal slices *in vitro*. This enabled the recording from, and stimulation of field EPSPs from two separate afferent pathways (P1 and P2), converging on the same group of CA1 pyramidal cells. Using this experimental configuration, Frey and Morris found that L-LTP could be induced in P1 (with P2 acting as a baseline control) under control conditions, and that L-LTP in both pathways could be blocked (returning to pre-tetanus levels over 8 hours) via bath application of the protein synthesis inhibitor anisomycin, during the tetanus. The authors also demonstrated that L-LTP could be induced in P2 in the presence of anisomycin if it had been preceded 50 minutes earlier by L-LTP induction of P1 in the absence of the drug. This suggested that proteins synthesised in response to L-LTP induction in P1 could be utilised for the induction of L-LTP in P2.

Further experiments showed that the ability to induce L-LTP was dependent on the history of activation. In the presence of anisomycin, L-LTP could be induced in previously potentiated neurons, whilst 'naïve' neurons in the same slice showed only E-LTP. In addition to experiments with anisomycin, Frey and Morris also found that E-LTP (produced by weaker tetanisation) in one pathway in a specific group of

neurons, could be converted into a L-LTP if given 30 minutes after an L-LTP inducing stimulus from a separate but convergent pathway. One interpretation of these results is that both E-LTP-inducing stimuli or L-LTP-inducing stimuli in the presence of anisomycin are sufficient to set a tag but not to induce protein synthesis, thus resulting only in E-LTP. If, however, a source of newly synthesised plasticity related proteins is provided to these neurons (i.e. via previous L-LTP induction), L-LTP occurs normally. Therefore, L-LTP may be dependent on protein synthesis in the cell bodies coupled with the protein synthesis-independent setting of a tag at activated synapses. In this way tagged synapses can capture newly synthesised proteins that are distributed randomly throughout the dendrites in response to L-LTP-inducing stimuli.

These results may also be explained by another mechanism, whereby, in response to L-LTP induction, a cell wide priming of synapses occurs (possibly due to increases in the levels of plasticity related proteins) resulting in the lowering of the threshold for subsequent L-LTP. Therefore stimuli that could previously only induce E-LTP can now induce L-LTP. Frey and Morris (1998b) addressed this concern using a 'weak before strong' experimental design. They showed that E-LTP (induced via weak tetanisation) that would normally decay to baseline levels over 8 hours, could be rescued and converted into L-LTP if it was followed within a limited time window (<2hours) by a strong, L-LTP inducing stimulus. The time window during which this rescue could occur may provide a measure of the lifetime of the tag, suggesting a rapid induction and a decay of 1-2 hours. These results rule out the possibility of a priming effect of strong stimulation in the induction of L-LTP and provide further evidence for the sequestering of plasticity-related proteins via a tag.

Further evidence for the presence of a synaptic tag has recently been reported during LTD in hippocampal organotypic cultures. Kauderer and Kandel (2000) demonstrated that a transient early-LTD (E-LTD) was transformed into a more persistent late-LTD (L-LTD) that was dependent on both transcription and translation, if it had been preceded by the induction of L-LTD at another input on the same population of cells. The existence of a synaptic tag has also been proposed for the production of

long-term facilitation in the marine snail *Aplysia*; these data are discussed in section 1.7.6.

Although good evidence exists in support of a synaptic tag, the nature of the tag is still unknown. A number of candidate mechanisms have been suggested. The tag could take the form of a morphological change such as an increase in spine neck diameter (allowing molecules easier access) or spine branching. Alternatively, the tag could represent covalent changes in an enzyme present at the synapses, such as the phosphorylation of a specific kinase. One could postulate that modification of newly synthesised plasticity-related proteins by this synaptically localised enzyme might be a prerequisite for subsequent long-term synaptic changes that result in L-LTP. In this way, plasticity-related proteins would only be utilised at specific synapses expressing the tag. Alternatively, the tag could act as an address marker that could direct plasticity related proteins to potentiated synapses once they had been distributed into the dendrites.

As previously discussed, the 'mail' and the 'tag' hypotheses have very different mechanisms by which plasticity related proteins could be directed to the synapses that require them. However, both hypotheses have a requirement for some form of message from the synapse back to the nucleus to induce gene expression and protein synthesis. To date, the identity of such a message remains a mystery. One possibility is that, a molecule could travel back to the nucleus, from specific synapses, in response to LTP induction. On arrival, this molecule could activate gene transcription, and translation of new plasticity related proteins. A number of proteins that have been shown to be required for L-LTP induction, have also been shown to translocate into the nucleus from the cytoplasm. Using fluorescence imaging techniques, catalytic subunits of PKA have been shown to dissociate from their regulatory subunits and translocate into the nucleus of cultured mammalian cells (accompanied by an increase in CREB phosphorylation), within 5 minutes of stimulation via application of the adenylate cyclase agonist, forskolin (Nigg *et al.*, 1985; Hagiwara *et al.*, 1993). Using confocal imaging techniques, real time images of cAMP and PKA migrations within living cells have been observed. PKA labelled with the fluorescent dyes fluorescein and rhodamine on the catalytic (C) and regulatory (R) subunits respectively, was

injected into *Aplysia* sensory neurons (Bacskai *et al.*, 1993). The ratio of fluorescein to rhodamine emissions (imaged using a confocal microscope) provide an indication of the extent of the dissociation of the PKA, R and C subunits and thus a measure of the cAMP levels (responsible for this dissociation) within the cell. In response to application of the drug serotonin (5-HT) (which induces synaptic facilitation in these preparations), cAMP levels increased, resulting in a translocation of the PKA catalytic subunits into the nucleus (Bacskai *et al.*, 1993). This technique has also been used to image cAMP/PKA levels in the intact neural circuit of the lobster stomatogastric ganglion (STG). Under conditions of repeated stimulation, cAMP, produced initially in fine neurites, diffused throughout the neuritic tree where it eventually reached the cell body (Hempel *et al.*, 1996). This result provides a mechanism whereby distal synaptic stimulation could lead to gene induction via the retrograde 'diffusion/transport' of cAMP to the soma.

In addition to cAMP and PKA, MAPK has also been shown to translocate to the nucleus in mammalian cell cultures in response to growth factors or serum treatment (Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993). More recently, increases in intracellular cAMP levels (via the application of forskolin), or induction of long-term facilitation (produced by repeated pulses of 5-HT) in *Aplysia*, have been shown to involve MAPK nuclear translocation (Martin *et al.*, 1997b). Furthermore, long-term, but not short-term facilitation is blocked by intracellular injection of either anti-MAPK antibodies, or inhibitors of MAPK (Martin *et al.*, 1997b). A rapid translocation (<1 min) of calmodulin into the nucleus, and a subsequent increase in CREB phosphorylation has also been seen following electrical stimulation of cultured hippocampal CA3/CA1 neurons (Deisseroth *et al.*, 1998). Even CREB protein itself has been proposed as a signalling molecule from the synapse to the nucleus (Crino *et al.*, 1998). Injection of a fluorescently tagged CREB protein into the proximal dendrites of cultured hippocampal neurons results in the unidirectional translocation of CREB to the cell bodies where it accumulates in the nucleus (Crino *et al.*, 1998). However the presence of endogenous CREB protein in dendrites has yet to be seen.

Although the translocation of various LTP related proteins into the nucleus has been shown, most of these studies have been performed using non-neuronal cells, or the

somatic regions of cultured hippocampal neurons. These experiments do not address the movement of specific messenger proteins from synapses on distal dendrites back to the cell bodies. In the single case where this has been shown (examining cAMP levels in the lobster STG) retrograde 'transport' is slow and dependent on artificially high levels of synaptic stimulation (Hempel *et al.*, 1996). A more rapid signal may therefore be required.

One candidate mechanism is the spread of Ca^{2+} ions from the synapse to the nucleus. In line with this view, LTP induction in hippocampal neurons, produced by the pairing of a back-propagating action potential with synaptic stimulation, gives rise to a supralinear Ca^{2+} transient at the dendritic spine that can spread to the soma (for a recent review see Emptage, 1999). The arrival of this Ca^{2+} signal could activate a number of pathways involved in gene expression. The supralinear Ca^{2+} signal has been shown to be dependent on the synergistic interaction of Ca^{2+} entry via voltage sensitive Ca^{2+} channels (VSCC) and IP_3 production via mGluR activation (Nakamura *et al.*, 1999). Recently, pharmacological or synaptic stimulation of group 1 mGluRs has been shown to produce an input specific conversion of a weak, decaying form of LTP, that was not dependent on protein synthesis, into a more persistent form that was blocked by inhibitors of translation but not transcription. These results suggest a role for group I mGluRs in the regulation of protein synthesis from pre-existing mRNA (Raymond *et al.*, 2000). Consistent with this result, mGluR activation increases the levels of [^{35}S] methionine incorporation in hippocampal slices, (Raymond *et al.*, 2000). Therefore, retrograde messages from the synapse to the nucleus could take the form of a transient Ca^{2+} wave (due in part to mGluR activation at specific synapses) which upon arrival at the soma, could activate both gene induction (via the translocation of certain proteins to the nucleus) or protein synthesis (via the action of translational regulator proteins) resulting in long-term changes in synaptic efficacy. It is important to note however, that a role for mGluRs in the regulation of protein synthesis in the dendrites cannot be ruled out (see section 1.7.4).

1.6.3 The 'Local Synthesis' Hypothesis

Both the mail and the tag hypothesis have been used to address the role of somatically synthesised proteins and their subsequent stabilisation of L-LTP at specific synapses. The 'local synthesis' hypothesis however, relies on protein synthetic machinery being present in the dendrites or even in single dendritic spines (Fig. 1.1c). This machinery is capable of translating mRNA that is produced in the soma and trafficked to the dendrites. Therefore, synaptic activity in individual spines could be coupled to nearby dendritic protein synthetic machinery, resulting in the local synthesis of plasticity related proteins from pre-existing dendritic mRNA. In this way, proteins required for long-term synaptic plasticity (i.e. L-LTP) could be rapidly and 'locally' produced, in response to synaptic activity. In addition, a single copy of an mRNA species has the potential to produce multiple copies of a protein, suggesting that dendritic targeting of mRNA, rather than protein may be a more efficient mechanism.

It is important to note however that L-LTP induction may also trigger the synthesis of new mRNA species in the nucleus, and their subsequent trafficking to the dendrites. If such a scenario exists then the specificity of this mRNA trafficking must be considered and the mail and tag hypotheses therefore remain important. These mRNA species could be transported specifically to activated synapses as proposed by the mail hypothesis. Alternatively, a synaptic tag may also be required for the capture, or site specific translation of globally distributed mRNAs. Furthermore, depending on the proximity of the protein synthetic machinery to the activated synapses, the capture of dendritically synthesised proteins by a synapse specific tag could still be important.

1.7 LOCAL PROTEIN SYNTHESIS

1.7.1 Dendritically Localised mRNA

The first evidence that mRNA may be localised in dendrites came from Steward and Levy (1982). Electron microscopy of the rat dentate gyrus, revealed that polyribosomes were preferentially localised at the base of dendritic spines, and in some cases were seen in the spine neck, or head (Steward & Levy, 1982). Further

evidence came when Garner *et al.* (1988), using *in situ* hybridisation, found that the microtubule-associated protein MAP2 was present in the dendrites of both cortical and hippocampal neurons. These results were later confirmed in intact hippocampal tissue and in cultured hippocampal and sympathetic neurons (Bruckenstein *et al.*, 1990; Kleiman *et al.*, 1990). Following these initial observations a small number of specific mRNA species have been shown (using *in situ* hybridisation) to display extensive dendritic localisation in hippocampal neurons. These comprise mRNA for the α-subunit of Ca^{2+}/CaM dependent kinase αCaMKII (Burgin *et al.*, 1990), the activity regulated cytoskeletal-associated protein, Arc (also termed Arg 3.1)(Link *et al.*, 1995; Lyford *et al.*, 1995), the NMDA receptor subunit NMDAR1 (Gazzaley *et al.*, 1997), and Dendrin (Herb *et al.*, 1997). In addition, a number of other mRNA have been shown to localise to proximal dendrites of hippocampal neurons, these include, brain-derived neurotrophic factor (BDNF), along with its receptor TrkB and the amyloid precursor protein (APP) (for a more complete list see Kuhl and Skehel, 1998).

In addition to *in situ* hybridisation, other techniques have been employed to identify dendritically localised mRNA species. Miyashiro *et al.* (1994) used patch pipettes to aspirate the cytoplasmic contents of individual dendrites of hippocampal neurons in culture. Using a combination of PCR based amplification, differential display and northern blot techniques, a relatively large number of mRNAs were identified, most notably those encoding for a number of different NMDA and AMPA receptor subunits. Using similar techniques, a large heterogenous population of mRNAs have also been shown to be present in the dendritic growth cones of cultured hippocampal neurons (Crino and Eberwine, 1996), although many of the mRNAs identified in these two studies, have yet to be detected by *in situ* hybridisation techniques. Whether this is because they are present at levels below the threshold for detection, or they are simply just absent, remains unclear. It does, however seem to be the case that a small number of structurally and functionally unrelated mRNA species are localised in the dendrites of hippocampal neurons (as well as other neuronal types; not discussed here). What is the mechanism by which these mRNAs reach the dendrites? It is certainly the case that the presence of mRNA in the proximal dendrites could be accounted for by simple diffusion from the cell bodies; however mRNA species seen

in relatively high levels at distal dendrites (αCaMKII and *Arc*) may require some kind of active transport mechanism.

1.7.2 Dendritic Transport of mRNA

Evidence that specific mRNA species may be actively targeted to the dendrites has been provided by a number of studies. The dendritic localisation of a number of mRNA species have been shown to be regulated by synaptic connectivity and growth. RNA for BC1, an RNA polymerase III transcript and component of a ribonucleoprotein particle is detectable in the dendrites of cultured hippocampal neurons in response to synapse formation. However, in low-density cultures, where synapse formation is delayed, BC1 levels in the dendrites were reduced. Furthermore, somatodendritic expression of BC1 could be reversibly blocked by the inhibition of neuronal activity (Muslimov *et al.*, 1998). In *Aplysia* sensory neurons, the expression levels of the somatodendritically localised mRNA, Sensorin A (detected by single-cell RT-PCR), are increased in neurites in response to contacts with appropriate target cells, but not to contact with inappropriate targets (Schacher *et al.*, 1999). These differences can even be seen in separate neurites of the same sensory cell (Schacher *et al.*, 1999). The results suggest a branch specific transport of mRNA to favourable connections for the formation of new synapses. Furthermore, the application of neurotrophin-3 (NT-3) has been show to cause a microtubule-dependent localisation of β-actin mRNA and protein to growth cones in cultured hippocampal neurons, via a mechanism dependent on PKA (Zhang *et al.*, 1999). In line with these results there is some evidence that NT-3 is involved in the translocation of RNA-containing granules into dendrites in cultured cortical neurons (Knowles and Kosik, 1997).

In addition to dendritic mRNA localisation in response to growth and synapse formation, changes have also been seen in response to synaptic activity. mRNAs for BDNF and its receptor TrkB, that normally localise to the proximal 30% of the dendrites in cultured hippocampal neurons, have been shown to extend their localisation up to an average of 68% of the total dendritic length, in response to potassium depolarisation. This increase is dependent on Na^+ channels, L-type Ca^{2+} channels and on glutamate receptor activation, but independent of new mRNA

synthesis (Tongiorgi *et al.*, 1997). Furthermore, dendritic localisation of αCaMKII mRNA has been seen to increase, 48 hours following LTP induction (Thomas *et al.*, 1994). Recently, interesting results have been published involving *Arc*, an immediate early gene previously shown to exhibit dendritic translocation of both its mRNA and protein, in response to repeated high-frequency stimulation (Link *et al.*, 1995; Lyford *et al.*, 1995); see section 1.5.3). Steward *et al.* (1998) showed that repeated high-frequency stimulation applied to the medial perforant path (MPP) caused newly synthesised *Arc* mRNA and protein to localise to the middle molecular layer of the dentate granule cell dendrites, the area that is directly innervated by the MPP. Furthermore, the dendritic targeting of the *Arc* mRNA was independent of protein synthesis, suggesting that the signal conferring specific localisation is present in the mRNA itself (Steward *et al.*, 1998; Wallace *et al.*, 1998). These results show that *Arc* mRNA can selectively localise to regions of recent synaptic activity. However, whether this *Arc* mRNA is specifically trafficked to these activated regions, or globally distributed and locally stabilised, is not clear.

In addition to studying changes in the distribution of mRNA in response to activity, attempts have been made to visualise, in real time, the dynamics of dendritic mRNA transport in living cells. In 1996 Knowles *et al.*, using the membrane permeable nucleic acid dye SYTO14, showed that RNA was present as discrete 'granules' in the dendrites of cultured cortical neurons. These granules were colocalised with poly (A^+) mRNA, the 60S ribosomal subunit and with elongation factor 1α, suggesting that the granules represent a translational unit. Furthermore, a number of these granules were seen to undergo a bi-directional microtubule-dependent transport in the dendrites, moving at an average rate of ~6μm/min. The movement and density of these RNA-containing granules has subsequently been shown to be increased by the application of NT-3 via a mechanism dependent on protein kinases (Knowles & Kosik, 1997). Similar dendritic delivery rates for RNA have been seen in other studies. Microinjection of a radiolabelled BC1 RNA into cultured sympathetic neurons, and its subsequent dendritic distribution (measured by autoradiography) provide a dendritic delivery rate of ~4μm/min (Muslimov *et al.*, 1997). Furthermore, in situ hybridisation shows an apparent dendritic rate of ~5μm/min for *Arc* mRNA (Wallace *et al.*, 1998).

1.7.3 Mechanisms for Dendritic Localisation and Transport of mRNA

The visualisation of dendritically motile, RNA-associated granules, has led to the hypothesis that certain mRNA species are bound by proteins, to form complexes that are then transported to the dendrites, maybe via the microtubules, where they undergo local translation. However, it is only recently that a number of candidate signals and molecules have been discovered that could be involved in the sorting and dendritic transport of these specific mRNAs. In 1996 Mayford *et al.* produced two mutant mouse strains in which the αCaMKII promoter was used to drive the expression of a lacZ transcript which either contained or lacked the 3'-untranslated region (UTR) of the αCaMKII gene. Mice containing the 3'-UTR showed dendritic localisation of lacZ whilst those lacking this 3'-UTR did not. This suggested that the 3'-UTR contained a 'zipcode' that was both necessary and sufficient for dendritic targeting. Recently, dendritic targeting signals have been found in the 3'-UTR of a number of other dendritically localised mRNAs, including MAP2 (Blichenberg *et al.*, 1999). However, there appears to be no homology between 3'-UTR primary sequences of different mRNA species, suggesting that the dendritic localisation characteristics are conferred by the complex secondary, and tertiary structures of the folded mRNA. These mRNA specific, secondary structures may be recognised by molecules that could mediate their transport to the dendrites.

A number of proteins have been identified that display RNA-binding properties and the role of some these as possible mRNA trafficking proteins has been investigated. Among these, three different families of RNA-binding proteins appear to be important: heterogenous nuclear ribonucleoproteins (hnRNPs), zipcode binding proteins (ZBP, Vera, or Vg1 RBP) and double stranded RNA binding proteins e.g. Staufen (for a recent review see Kiebler and DesGroseillers, 2000). The majority of work on these proteins has been carried out in oocytes, embryos, and fibroblasts. Binding of hnRNPs to *fushi tarazu* mRNA is responsible for the subsequent localisation of this mRNA in *Drosophila* embryos (Lall *et al.*, 1999). Similarly, in fibroblasts, ZBP binds to the 'zipcode' within the 3'-UTR of transported chick β-actin mRNA (the region responsible for its cellular localisation) (reviewed by Oleynikov and Singer, 1998). Additionally, the double stranded RNA-binding protein Staufen

has been shown to be involved in the binding and localisation of a number of different mRNA species to different regions during *Drosophila* oocyte activation and fertilisation (reviewed by St Johnston, 1995). Data suggesting an involvement of hnRNPs and ZBP in dendritic transport in neurons is limited, however there is some circumstantial evidence suggesting a role for Staufen in neuronal mRNA transport. Using immunohistochemical techniques, a Staufen rat homologue (mStau) has been found that shows a somatodendritic localisation in cultured rat hippocampal neurons (Kiebler *et al.*, 1999). Furthermore, mStau is associated with large RNA-containing granules, rough endoplasmic reticulum, and microtubules, in the dendrites of these neurons (Kiebler *et al.*, 1999). The functional significance of these granules in mRNA transport (if any) was examined by Kohrmann *et al.* (1999). The authors transfected a mammalian Staufen construct tagged to green fluorescent protein (Stau-GFP), into hippocampal neurons. The Stau-GFP assembled into granules that colocalised with the fluorescent RNA dye, SYTO14. Some of these granules were seen to move in a bi-directional manner between the cell bodies and the dendrites of living neurons. The average speed of these particles was 6.4μm/min and their movement was dependent on microtubules. These findings were in agreement with previous studies looking at the movement of RNA-containing granules in cortical neurons (Knowles *et al.*, 1996). Although these results are encouraging, a role for Staufen in the dendritic trafficking of any specific mRNA has yet to be confirmed.

1.7.4 Local Translation in Dendrites

As we have seen, good evidence exists for the transport and subsequent dendritic localisation of certain mRNA species. What is the evidence that this mRNA can be translated in these distal processes? Although the presence of polyribosomes at dendrites has been known for about 20 years, it is only relatively recently that other elements of the translational machinery have been shown to be present in the dendrites. In 1996 Tiedge and Brosius, using *in situ* hybridisation and immunocytochemistry, revealed that ribosomes, tRNAs, elongation factors (eIF2β and eEF2) and elements of the cotranslational recognition mechanism (TRAPα) were present throughout the dendrites of cultured hippocampal neurons. Around the same time, elements of the endoplasmic reticulum (ER) and the Golgi apparatus (GA) were

also seen to be localised in the proximal and middle dendrites of cultured hippocampal neurons (Torre and Steward, 1996). Recently, translocation machinery necessary for the synthesis of integral membrane proteins, has been shown to be present in both dendrites and in individual dendritic spines (Pierce *et al.*, 2000).

In line with the previous observations, protein synthesis as been shown to occur in living dendrites of cultured hippocampal neurons that have been isolated from their cell bodies (Torre and Steward, 1992). Furthermore, transfection of isolated dendrites from hippocampal neurons with mRNA for both glycogen synthase kinase (GSK) and CREB, following their treatment with BDNF or NT-3 to stimulate translation, results in the subsequent appearance of GSK and CREB protein between 1 and 5 hours later (Crino & Eberwine, 1996; Crino *et al.*, 1998). All of these experiments suggest that the translational machinery seen in dendrites is indeed functional.

Can local protein synthesis in dendrites be regulated by synaptic activity? Early attempts to address this issue have used synaptosomal preparations, i.e. subcellular fractions that are enriched in isolated pre and postsynaptic nerve endings. In 1993 Weiler *et al.* observed a rapid increase in the size of synaptosomal polyribosomal aggregates in response to K^+ depolarisation or glutamate application. Furthermore, this apparent increase in synaptic translation, appeared in part to be regulated via metabotropic glutamate receptor activation coupled to the phosphatidylinositol system. In line with these findings, stimulation of mGluRs (via application of the mGluR agonist DHPG), in rat hippocampal slices, results in a rapid (<90 seconds), PKC-dependent translocation of the serine-threonine kinase p90rsk, to polyribosomes. A known substrate of p90rsk, is glycogen synthase kinase 3β (gsk-3β), which is involved in regulating translation efficiency. This result therefore suggests a possible role for mGluRs (via p90rsk and gsk-3β) in the regulation of synaptic activity-dependent translation (Angenstein *et al.*, 1998).

Increases in dendritic protein synthesis in response to synaptic activity have also been shown in intact tissue. In the CA1 region of hippocampal slices, electrical stimulation of Schaffer collateral afferents paired with application of the cholinergic agonist carbachol, resulted in a three fold increase in the levels of dendritic [^{3}H] leucine

incorporation (Feig and Lipton, 1993). This dendritic protein synthesis was dependent on NMDA and muscarinic receptor activation and did not occur in response to either stimulation or carbachol application alone. Furthermore, the fact that slices were labelled and fixed within 3 minutes of the end of the stimulation suggests that dendritic translocation of either protein or mRNA from the soma would not be fast enough to impact on the observed findings. These results therefore provide strong evidence for NMDA receptor-dependent protein synthesis from pre-existing mRNA within the dendrites in response to synaptic activity. Recently, NMDA receptor activation has been linked to increases in the synthesis of specific proteins. Activation of NMDA receptors in synaptosomal preparations from rat superior colliculus, results in a rapid increase in both the rate of synthesis and overall levels of expression of αCaMKII protein, paralleled by a reduction in total synaptic protein synthesis (Scheetz *et al.*, 2000)(see section 1.7.5). Indeed, increases in αCaMKII levels have been seen in dendrites of hippocampal CA1 neurons in response to tetanic stimulation of the Schaffer collateral pathway (Ouyang *et al.*, 1997; Ouyang *et al.*, 1999). These increases were blocked by the protein synthesis inhibitor anisomycin and could be seen within 5 minutes of tetanic stimulation, suggesting that they were due to dendritic protein synthesis (Ouyang *et al.*, 1997; Ouyang *et al.*, 1999). *In vivo* studies have produced similar results. Proteins for *Arc*, CaMKII and MAP2 have all been shown to display distinct localisation patterns in and around specific regions on the dendrites of dentate granule cells that have undergone recent synaptic activation (Steward *et al.*, 1998; Steward & Halpain, 1999). In addition, dendritic localisation for both *Arc* and MAP2 (but not CaMKII) was blocked by the protein synthesis inhibitor cycloheximide; however, the locus of this protein synthesis was not addressed (Steward *et al.*, 1998; Steward & Halpain, 1999). Overall, good evidence exists that dendrites are capable of protein synthesis and that this may be regulated by synaptic activity.

1.7.5 Mechanisms for Dendritic Translation

Recently, a number of molecules have been identified which may play a role in the regulation of dendritic translation. These candidates include the cytoplasmic polyadenylation element binding protein (CPEB), eukaryotic elongation factor (eEF2)

and the fragile X mental retardation protein (FMRP). In oocytes, CPEB has been shown to bind to mRNA containing cytoplasmic polyadenylation elements (CPEs). Once activated by phosphorylation, CPEB is thought to initiate polyadenylation of these mRNAs, resulting in their translation during oocyte maturation (recently reviewed by Wells *et al.*, 2000). The role of this CPE protein in mammalian systems has recently been tested. CPEB mRNA is widely expressed in rodent brains including the cell bodies of the dentate gyrus and CA1 regions of the hippocampus. Furthermore, CPEB protein is present in both dendritic and cell body regions of the hippocampus and localised to dendritic spines where it is associated with the post synaptic density (PSD) (Wu *et al.*, 1998). CPEB has been shown to bind two CPE sequences present in the 3'-UTR of rat αCaMKII, and can initiate polyadenylation induced translation of rat αCaMKII 3'-UTR complexes, in injected *Xenopus* oocytes (Wu *et al.*, 1998). Furthermore αCaMKII mRNA polyadenylation and translation have been seen in response to activity driven reorganisation of the visual cortex following the exposure to light, of previously dark reared rats (Wu *et al.*, 1998). These results suggest mRNA encoding αCaMKII can undergo CPEB meditated polyadenylation and translation in the brain, in response to synaptic activation. Although not confirmed, it is also suggested (due to the localisation of CPEB) that this may occur in the dendrites.

Other mechanisms by which local translation may be regulated in response to synaptic activity have also been proposed. In the tadpole tecta, a number of proteins undergo phosphorylation in response to NMDA receptor activation. One of these proteins is the dendritically localised, eukaryotic elongation factor 2 (eEF2) (Scheetz *et al.*, 1997). Phosphorylation of eEF2 reduces peptide chain elongation during translation and is associated with a reduction in protein synthesis. Furthermore, eEF2 can undergo regional specific phosphorylation in adult frog tectum, in response to synaptic activity associated with visual stimulation (Scheetz *et al.*, 1997). Recently, NMDA receptor activation in synaptosomal preparations from the rat superior colliculus has been shown to rapidly increase αCaMKII translation, whilst reducing total protein synthesis (Scheetz *et al.*, 2000). NMDA receptor activation also resulted in an increase in the phosphorylation of eEF2. When low concentrations of the protein synthesis inhibitor cycloheximide were applied to these preparations (to mimic the

effects of phospho-eEF2), the translation rate of αCaMKII was seen to increase while overall protein translation decreased (Scheetz *et al.*, 2000). These results suggest that eEF2, by slowing chain elongation, may favour the translation of αCaMKII, providing a possible mechanism by which synaptic activity could be coupled to the local translation of specific proteins.

Finally, there is some evidence that the fragile X mental retardation protein (FMRP) may be involved in the regulation of local translation. FMRP has been shown to localise at the dendrites, or in individual spines of cortical and hippocampal neurons (Weiler *et al.*, 1997). Consistent with this, mice lacking FMRP, posses thin lengthened dendritic spines, suggesting this protein is required for the development of normal spine morphology. Weiler *et al.* (1997), using synaptosomal preparations, found a rapid association of FMR mRNA with polyribosomes, following mGluR stimulation. This association was accompanied by the increased expression of FMRP and its association with synaptic ribosomal complexes. Taken together, these results suggest a role for FMRP, in activity dependent local translation that may be required for normal synaptic development.

1.7.6 Local Protein Synthesis and Synaptic Plasticity

Although a growing body of data suggests the presence of dendritic mRNA and their local translation, there is at present no evidence that this local protein synthesis is required for hippocampal LTP. However, in the last five years, local protein synthesis has been shown to be involved in a number of different forms of synaptic plasticity. In 1996 Kang and Schuman showed that local protein synthesis was involved in neurotrophin induced synaptic plasticity in the hippocampus. Application of either BDNF or NT-3 produced a slow onset enhancement of the field EPSP slope of CA1 pyramidal cells in hippocampal slices, that lasted for at least 3 hours. In addition, this enhancement was immediately and significantly attenuated by the protein synthesis inhibitors anisomycin and cycloheximide, suggesting a local rather than somatic protein synthesis. Using this preparation, the authors were able to examine the locus of neurotrophin induced protein synthesis. They found that when either the presynaptic or postsynaptic cell bodies (or both) were isolated from the synaptic

neuropil, the enhancement was unaffected (Kang & Schuman, 1996). These results indicate that the protein synthesis is not somatic and strongly suggest a dendritic origin.

Recently, a form of chemically induced hippocampal long-term depression (LTD) has been shown to be dependent on local protein synthesis. A rapid onset depression of the field EPSP slope in the CA1 region of rat hippocampal slices, occurred in response to application of the selective group 1 mGluR agonist DHPG. This LTD was blocked by the mGluR antagonist LY341495 and was dependent on translation but not transcription (Huber *et al.*, 2000). To confirm this requirement for mRNA translation, postsynaptic cells were loaded with an mRNA cap analogue (m^7GpppG) prior to application of DHPG. This cap analogue competes with endogenous capped RNA for the cap binding protein eIF-4E (a translation initiation factor) thereby inhibiting translation. Following m^7GpppG application, DHPG produced a decremental LTD that returned to baseline levels over 1 hour (Huber *et al.*, 2000). Finally, DHPG induced depression also occurred in dendrites that had be separated from their cell bodies. Taken together, these results suggest that chemically induced mGluR LTD, is dependent on the local postsynaptic translation of pre-existing, dendritically localised mRNAs (Huber *et al.*, 2000). Although the data from the two previous studies is encouraging, similar results showing no requirement for nuclear involvement, have yet to be shown with electrically induced homosynaptic LTP. Indeed, in the CA1 region of hippocampal slices, tetanic stimulation has been shown to fail to induce L-LTP (potentials returned to pre-tetanus levels over 4-5 hours), when dendrites were separated from their cell bodies (Frey *et al.*, 1989). However, the results from Frey *et al.* (1989), although attributed to an involvement of somatic protein synthesis in L-LTP could equally be due to a block in mRNA trafficking to the dendrites.

To date, the most compelling evidence for local protein synthesis in synaptic plasticity comes from a non-mammalian system. In 1997 Martin *et al.* used a modified *Aplysia,* sensory to motor neuron culture system, in which a single bifurcated sensory neuron made synaptic contact with two separate motor neurons. Topical application of 5 pulses of serotonin to one of these sensory-motor synapses

induced a specific long-term synaptic facilitation (LTF) in that synapse, whilst the other remained unaffected. This synapse-specific facilitation (lasting at least 24 hours) was dependent on transcription and on CREB activation and was accompanied by growth of new synaptic connections (Martin *et al.*, 1997a). In addition, local application of the protein synthesis inhibitor emetine to one synapse prior to, and during the application of 5 pulses of serotonin, blocked LTF specifically within that synapse. This branch specific local protein synthesis was shown to have a presynaptic locus and could occur in the neurites of sensory cells that had been separated from their cell bodies, increasing 3 fold in response to application of serotonin (Martin *et al.*, 1997a). Most importantly, application of one pulse of serotonin to a synapse that would normally produce a short-term facilitation could be converted into a long-term facilitation, if preceded by 5 pulses of serotonin applied to the other synapse on the same cell. Therefore a single serotonin pulse serves to mark or tag, this synapse enabling the 'capture' of products induced by gene expression in response to 5 pulses of serotonin at the other synapse. These 'synaptic capture' results are similar to those seen in the synaptic tagging experiments of Frey and Morris (Frey & Morris, 1997). Finally, synapse-specific facilitation lasting 24 hours was found to require protein synthesis; while synaptic capture lasting 24 hours did not (Martin *et al.*, 1997a). These results suggest that a retrograde signal travels back to the nucleus to initiate gene transcription and may be dependent on protein synthesis, whilst the tag is not.

Synapse-specific LTF has recently been further investigated in the same laboratory. Using the same cell culture preparation, Casidio *et al.* (1999) investigated the time course and the nature of the tag. The tag set by a single serotonin pulse could persist for between 4 to 5 hours, whilst the nuclear products of gene expression initiated by 5 pulses of serotonin persisted for between 1 and 4 hours. Furthermore, LTF normally produced by synaptic capture, was blocked at 24 hours post-induction, by the PKA inhibitor Rp-cAMPS, suggesting that initially the tag takes the form of a PKA-mediated covalent modification (Casadio *et al.*, 1999). Importantly, extending the length of time over which long-term facilitation was followed, produced an interesting result. LTF occurring from synaptic capture, although previously shown to be protein synthesis independent for a least 24 hours (Martin *et al.*, 1997a), was seen to return to baseline levels at 72 hours during protein synthesis inhibition (Casadio *et*

al., 1999). Additionally, local application of the immunosuppresant rapamycin (known to block serotonin-induced translation), blocked LTF produced by synaptic capture at 72 hours, although synaptic growth over 24 hours was unaffected. Surprisingly, therefore, rapamycin-sensitive local protein synthesis was not required to initiate synaptic growth but was required for the stabilisation and persistence of that growth. Furthermore, rapamycin did not block synapse-specific facilitation over 24 hours (unlike emetine) but did block it over 72 hours. These results suggest that local synthesis at the synapse may be required for two different mechanisms: 1) a rapamycin-insensitive, emetine-sensitive generation of a retrograde signal at the site of LTF, that can travel to the nucleus to initiate gene expression 2) a rapamycin sensitive stabilisation of synaptic growth (maybe due to stabilisation of the initial PKA dependent covalent tag) leading to the persistence of LTF for greater then 72 hours.

In addition to 'capture' occurring from synapse-specific facilitation, it has also been shown to occur from cell-wide facilitation. Casidio *et al.* (1999) showed that 5 pulses of serotonin applied to the cell bodies of *Aplysia* cultured sensory neurons, produced a cell-wide long-term facilitation that was dependent on transcription, translation and CREB activation. This facilitation was different from synapse-specific long-term facilitation, in that it showed no short-term facilitation, was not dependent on synaptic growth and did not exist beyond 48 hours (Casadio *et al.*, 1999). However, coupling of this cell-wide facilitation, with a single pulse of serotonin at a specific synapse, could convert this synapse into one that showed growth and in which LTF was persistent for at least 72 hours (Casadio *et al.*, 1999). This situation occurred even in cases in which subthreshold stimulation, that produced no detectable facilitation, was applied to the cell bodies (Casadio *et al.*, 1999). Around the same time, it was shown in *Aplysia*, *in vivo*, that the duration of serotonin application to either the soma or the synapses could be titrated so as to produce a stimulus that was below the threshold for long-term facilitation. However, if synaptic, as well as somatic serotonin stimulation occurred together, within a stringent time window, LTF occurred (Sherff and Carew, 1999). This coincident LTF was dependent on immediate local protein synthesis at the synapses as well as somatic protein synthesis occurring 1-3 hours later (Sherff & Carew, 1999).

Although a role for local protein synthesis in *Aplysia* long-term facilitation is in no doubt, the mechanisms by which these changes are produced remain unclear. Is local synthesis required to generate a retrograde signal that can travel back to the nucleus? Furthermore, are the products trafficked back to the synapses, proteins, mRNAs or both?

1.8 SUMMARY

This introduction has attempted to summarise the major mechanisms underlying hippocampal long-term potentiation. LTP remains the dominant model of activity dependent synaptic plasticity within the mammalian brain. Like behavioural memory, LTP consists of mechanistically distinct temporal components, most importantly, E-LTP (early LTP) lasting 3-6 hours, and L-LTP (late LTP) lasting 8 hours or more. E-LTP depends primarily on short-term kinase activity, whilst L-LTP, presumably involved in the long-term storage of memories, is dependent on mRNA and protein synthesis. The exact locus of protein synthesis has still not been elucidated. Although historically, translation was thought to occur at the soma, a growing body of recent evidence suggests that in response to synaptic activity, protein synthesis may occur locally in the dendrites, from either pre-existing mRNA or from newly synthesised mRNA, dendritically trafficked from the nucleus. Retrograde signals from synapse to nucleus to activate gene expression, as well as tagging mechanisms to direct these nuclear products to specific synapses, have been proposed in a number of systems. Recent evidence suggests that local protein synthesis is involved a number of different forms of synaptic plasticity in both invertebrate and mammalian systems. However, a requirement for local translation in hippocampal L-LTP has yet to be shown.

1.9 PROJECT AIMS

This study attempts to address the involvement of both transcription and translation during L-LTP in the CA1 region of mouse hippocampal slices. The onset of L-LTP in hippocampal slices occurs ~3-6 hours following tetanisation, but due to problems

associated with slice health and stability of recordings, conventional LTP experiments usually follow LTP for only 1-2 hours. In order to study L-LTP, it is therefore necessary to prolong the life span of hippocampal slice preparations. The initial stages of this project involved the optimisation of experimental conditions necessary for the continued maintenance and recording of LTP for up to 8 hours. Once L-LTP could be induced with reasonable reliability, the following questions were addressed. Firstly, is the maintenance of L-LTP dependent on both transcription and translation? Secondly, what is the locus of each of these processes in L-LTP (i.e. presynaptic or postsynaptic, somatic or dendritic)? Finally, what molecules are involved in the regulation of these processes?

MATERIALS AND METHODS

2.1 ELECTROPHYSIOLOGY

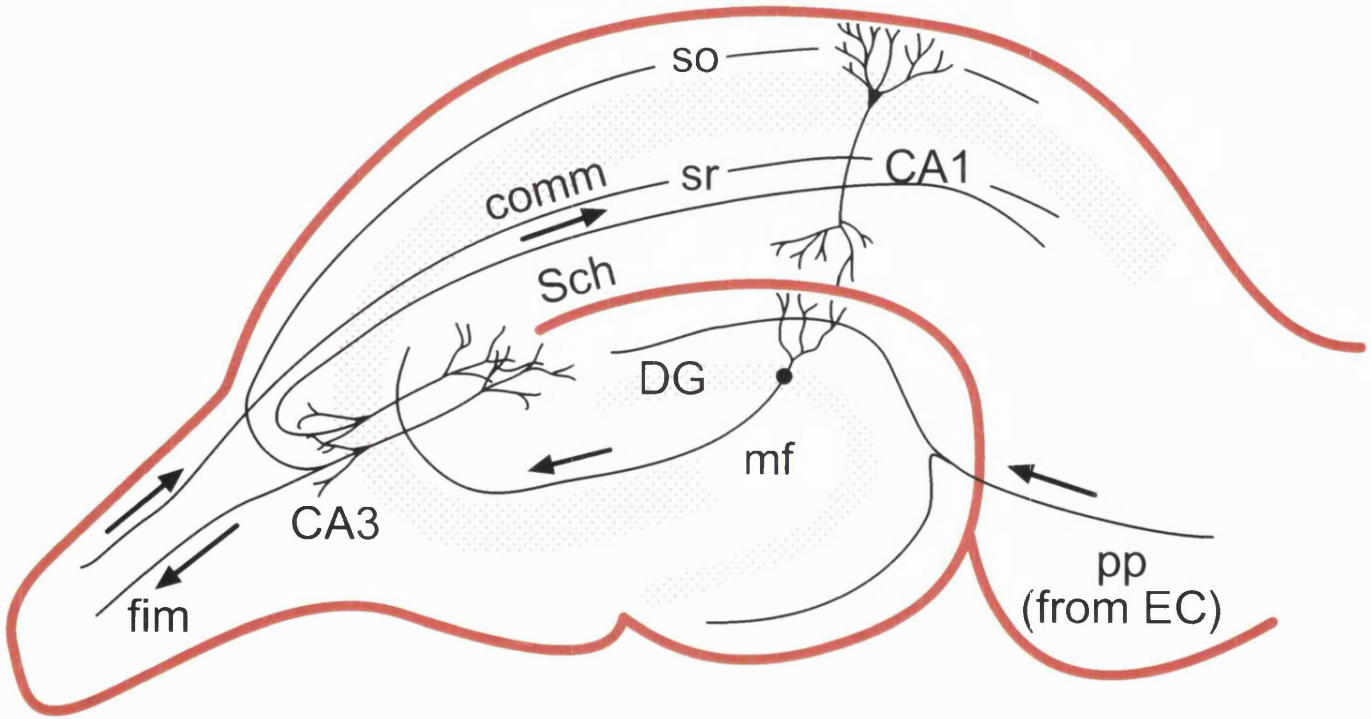
2.1.1 In vitro Hippocampal Slice Preparation

Since its development over 40 years ago (Li and McIlwain, 1957), the *in vitro* brain slice preparation has become a valuable method for studying the electrical properties of CNS neurons within living tissue. The technique, in which thin sections of tissue (100-700μm) are taken from the brain of adult mammals and maintained for many hours *in vitro*, has a number of advantages over *in vivo* studies. Brain slices allow for good accessibility to the cells, and permit control of the external environment, two properties that are difficult to achieve *in vivo*. The brain slice strategy was first applied to the hippocampus by Yamamoto and Kawai (1967) and also by Bliss and Richards (1971). This technique was subsequently refined into the transverse hippocampal slice preparation by Skrede and Westgaard (1971). The hippocampus is a brain structure well suited to the *in vitro* slice preparation as it is composed of a series of parallel lamellae, oriented transversely to its longitudinal axis. This structure allows for minimal disruption in synaptic connectivity, when cut along a defined plane.

The main regions of the hippocampus form what has come to be known as the 'trisynaptic loop' (see Fig. 2.1). Fibres of the perforant path project from the entorhinal cortex to form synaptic connections with the granule cells of the dentate gyrus. Mossy fibre projections from these cells then synapse onto pyramidal cells in the CA3 region. Axons of these cells in turn project both to the contralateral hippocampus as commissural fibres, and as Schaffer collaterals to the CA1 pyramidal cells of the ipsilateral hippocampus. The CA1 pyramidal cells then send projections back to the entorhinal cortex.

Transverse hippocampal slices were prepared from male C57BL6 mice (8-10 weeks, 20-30g). Animals were stunned by a blow to the thorax, followed by cervical

Figure 2.1 Schematic representation of a transverse section through the hippocampus. The 'trisynaptic loop' is shown. Abbreviations: EC-entorhinal cortex; pp-perforant path; DG-dentate gyrus; mf-mossy fibres; fim-fimbria; comm-commissural fibres; Sch-Schaffer collateral fibres; sr–stratum radiatum; so–stratum oriens.


so
comm
sr
CA1
Sch
DG
mf
CA3
fim
pp
(from EC)

dislocation and decapitation. The brain was removed from the skull case and placed in a Petri dish containing cold (0-4°C), oxygenated (95% O_2 / 5% CO_2), artificial cerebrospinal fluid (ACSF) of the following composition: NaCl 120mM, KCl 3mM, $MgSO_4$ 2mM, $CaCl_2$ 2mM, NaH_2PO_4 1.2mM, $NaHCO_3$ 23mM, Glucose 11mM. To maintain the ACSF at the required temperature (0-4°C) throughout the dissection, the ACSF-containing Petri dish was encased in an aluminium cooling block that had previously been chilled to -20°C in the freezer.

The dissection to remove the hippocampus from the brain was performed in ACSF, in the cooled Petri dish. The brainstem and cerebellum were removed and the brain placed on its ventral surface. A transverse cut was made to free the forebrain. Following this, a sagittal cut was made along the midline between the left and right cortex (but not deep enough to separate the two hippocampi). The cortex of the right hemisphere was then gently rolled away to expose the underlying hippocampus. A further sagittal cut was made to divide the two hippocampi and the right hippocampus was separated from the remaining cortex by rolling it out with the aid of a spatula.

The right hippocampus was removed from the ACSF and placed on a layer of moistened filter paper on a perspex chopping block. This chopping block was placed onto a McIlwain tissue chopper and the position of the block adjusted to ensure that the longitudinal axis of the hippocampus was oriented at 30-40° to the blade. 3-4 transverse slices 400-450μm thick, were serially cut, from the middle region of the right hippocampus. Each individual slice was gently removed from the blade in a rolling motion, using a small sable paint brush (N°1), and transferred immediately to a Petri dish containing cold oxygenated ACSF (0-4°C). In some cases, the slices were further microdissected; a scalpel blade was used to cut along the CA1-CA3 border and the CA3 subfield was excised (the resulting preparation being termed a CA1 mini-slice, see Fig. 5.11)(Lynch *et al.*, 1982). Finally, three hippocampal slices were transferred directly to an *interface* type slice chamber (Scientific Systems Design Inc., UK) where they were maintained at 27-28°C and allowed to incubate for 2-3 hours before recording, a period shown to permit optimal metabolic recovery of hippocampal slices following cutting (Whittingham *et al.*, 1984). The complete slice

preparation (from brain removal to transfer of slices into the recording chamber) was performed in less than 5 minutes.

2.1.2 Slice Chamber Design

Chamber design considerations are of utmost importance when attempting to record from living tissue for a number of hours and are *critical* in L-LTP experiments where recordings may be made for up to 10 hours. Good slice health is generally assured by a slice chamber that provides a moist, oxygenated environment, at a stable temperature, and adequate perfusion of slices with ACSF. It is, however, a delicate balance to achieve each of these conditions and thus maintain slices that are healthy enough for the study of L-LTP.

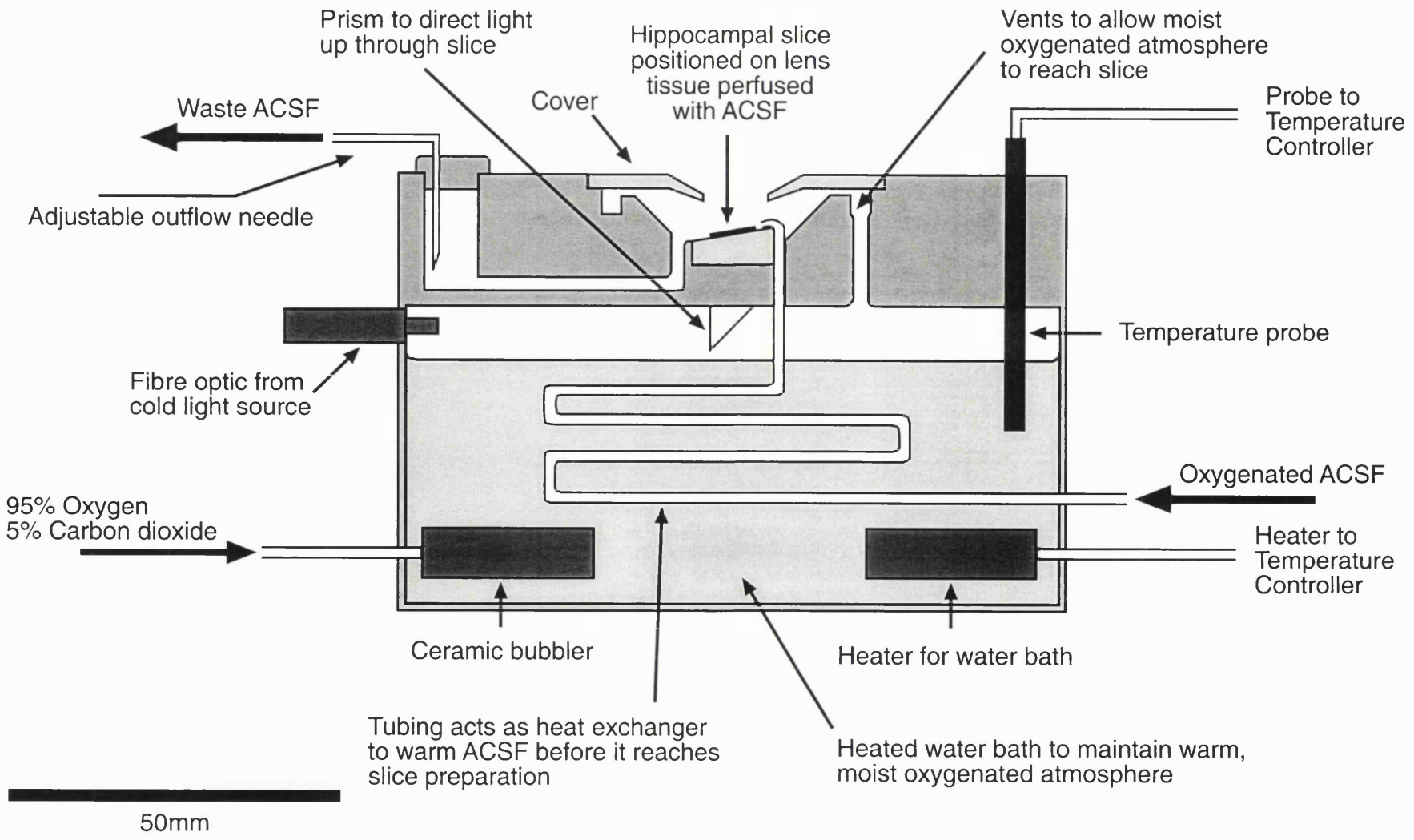
Slice chambers can be broadly classified into two groups. The first of these are *interface* chambers in which the slices rest on a layer of lens tissue or nylon net at the interface between slow flowing ACSF (0.1-1ml/min) and a moist 95% O_2/5% CO_2 saturated atmosphere. The second group are the *submerged* chambers in which slices are covered by a layer of oxygenated ACSF flowing at ~2-4 ml/min. Each of these chamber types has advantages and disadvantages associated with their design. Interface chambers use less ACSF due to their slower flow rates and smaller dead space, and thus reduce the cost of pharmacological experiments. However, at higher temperatures (above 30°C) they are prone to produce condensation on the metal stimulating electrodes, thus affecting the stability of the recordings. In contrast, condensation is not a problem in submerged chambers, and as a consequence the electrode placement is more stable. Slices in these chambers are also better protected from temperature changes in the external environment. Submerged chambers do, however, have some disadvantages. Visualisation is poor, making it difficult to place electrodes. Also the size of the electrical potentials recorded from the slices are reduced, due to shunting of the signal current to the surrounding fluid. In addition, large fluid volumes, due to high flow rates and large dead spaces significantly increase the quantity of pharmacological agents required for an experiment. It is also more difficult to achieve the required oxygen tension in the ACSF, due to out-gassing and a lower level for oxygen saturation at the higher temperatures.

One of the first aims of this project was to develop a slice chamber that would maintain healthy hippocampal slice preparations for time periods far in excess of those typically used. An interface chamber of the type shown in figure 2.2 was found to be best suited. However, it should be noted that the interface design highlighted here is the result of extensive experimentation with different chamber designs and experimental conditions. The problems associated with maintaining hippocampal slices for these extended time periods and the methods developed to solve them are further discussed in chapter 3.

The interface chamber outlined in figure 2.2 is a modification of a model supplied by Scientific Systems Design Inc., UK. The hippocampal slices (usually 3-4) sit on a layer of moist lens tissue on top of a sloped circular chamber insert. The slices are perfused with a continuous supply of oxygenated (95% O_2/5% CO_2) ACSF that is warmed as it passes through the base of the chamber. The lens tissue acts to reduce the surface tension of the ACSF and distribute it evenly as it flows over and around the slices, down a gradient and drains into the outflow chamber. The fluid height of the outflow chamber is controlled by aspiration via a hypodermic needle connected to a peristaltic pump. In addition to their perfusion with ACSF, slices are enclosed within a moist oxygenated micro-atmosphere, created by the passage of pre-moistened 95% O_2/5% CO_2 gas through a ceramic bubbler in the base of the chamber. The gas is warmed and further moistened as it passes through the water bath, and is distributed over the slice by vents connecting the base and top of the chamber. Good slice visualisation is achieved using a cold light source, directed via a prism up through the slices.

Slices were perfused with ACSF warmed to 27-28°C at a flow rate of 100-150µl/min. These conditions were found to maintain healthy stable slices for at least 12 hours and in some cases up to 24 hours. It is important to note that due to the slow flow rate, the upper surface of each slice receives ACSF only by capillary flow. This allows for diffusion of oxygen from the surrounding saturated atmosphere into the slices. In this configuration slices receive the majority of their oxygen from the environment, rather than through the perfusing solution.

Figure 2.2 Schematic representation of an interface slice chamber. Warmed, oxygenated ACSF, perfuses across hippocampal slices positioned on a lens tissue insert. The slices are partially enclosed in a warm, moist, oxygenated (95% O_2 / 5% CO_2) atmosphere.


Prism to direct light up through slice
Hippocampal slice positioned on lens tissue perfused with ACSF
Vents to allow moist oxygenated atmosphere to reach slice
Waste ACSF
Cover
Probe to Temperature Controller
Adjustable outflow needle
Temperature probe
Fibre optic from cold light source
Oxygenated ACSF
95% Oxygen
5% Carbon dioxide
Heater to Temperature Controller
Ceramic bubbler
Heater for water bath
Tubing acts as heat exchanger to warm ACSF before it reaches slice preparation
Heated water bath to maintain warm, moist oxygenated atmosphere
50mm

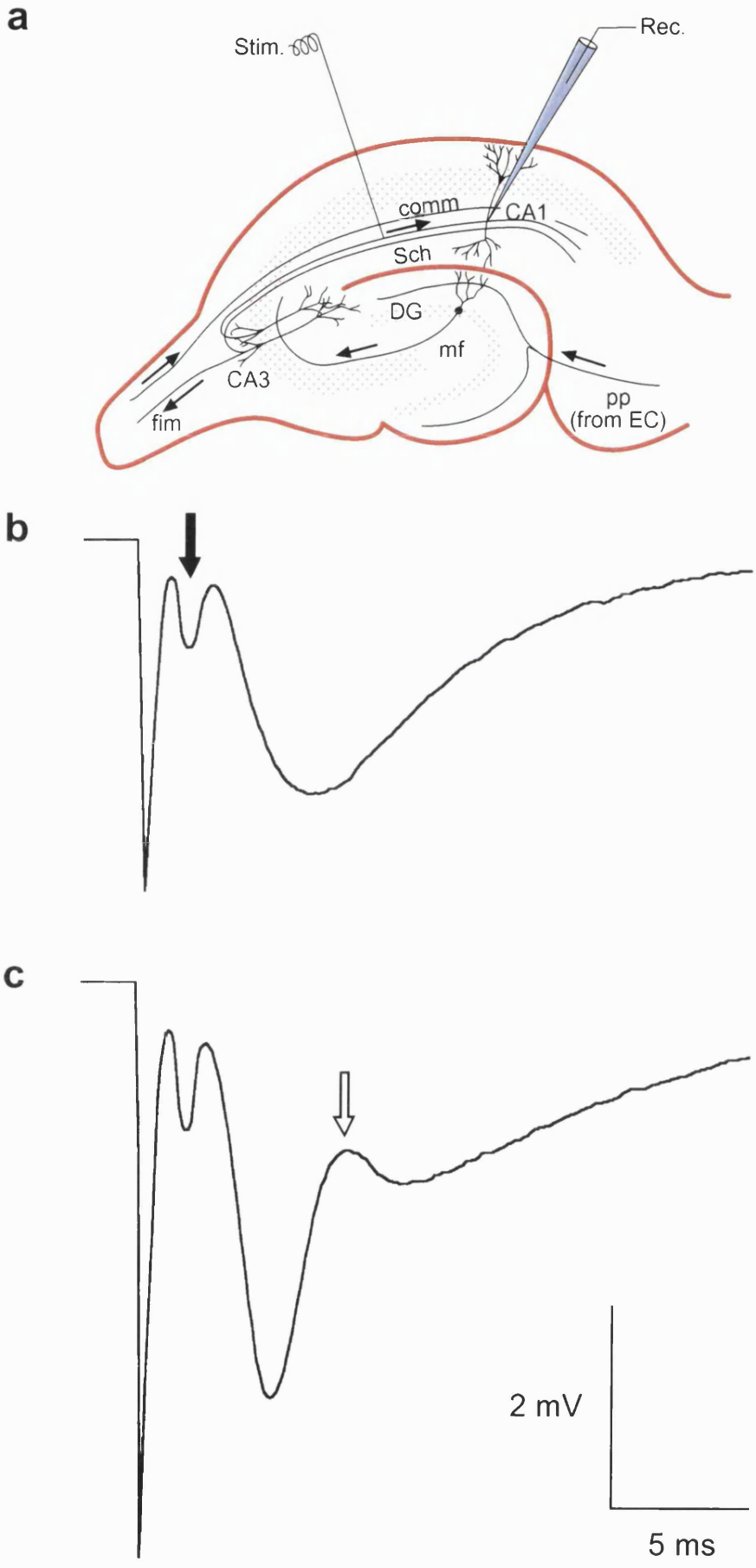
The slow flow rates used in this chamber are well suited to the experimental conditions required for the study of L-LTP. Typically an individual L-LTP experiment lasts ~12-13 hours. This time includes 3 hours slice incubation, 1 hour for electrode positioning and the attainment of stable recordings, plus a further 9 hours of experimental recording. Throughout this period the hippocampal slices must be provided with a continual supply of ACSF. The slow flow rates of this chamber are ideal, allowing for a reduction in the ACSF reservoir volume, in addition to limiting the amount of drug required in pharmacological experiments. Furthermore, recirculation of the ACSF is not required, thereby eliminating problems associated with bacterial contamination and glucose depletion of the perfusing solution. A slow flow rate also helps to maintain mechanical stability, an extremely important factor when recording for long time periods.

2.1.3 Field Potential Recording

Throughout this study, extracellularly recorded field excitatory postsynaptic potentials (field EPSPs) were measured. These field EPSPs reflect changes in voltage caused by the near synchronous activation of a population of postsynaptic cells, in response to extracellularly applied stimulation to presynaptic cells. Recordings were made from the dendritic region of pyramidal cells in area CA1, and stimulation was delivered to the Schaffer collateral/commissural axons. Electrodes were placed within either the stratum radiatum (apical dendrites), or the stratum oriens (basal dendrites), or in some cases both (see Fig. 2.1).

The basic synaptic response *in vitro* is similar to that seen in the intact animal. Figure 2.3 illustrates the profile of EPSPs recorded *in vitro* in the stratum radiatum of the CA1 region, in response to Schaffer collateral/commissural stimulation. An extracellular electrode located in this region records a monophasic negative potential (Fig. 2.3b) that is indicative of an extracellular inward current associated with dendritic synaptic activity. When recording in the dendritic region *in vitro* it is also usually possible to detect a small negative response that precedes the postsynaptic potential (see Fig. 2.3b, closed arrow). This negative deflection is calcium independent and is termed the presynaptic fibre volley (also termed afferent

Figure 2.3 Field EPSPs recorded in the CA1 region of the hippocampal slice. a, Schematic representation of electrode placement within the hippocampal slice. The stimulating electrode (Stim.) is placed in the path of the Schaffer collateral/commissural projections, whilst the recording electrode (Rec.) is positioned within the stratum radiatum of the CA1 pyramidal cells. **b**, A typical EPSP, obtained using the recording configuration shown in a. Closed arrow indicates fibre volley. **c**, The EPSP corresponds to that shown in b, in response to increased stimulation strength. Note that the amplitude of the afferent volley has increased, whilst a population spike (open arrow) has also been recruited.


a
Stim.
Rec.
comm
CA1
Sch
DG
mf
CA3
fim
pp
(from EC)
b
c
2 mV
5 ms

volley)(Andersen *et al.*, 1977). The afferent volley results from extracellular currents produced in response to the synchronous activation of unmyelinated afferent fibres running through the dendritic layers of this region (Andersen *et al.*, 1978). As the stimulation strength is increased, more presynaptic fibres are recruited, consequently the afferent volley size increases (see Fig. 2.3c). In addition, the slope of the postsynaptic potential becomes steeper and at some point the negative dendritic response is broken by a positive deflection on the rising phase of the EPSP (see Fig 2.3c, open arrow). This is due to the synchronous generation of action potentials in the postsynaptic cells in the recording region and is termed the population spike. Although the stratum radiatum has been used as the example of the basic synaptically evoked response, a similar profile is also seen for EPSPs recorded in the stratum oriens.

Field EPSPs were recorded with extracellular glass microelectrodes, produced from capillary tubing (1.0mm OD x 0.58mm ID, Clark Electromedical Instruments, UK), using a vertical pipette puller (Model 700c Kopf Instruments). Electrodes were broken back under visual control to give a final impedance of 8-12MΩ when filled with ACSF. Electrodes were coupled via a chlorided silver wire to electrode holders (Clark Electromedical Instruments, UK), which were in turn attached to recording headstages (Digitimer Ltd, UK). EPSPs were amplified, and filtered @ 5kHz (Neurolog), then digitised using an ITC-16 Interface (Instrutech Corp., USA) for display and storage on a Power Macintosh 7100/66AV. Off line analysis of the EPSP slope (V/s) was performed with A/Dvance 3.61j software (Fine Scientific Tools, USA).

Potentials were evoked using monopolar stainless steel electrodes with a 250μm tip diameter, tapered at 12°, with a resistance of 5MΩ (A-M Systems Inc. USA). The timing and duration of the test stimuli (monophasic, pulse width 40μs, intensity adjusted in the range of 20-200μA) were under software control. The intensity of the test shock was set to evoke a response with an initial EPSP slope that was ~30% of the maximum slope attainable. In the stratum radiatum at 30% of the maximum EPSP slope, the potentials ranged from 1.5-3.0mV in amplitude, with a gradient of 0.80-1.30V/s. In stratum oriens, under identical conditions, potentials were typically

smaller in both amplitude (0.7-1.5mV) and gradient (0.3-0.6V/s). Each pathway received a test stimulus every 30 seconds, with stimuli to the two pathways interleaved at 15 second intervals. In all experiments the protocol to induce L-LTP was 20 pulses @ 100Hz repeated 6 times with an inter-train interval of 3 seconds. Experiments with a drift in the control pathway of >30% from baseline over a period of 8 hours were discarded. Results are shown as the time course of the change in the field EPSP gradient, expressed as a percentage change from the baseline. Baseline is taken as zero percent and corresponds to the mean of the EPSP gradient values recorded 10 minutes prior to tetanisation. Data were statistically evaluated using an unpaired, two tailed, Student t-test.

In pharmacological experiments involving bath application, drugs were added directly to the existing ACSF reservoir and were therefore applied for the remainder of the experiment. This protocol avoided 'wash-on' artefacts caused by switching between different reservoirs, a problem which had proved difficult to eliminate. The time taken for the drug to reach the slices (termed *dead time*) was 5 minutes. The topical application of drug was achieved by a different method. Glass recording microelectrodes were filled with ACSF containing the drug at the desired concentration. Following electrode insertion into the hippocampal slice, the ACSF/drug mixture could diffuse freely into the tissue, at a rate determined by the diameter of the electrode tip. When applying drugs to the dendritic regions of either the stratum radiatum or the stratum oriens, the same electrodes were also used for recording, ensuring drug delivery to the same population of dendrites that were being sampled. In the case of focal application to the somata, a separate drug-containing electrode was used.

2.2 IN VITRO PROTEIN SYNTHESIS ASSAYS

2.2.1 Slice Holding Chamber Design

For both the *in vitro* translation and transcription assays, radioactive isotopes were used. To avoid radioactive contamination of the interface chamber, an alternative chamber for maintaining healthy hippocampal slices without recording was employed. A schematic diagram of the chamber used in these experiments (Scientific Systems Design Inc., UK) is outlined in figure 2.4. Hippocampal slices rest on a nylon net suspended between a pair of closely fitting acrylic rings. This structure is completely submerged within an ACSF filled Buchner funnel with a sintered glass base. An oxygen rich, gas mixture (95% O_2/5% CO_2), applied to the base of the funnel, passes up through the sintered glass and is dispersed as tiny bubbles which saturate the ACSF and ensure a constant circulation of oxygen rich solution around the slices. The deflector shield below the lower acrylic ring prevents mechanical damage caused by rising gas bubbles and reduces impairments in fluid circulation caused by trapping of gas bubbles under the net. This chamber can keep slices viable for many hours, whilst the small volume of ACSF required (30ml) once again minimises the cost of pharmacological experiments. Figure 2.5a shows potentials from two separate slices that had been incubated in this holding chamber for 5 hours, prior to transfer to an interface chamber for recording (electrode positioning as seen in Fig. 2.3a). These slices can support stable LTP in the stratum radiatum of the CA1 region for at least 1.5 hours (Fig. 2.5b). At the end of the experiment the total time elapsed since slice dissection was in excess of 8 hours.

2.2.1 In vitro Translation Assay

In addition to studying the effects of various translational inhibitors on L-LTP, it was important to have some indication that at the concentration required to block L-LTP these drugs were producing a block of protein synthesis in the slices. The method outlined below was designed as a simple, *in situ* assessment of translation in hippocampal slices, by monitoring the incorporation of [^{3}H] valine into newly synthesised proteins.

Figure 2.4 Schematic representation of a hippocampal slice holding chamber. Hippocampal slices rest on a nylon net, submerged within constantly bubbled oxygen-rich ACSF. Slices are protected from mechanical damage by the surrounding acrylic rings and the deflector shield below.

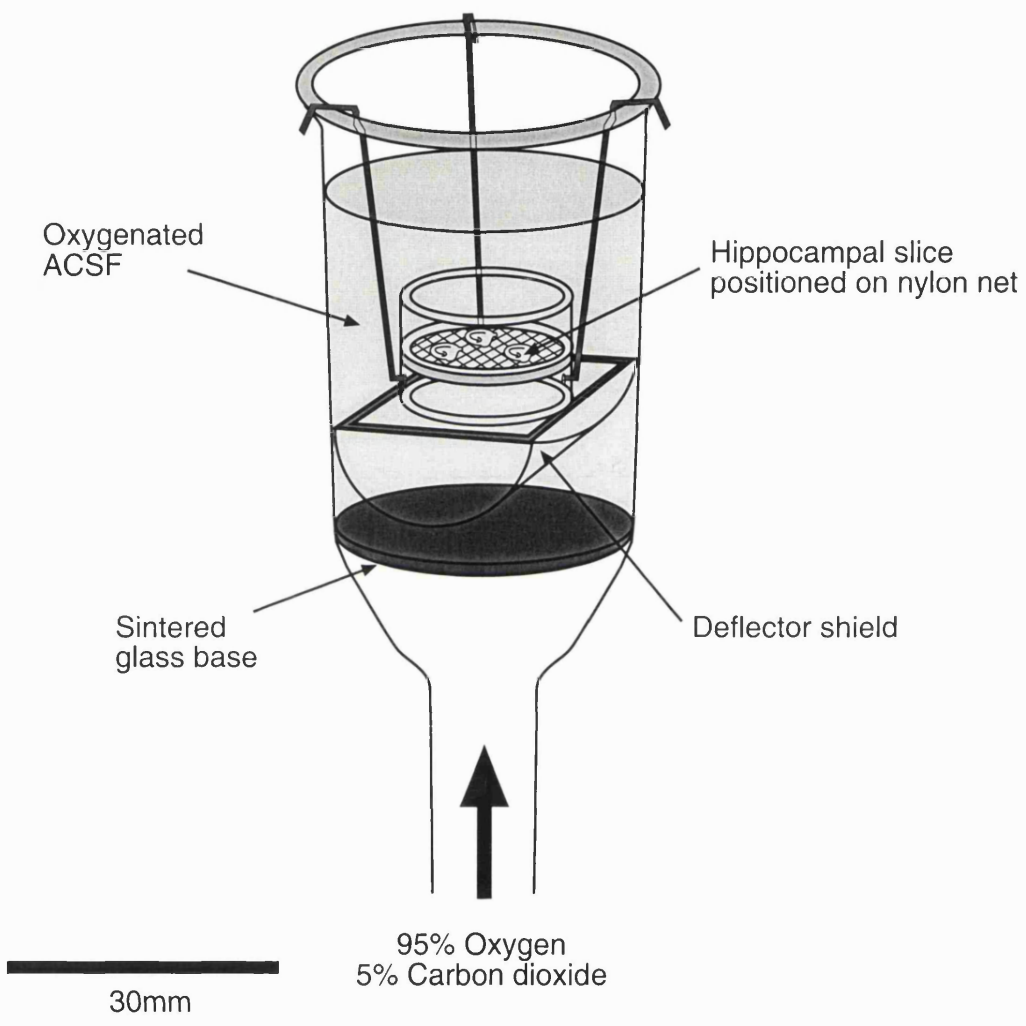
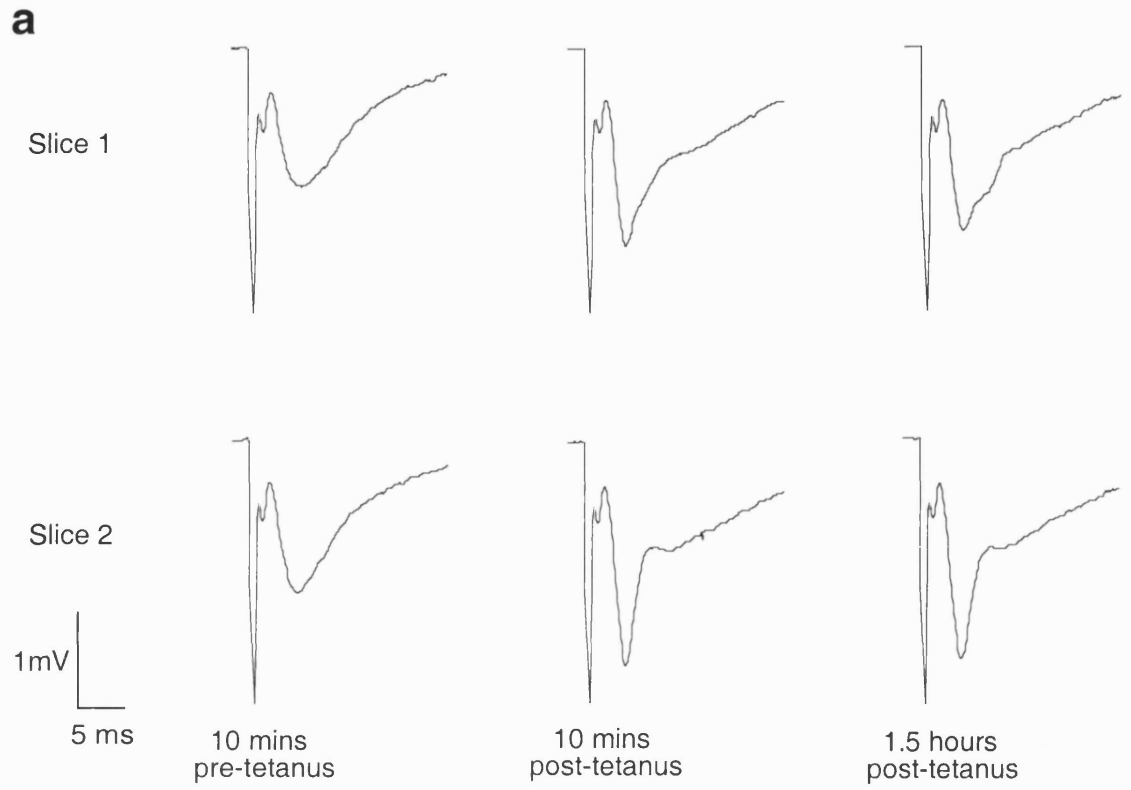
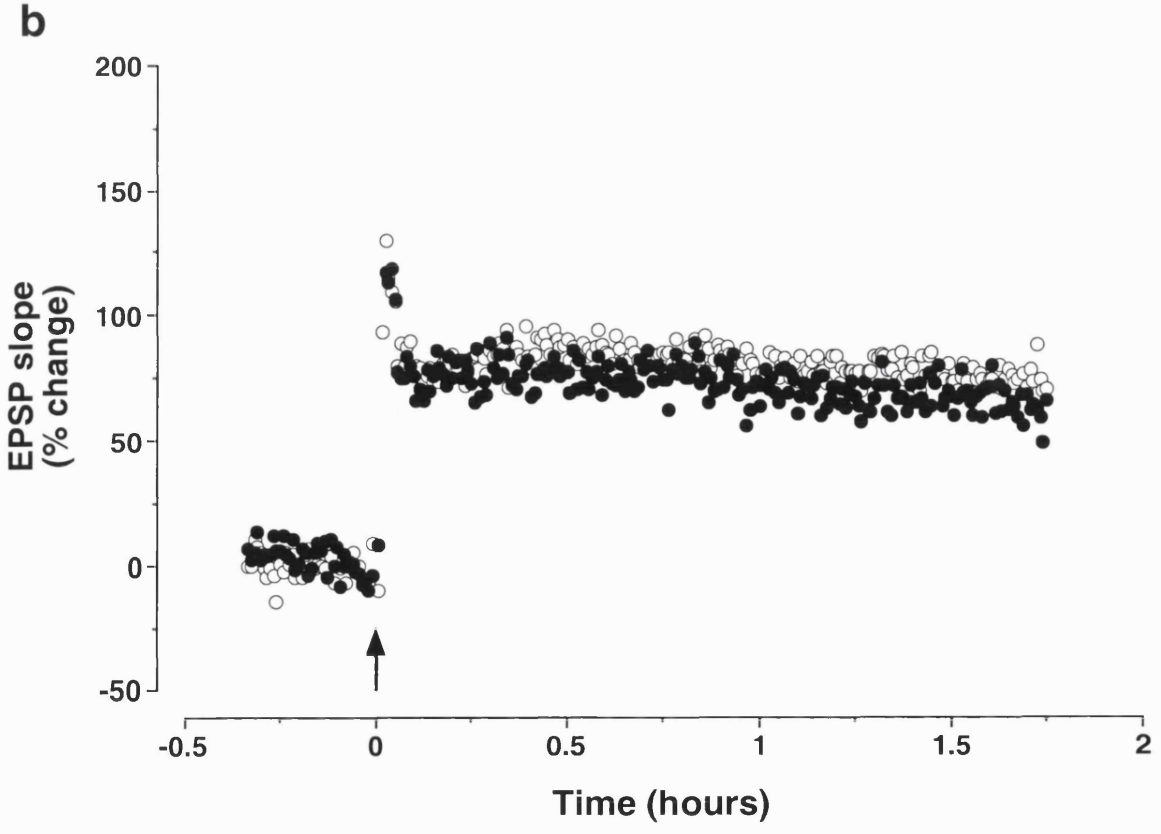

Oxygenated
ACSF
Hippocampal slice
positioned on nylon net
Sintered
glass base
Deflector shield
95% Oxygen
5% Carbon dioxide
30mm

Figure 2.5 Induction of LTP following incubation of slices in a holding chamber. a, Sample Field EPSPs from two separate slices, following 5 hours incubation within the holding chamber. EPSPs are taken at various times before and after LTP induction and correspond to the data shown in b. **b**, Field EPSP slope, expressed as percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Symbols: ● Slice 1; **O** Slice 2.


a
Slice 1
Slice 2
1mV
5 ms
10 mins
pre-tetanus
10 mins
post-tetanus
1.5 hours
post-tetanus


b
EPSP slope
(% change)
200
150
100
50
0
-50
-0.5
0
0.5
1
1.5
2
Time (hours)

Transverse hippocampal slices were prepared from male C57BL6 mice (8-10 weeks, 20-30g) as described previously. For each experiment, four slices were taken from the right hippocampus of two different mice. Immediately after cutting, these slices were transferred to two separate slice holding chambers (see section 2.2.1) and were left to incubate for 2 hours. Slices were distributed so that a single chamber contained four slices (two from each animal). Following incubation, the drug to be tested was added to one of the chambers whilst the other remained as a control. Thirty minutes later [^{3}H] valine (specific activity 27.0Ci/mmol, Amersham International plc, UK) was added to both chambers at a final concentration of 1μCi/ml. After an additional 90 minutes, incorporation was terminated by fixing the slices in 4% paraformaldehyde in phosphate buffered saline (PBS: NaCl 171mM, KCl 3.4mM, Na_2HPO_4 10.1mM, KH_2PO_4 1.8mM, pH 7.4) for 1 hour. Slices were then washed four times for 20 minutes in PBS, and stored in PBS at 4°C overnight. The following day, slices were transferred onto filter paper (Whatman International Ltd., UK) and allowed to dry in air. They were then placed in contact with [^{3}H] sensitive, autoradiographic film (Hyperfilm MP, Amersham International plc, UK) and in some cases an intensifying screen (Kodak Biomax Transcreen LE, Amersham International plc., UK). Preparations were stored at –70°C for 4 months, or for a shorter period of 2 months, in cases where an intensifying screen was used.

2.2.2 In vitro Transcription Assay

A transcriptional assay was also developed to allow the quantitative assessment of mRNA synthesis in hippocampal slice preparations.

Transverse hippocampal slices were prepared from male C57BL6 mice (8-10 weeks, 20-30g) as described previously. For each experiment three slices were taken from the right hippocampus of three different mice. Immediately after cutting, these slices were transferred to three separate slice holding chambers (see section 2.2.1) and were left to incubate for 2 hours. Slices were distributed so that a single chamber contained three slices (one from each animal). Following incubation, drugs to be tested were added to two of the chambers whilst the third remained as a control. One hour after the start of drug incubation, [^{3}H] uridine (specific activity 37.0Ci/mmol, Amersham

International plc., UK) was added to each holding chamber at a final concentration of 1μCi/ml. Slices were incubated for a further 2 hours in the presence of both the drugs and the uridine, at which time, incorporation was terminated by dilution of the isotope in unlabelled uridine (final concentration 0.2mg/ml). Slices were then snap frozen in liquid nitrogen and stored at –70°C.

The following day, mRNA was extracted from these hippocampal slices using a commercially available kit (Qiagen Ltd., UK). This process takes advantage of the characteristic poly A tail, a homopolymer of 20-250 adenosine nucleotides, added only to mRNA following transcription in the nucleus. Separation of poly A^+ mRNA can be achieved by hybridising the polyadenylated tails of mRNA molecules to oligo-dT primers coupled to small beads. RNA species lacking poly A (principally rRNA and tRNA) fail to bind oligo-dT and hence can be washed away.

The protocol is as follows. Slices were homogenised on ice in lysis buffer containing β-mercaptoethanol, using a rotor-stator homogeniser (Polytron PT-MR-3000, Kinematica AG, Switzerland). Cell debris was removed by centrifugation. Oligotex beads were added and left to incubate to allow for hybridisation between oligo-dT on the beads and the poly A tails of the mRNAs. Bead/mRNA complexes were harvested by centrifugation and washed repeatedly. Finally, mRNA was eluted from the oligo-dT beads, in low ionic strength buffer, and collected for further analysis. The level of radioactivity was determined by liquid scintillation counting in 5ml, using a Beckman LS 5000 CE counter. Inhibition of mRNA synthesis (%) was determined by calculating the ratio of [^{3}H] uridine incorporation between control and drug samples.

2.3 PHARMACOLOGY

A list of all drugs used, their method of preparation and their mode of action is provided below.

Anisomycin

Anisomycin inhibits protein synthesis during translation by blocking the peptidyl transferase reaction on ribosomes. Anisomycin (Sigma, UK), was dissolved in equimolar HCl, adjusted to pH 7.2. It was stored as a stock solution at –20°C and diluted to the final concentration in the perfusate. It was also prepared by dissolving in ACSF via sonication.

Emetine

Emetine irreversibly blocks the translation step of protein synthesis by inhibiting the movement of ribosomes along the mRNA. Emetine, Dihydrochloride (Calbiochem, UK) was prepared each day and diluted to the final concentration in the perfusate.

Actinomycin-D

Actinomycin-D is an RNA synthesis inhibitor. It blocks transcription by complexing with DNA and inhibiting the movement of DNA-primed, RNA polymerases. Actinomycin-D (Sigma, UK) was dissolved in methanol and stored as a stock solution at –20°C. It was diluted to the final concentration in the perfusate.

α-Amanitin

α-Amanitin is a highly selective inhibitor of RNA polymerase II and consequently it inhibits messenger RNA synthesis. It does not block RNA polymerase I and has a moderate effect on RNA polymerase III at higher concentrations (see section 5.2 for more detail). α-Amanitin (Sigma, UK) was dissolved in H_2O and stored as a stock solution at –20°C. It was diluted to the final concentration in the perfusate.

Bisindolylmaleimide I

Bisindolylmaleimide I (BDM I) is a cell-permeable kinase inhibitor displaying increased selectivity for PKC compared to other kinase inhibitors such as H7 and

staurosporine (BDM I, IC_{50} values: Mixed Rat Brain PKC 0.031μM, phosphorylase kinase 0.7μM, PKA 2μM). BDM I is a staurosporine analogue and acts as a competitive inhibitor for the ATP-binding site of PKC. Bisindolylmaleimide I, Hydrochloride (Alexis Corporation, UK) was dissolved in H_2O and stored as a stock solution at –20°C. It was diluted to the final concentration in the perfusate.

LATE-PHASE LTP: OPTIMISATION OF EXPERIMENTAL CONDITIONS

3.1 FACTORS THAT DETERMINE HIPPOCAMPAL SLICE HEALTH

The precise conditions used for maintaining healthy hippocampal slices, capable of expressing L-LTP, have been discussed in the previous chapter. However, due to the technical difficulties associated with maintaining stable field recordings in these slices, coupled with a lack of published data addressing this problem, I shall provide a discussion of this subject. The technical aspects of brain slice methodology that have proved critical for the recording of stable L-LTP in my experiments, are highlighted below.

3.1.1 Animals

Hippocampal slices were initially prepared from male Sprague Dawley rats (80-150g). However, problems with slice health and stable recordings were never adequately resolved. For these reasons it was decided to switch to mouse (C57BL6) hippocampal slice preparations. The condition of the mouse slices in the recording chamber seemed greatly enhanced compared to slices from the rat. In addition, field EPSP stability in the mouse slices was much improved. However, whether these observations are indicative of a true difference between species, or that the conditions met by the chamber were more suited to those required by mouse slices, is not clear. Hippocampal slice preparation is more difficult in the mouse, due the size of the brain, coupled with a reduction in the number of slices that can be taken from an individual hippocampus. However, perfecting the dissection technique, and optimising the chamber for mouse slices, allows the experimenter access to the vast number of transgenic mice that are now available.

3.1.2 Temperature

Although ideally one would like to maintain hippocampal slices at, or near normal body temperature (38-39°C for small animals like mice or rats), preparations generally survive longer and in a healthier state, when lower temperatures are used. It is for this reason that L-LTP experiments are traditionally performed at temperatures between 28°C and 32°C (Frey *et al.*, 1993; Huang & Kandel, 1994; Nguyen *et al.*, 1994; Abel *et al.*, 1997; Frey & Morris, 1997; Baranes *et al.*, 1998; Impey *et al.*, 1998a; Korte *et al.*, 1998; Winder *et al.*, 1998; Lu *et al.*, 1999; Wong *et al.*, 1999).

I found that incubation of mouse slices at 32°C gave cellular responses that were large and hyperexcitable. Field recordings would show multiple population spikes within a single EPSP whilst in some cases epileptiform discharges could also be seen. Despite this, slices maintained at 32°C were able to exhibit limited LTP (however in some cases a small depression was seen following the tetanus), where EPSPs would return to pre-tetanus levels over the course of about 2–6 hours. In many cases this was coupled with a decrease in the EPSP gradient in the non-tetanised (control) pathway over the same time period, suggesting that the 'loss of LTP' was due to a deterioration in slice health. Ultimately, I concluded that at 32°C, my chamber would not sustain slices capable of expressing L-LTP. I therefore decided to lower the temperature to 27-28°C, a temperature which had previously been shown to support L-LTP in mouse hippocampal slices (Nguyen & Kandel, 1997). At this temperature slices did not appear hyperexcitable, were not depressed after potentiation and could support L-LTP for 8 hours or more.

3.1.3 Stability

Although a recording chamber may provide the conditions required to maintain hippocampal slices for extended lengths of time (i.e. oxygenation, temperature, humidity), it is frequently still difficult to obtain stable recordings for long time periods. Typically, stability is not a problem when recording from hippocampal slices for up to 2 hours, since small changes in the profile of the potentials (termed 'drift') are modest. However, during L-LTP experiments where the time base is extended to 9

hours or more, a slow drift can become significant, often requiring the exclusion of experiments from the data pool.

The two main causes of EPSP drift, were electrode movement and chamber flooding. Small changes in the position of the stimulating or recording electrodes, can lead to changes in the EPSP profile. Electrode movement occurs when condensation collects on the stainless steel stimulating electrodes, the additional load causing them to move, and change position in the slice. In extreme cases, droplets of condensation fall onto the slice, abolishing the potentials due to mechanical and osmotic trauma. Clearly, condensation worsens as the incubation temperature in the chamber increases. To minimise movement, electrodes can be strengthened by sheathing them in glass capillary tubing, however this increase in electrode thickness limits the number of electrodes that can be positioned within a small area. This is a particular concern when recording from mouse slices. Blotting condensation from the electrodes using an absorbent wick provides some relief from the problem, however this practise does increase the risk of electrode displacement during droplet removal. A further solution, and the one employed in this project, was to increase the temperature of the external environment to a level just below that of the chamber, thereby reducing the amount of condensation formed.

Drifting potentials can also result from changes in the level of ACSF in the chamber. The ACSF level is critical for slice health as it determines the amount of oxygen that can be absorbed from the environment. Changes in slice oxygenation cause a drift in both the afferent volley and the EPSP profile, rendering the experiment uninterpretable. In extreme cases these changes in solution levels can lead to slice death. In conventional chambers the slices rest on a layer of lens tissue that protrudes into the outflow duct making direct contact with the waste ACSF. This arrangement produces a wick, which reduces the surface tension of the ACSF and enables free flow and drainage of fluid into the outflow chamber. This system seems to work well over the first 4-6 hours, however the lens tissue ultimately becomes saturated, resulting in an increase in the ACSF level around the slices. To overcome this problem the lens tissue wick was replaced with a silver wire that formed a channel between itself and the perspex of the chamber, thereby aiding drainage. This wire

maintained the same properties throughout the duration of the experiment, thereby eliminating changes in solution levels.

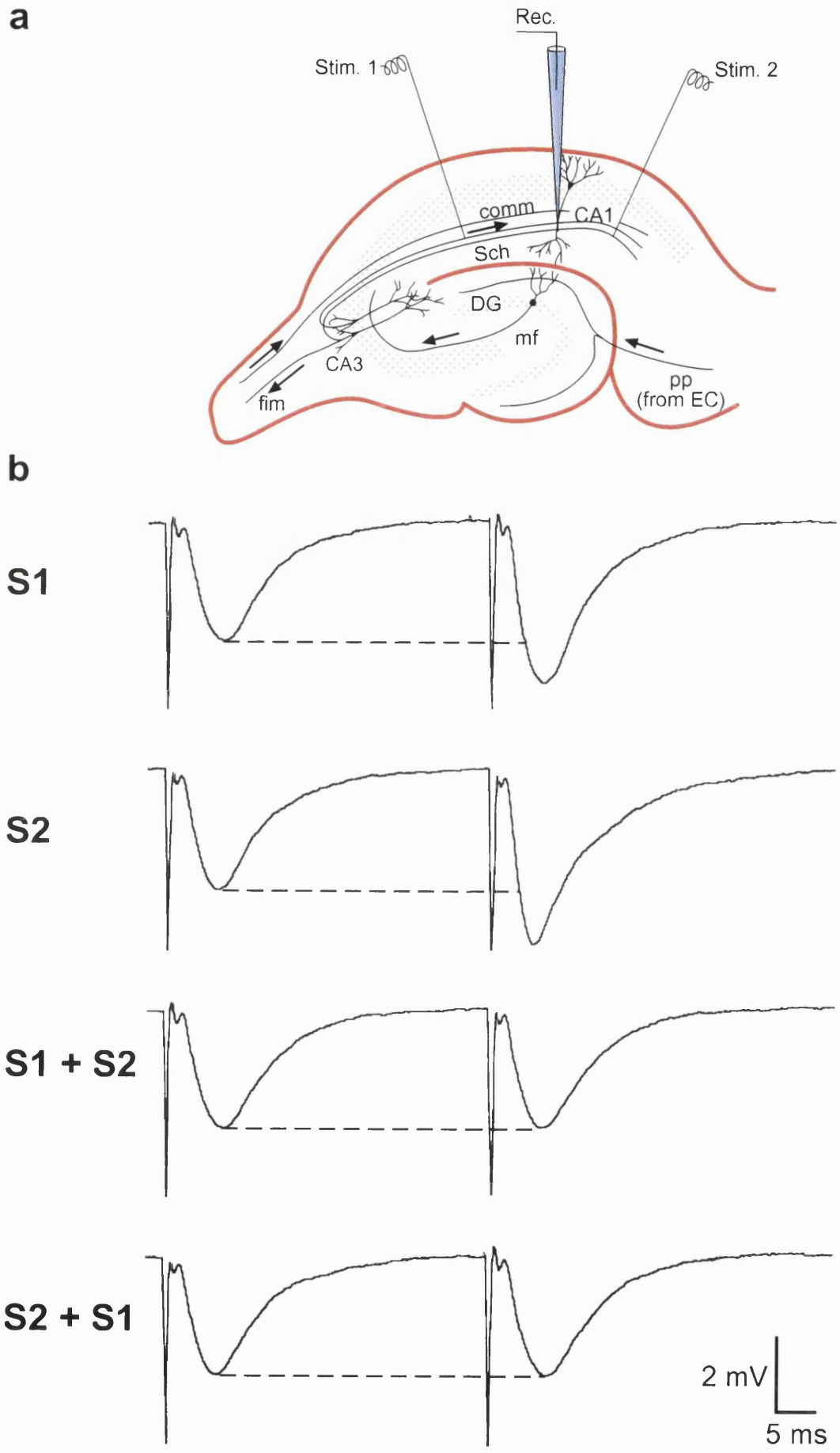
3.2 DETERMINATION OF SEPARATE PATHWAYS

3.2.1 Experimental Design

When studying LTP for extended time periods it is important to also have some indication of slice health. This can be achieved by recording from two separate pathways within the same region of the slice. Each pathway corresponds to a distinct population of synapses, within an overlapping population of postsynaptic cells. The arrangement makes use of the input specific nature of LTP and permits the tetanisation of one pathway (or group of synapses), and the subsequent monitoring of LTP in that pathway, whilst the other pathway is not tetanised, and remains as an unpotentiated control. This experimental configuration allows a comparison to be made between the two pathways, ultimately permitting experiments to be accepted or rejected on the basis of the stability of the non-tetanised pathway.

The recording configuration for a two pathway experiment is shown in figure 3.1a. Two monopolar stainless steel electrodes were placed either side of the recording microelectrode, ensuring the stimulation of independent synaptic populations. The independence was tested in each experiment by measuring paired pulse facilitation (the increase in a second EPSP, when it is elicited shortly after a first, to the same population of synapses). Paired pulses (two single pulses each of 40μs and with an inter-stimulus interval of 40ms) were delivered to the first pathway, S1, eliciting an increase in the amplitude of the response to the second pulse compared to the first (facilitation) (Fig. 3.1b). This was repeated for the second pathway, S2. A single pulse was then applied to S1, followed 40ms later by a single pulse to S2. This was repeated with a single pulse to S2 followed 40ms later by a single pulse to S1. If no facilitation was seen to the second pulse when S1 and S2 were each stimulated singly, then these pathways were said to be independent.

Figure 3.1 Determination of independent pathways in the hippocampal slice. a, Schematic representation of electrode placement within the hippocampal slice. Two stimulating electrodes (Stim.) are positioned within the stratum radiatum of the CA1 region, on either side of the recording electrode (Rec.). This maximises the chances of stimulating two populations of convergent but non-overlapping Schaffer collateral/commissural fibres, thereby ensuring the stimulation of independent synaptic populations. For key to abbreviations, see figure 2.1. **b**, Testing for pathway independence. Field EPSPs were elicited by application of a paired pulse (2 single pulses of 40μs duration @ test shock intensity, separated by 40ms) through stimulating electrode 1 (S1). This is repeated to stimulating electrode 2 (S2). A single pulse is then applied to S1, followed 40ms later by a pulse to S2. This is repeated by applying a single pulse to S2 followed 40ms later by a pulse to S1. The dotted lines aid comparison between first and second responses.


a
Rec.
Stim. 1
Stim. 2
comm
CA1
Sch
DG
mf
CA3
fim
pp
(from EC)
b
S1
S2
S1 + S2
S2 + S1
2 mV
5 ms

3.3 INDUCTION OF LATE–PHASE LTP

3.3.1 Experimental Design

Control experiments were performed, in order to determine the magnitude and duration of the potentiation in ACSF solutions using 400-450μM hippocampal slices from 8-10 week old C57BL6 mice, maintained at 27-28°C. Electrode placement is illustrated in figure 3.1a. Field EPSPs evoked by activation of the Schaffer collateral/commissural afferents, were recorded from two independent pathways in the stratum radiatum. Test stimuli were delivered alternately to each pathway at 15 second intervals and responses were monitored for in excess of 8 hours. Following a 1 hour baseline, a tetanus (20 pulses @ 100Hz repeated 6 times with an ITI of 3 seconds) was applied to one pathway whilst the other provided a non-tetanised control. Experiments in which drift in the non-tetanised pathway was <30%, over the course of 8 hours, were selected for further analysis.

3.3.2 Results

Stable field EPSPs were recorded in excess of 8 hours, for both the tetanised and non-tetanised pathways in the stratum radiatum. Field EPSPs taken from a sample experiment, are shown in figure 3.2. Note that the profile of the afferent volley is largely unchanged over the course of the experiment. The afferent volley reflects the number of presynaptic fibres activating the postsynaptic cells. LTP is an increase in the efficacy of synapses and not the recruitment of axonal projections. Consequently, changes in the size or latency of the afferent volley should not occur in either the tetanised or non-tetanised pathway. The afferent volley therefore serves as a useful tool in identifying changes in the EPSP slope, that could be interpreted as changes in synaptic efficacy, but are in fact due other phenomena, such as electrode drift or cell death. Therefore, experiments are also selected on the stability of the afferent volley throughout the experiment, in addition to selecting experiments on the amount of drift seen in the non-tetanised control pathway.

Figure 3.2 Representative examples of field EPSPs taken from a single L-LTP induction experiment. Individual EPSPs from both the tetanised and non-tetanised pathways in the stratum radiatum, are shown at various time points prior to, and following the tetanus. Enlarged boxes allow for the direct comparison of the EPSP afferent volleys in each pathway. The potentials are taken from the experiment shown in figure 3.3.

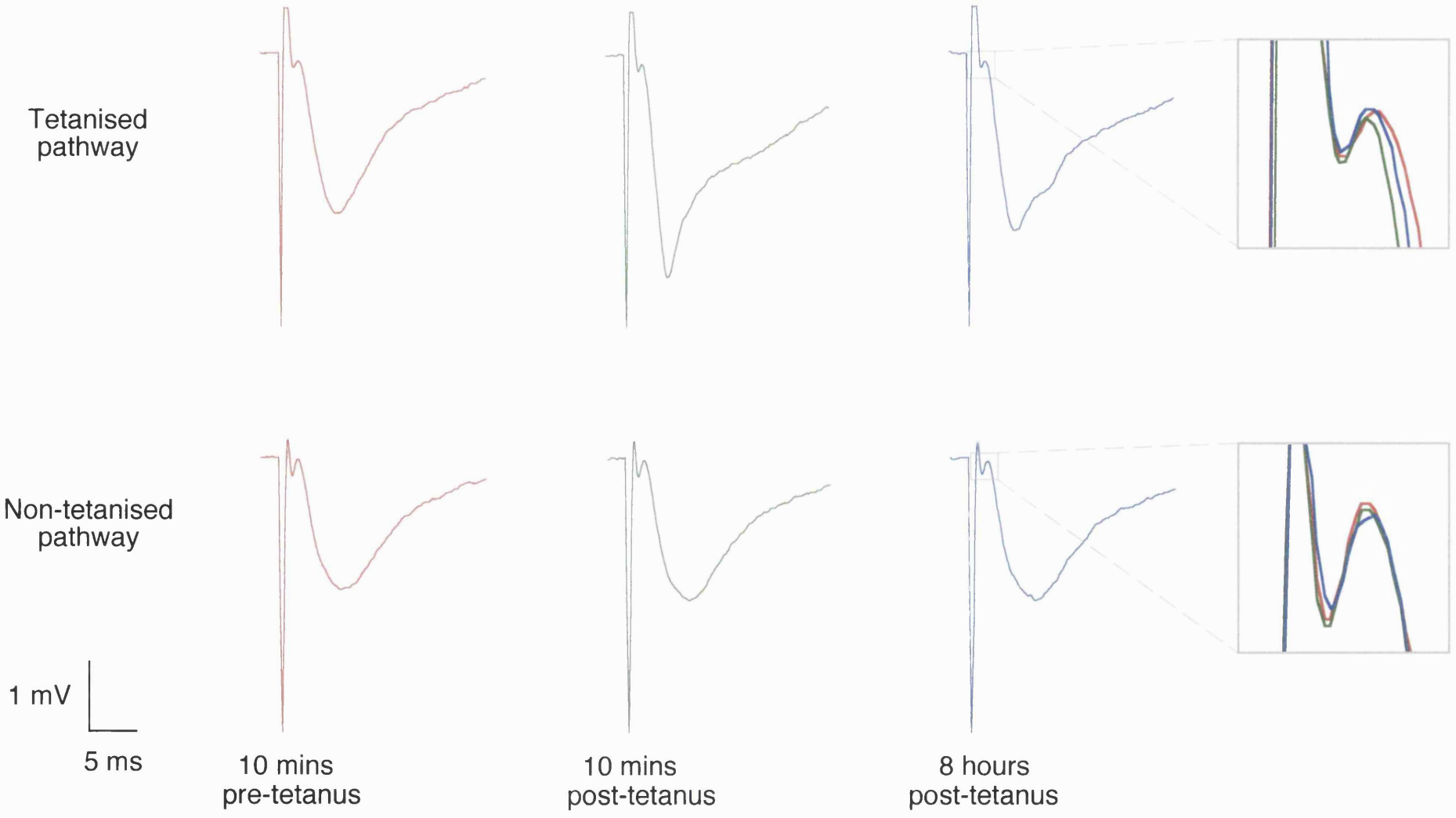

Tetanised pathway
Non-tetanised pathway
1 mV
5 ms
10 mins pre-tetanus
10 mins post-tetanus
8 hours post-tetanus

Figure 3.3 shows, in a representative experiment, the time course of the change in the field EPSP slope (expressed as percentage change from the baseline), in two independent pathways. Following the tetanus, LTP in the tetanised pathway declines from a PTP value of 165%, over ~5 hours, to reach a stable level of 38% and sustains this level of potentiation up to the 8 hour time point (35%). The non-tetanised pathway shows no change, either immediately following the tetanus (-3%), or after 8 hours (4%).

The results of 11 L-LTP induction experiments are shown in figure 3.4. The data are averaged and error bars are shown for every 20th point (i.e. every 10 minutes). The tetanised pathway exhibits robust potentiation reaching a PTP value of 156±14% (SEM). LTP subsequently declines to a stable level over ~3 hours (51±6%) and sustains this level of potentiation for at least 8 hours; reaching a value of 47±6% at 8 hours post-tetanus. Similarly, the non-tetanised pathway remains relatively stable over the same time frame (14±4% at 8 hours). Statistical comparisons between the potentiated and control pathways at various time points throughout the experiment (Fig. 3.5), show significant differences during PTP ($t=14.50$, $P<0.0001$), and 30 minutes post-tetanus ($t=10.27$, $P<0.0001$). A difference between the two pathways is also maintained at the 8 hour time point ($t=4.86$, $P\leq0.0001$)(Fig. 3.5).

3.4 DISCUSSION

3.4.1 Technical Aspects of Hippocampal Slice Health

Over the last 20 years a number of attempts have been made to define the optimum conditions required for healthy hippocampal slices (Skrede & Westgaard, 1971; Richards, 1981; Alger *et al.*, 1984); however, these were concerned with maintaining slices for periods of only a few hours. In both this and previous chapters, a great deal of emphasis has been placed on the requirements of hippocampal slices, for the induction and subsequent maintenance of L-LTP. Although some of these requirements may seem minor, they can make the difference between a successful and an unsuccessful experiment, and are usually omitted from the methods sections of published papers. Within the field, many different slice chambers are used, often

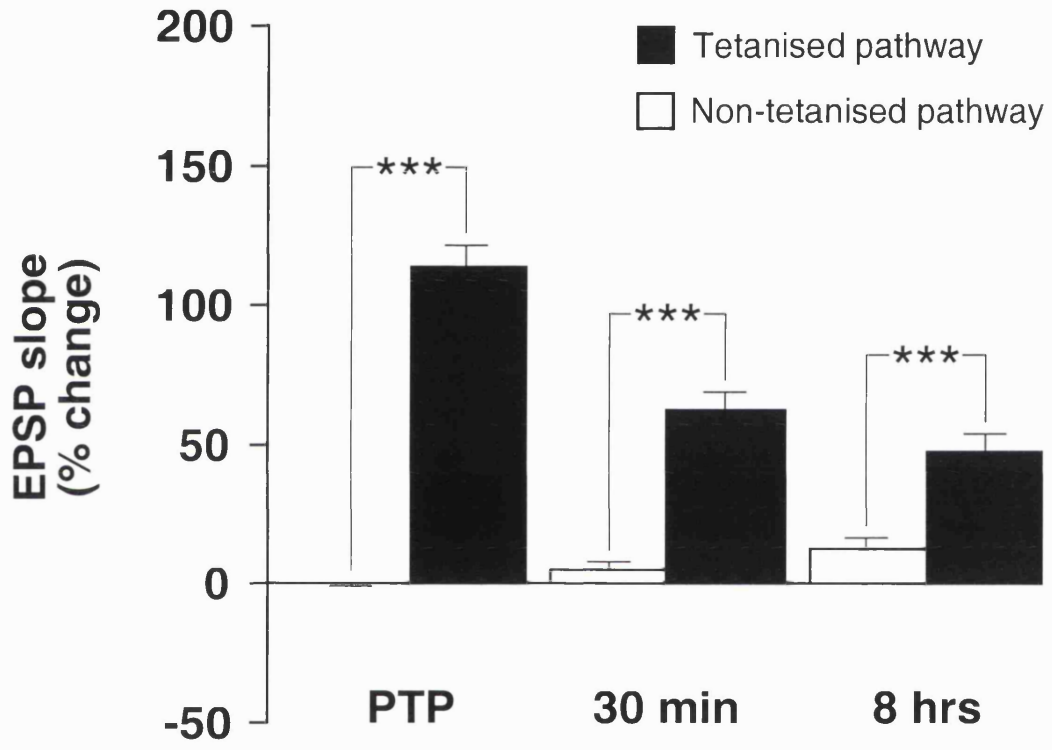
Figure 3.3 An example of L-LTP in the CA1 region of the hippocampus. Electrodes were positioned within the stratum radiatum. Field EPSP slope, expressed as the percentage change over time is shown. Baseline (zero percent) was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains of 20 pulses @ 100 Hz ITI 3 secs). Symbols: ● tetanised pathway; O non-tetanised pathway.

EPSP slope
(% change)
-50
0
50
100
150
200
Time (hours)
-1
0
1
2
3
4
5
6
7
8

Figure 3.4 Induction of L-LTP in the CA1 region of the hippocampus. Electrodes were positioned within the stratum radiatum. Field EPSP slope, expressed as the percentage change over time is shown. Baseline (Zero percent) was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Symbols: ● tetanised pathway; **O** non-tetanised pathway. Error bars indicate ±SEM (n=11).

EPSP slope
(% change)
-50
0
50
100
150
200
-1
0
1
2
3
4
5
6
7
8
Time (hours)

Figure 3.5 Statistical comparison between tetanised and non-tetanised pathways during L-LTP in the CA1 region of the hippocampus. Comparisons were made between the tetanised and non-tetanised pathways, during 3 distinct time periods following L-LTP induction. **PTP** (post-tetanic potentiation) corresponds to the 5 minute time period immediately following tetanisation; **30 min** corresponds to the 10 minute time period, between 25-35 minutes post-tetanus; and **8 hrs** corresponds to the 10 minute time period, between 7 hours 50 minutes-8 hours post-tetanus. Values represent the mean EPSP slope (expressed as the percentage change) measured during each time period. ***P<0.0001 (unpaired Student t test, two tailed). Error bars indicate ±SEM (n=11). Raw data sources are identical to those used for figure 3.4.


200
150
100
50
0
-50
EPSP slope
(% change)
Tetanised pathway
Non-tetanised pathway
***
***
***
PTP
30 min
8 hrs

making the exact parameters used in an experiment rather ambiguous. With this in mind it should be recognised that the conditions that I describe are those that I have optimised for the Scientific Systems Design chamber used in these experiments. It is however felt that some of the principles described are generally applicable.

3.4.2 Data Selection Procedure

Despite extensive experimentation in an attempt to optimise the parameters for healthy slices, stable L-LTP is still achieved in less than 20% of all cases. Consequently it became necessary to develop strict criteria for the inclusion of individual experiments into the data pool. These were: 1) the afferent volley of the EPSPs in each pathway did not change by more than 30% over the course of the experiment; 2) the drift in the non-tetanised pathway of the experiments did not exceed 30% during the 8 hour period following the tetanus.

In addition to the problems discussed for any single experiment, difficulties were also encountered in achieving consistent L-LTP induction between experiments. Extended periods of time (1 week, up to 3 months) would pass where slices did not show L-LTP. Although in some cases sub-optimal slice health as a result of chamber conditions was almost certainly the cause, on other occasions failure to induce L-LTP reflected seasonal changes and may therefore have been due to the animals themselves. During these extended periods, data was only included into the data pool once L-LTP could be induced and maintained in slices from different mice on two consecutive days. At this point, experiments fulfilling the relevant criteria (i.e. stable baseline and afferent volley), were included as part of the data set. During this time period, drug experiments were interleaved with control experiments on a day to day basis. Inclusion into the data set was terminated when two consecutive interleaved control experiments did not show L-LTP. Drug experiments were resumed again, only after L-LTP could be induced on two consecutive days.

3.4.3 Tetanisation Requirements for the Induction of Late-Phase LTP

In this chapter I have demonstrated that L-LTP can be induced in the stratum radiatum of the CA1 region of hippocampal slices. This L-LTP can remain stable and be maintained for at least 8 hours.

In the majority of the literature, L-LTP is induced using tetanisation procedures consisting of 3-4 trains of 100Hz stimulation (1 second duration) with an inter-train interval (ITI) of 5-10 minutes (Frey *et al.*, 1988; Nguyen *et al.*, 1994; Impey *et al.*, 1996; Abel *et al.*, 1997; Winder *et al.*, 1998; Lu *et al.*, 1999). However, here I show that L-LTP could be induced reliably in the stratum radiatum of the CA1 region of mice, in response to a tetanus consisting of 20 pulses @ 100Hz, repeated 6 times with an inter-train interval of 3 seconds. These results are consistent with other groups that have shown that L-LTP is not exclusively induced by multiple trains of 100Hz. Both 15 trains of 4 pulses @100Hz ITI 200msec(Nguyen & Kandel, 1997) or 3 trains of 30 pulses @100Hz ITI 5s (Korte *et al.*, 1998) will induce Schaffer collateral/CA1 L-LTP in mice. Furthermore, L-LTP in the CA1 region of rats has been induced using a tetanisation pattern, consisting of 4 trains of 20 pulses @ 100Hz with a 6 second ITI (Wong *et al.*, 1999) or even via a single 100Hz train for one second (U. Frey, personal communication).

In conclusion, I have optimised the interface slice chamber design, and investigated the conditions required for the maintenance of hippocampal slices for extended periods of time. This has enabled the induction and subsequent recording of stable L-LTP in the CA1 region of these slice preparations for at least 8 hours. The development of this preparation now permits a number of issues to be addressed, regarding the dependence of L-LTP on transcription and translation.

THE DEPENDENCE OF L-LTP ON TRANSLATION

4.1 IS L-LTP DEPENDENT ON PROTEIN SYNTHESIS?

Since the 1960's a large body of behavioural evidence has been amassed, suggesting that *de novo* protein synthesis is required for the formation of long-term memory, whilst short-term memory is protein synthesis independent (reviewed by Davies & Squire, 1984). The requirement for protein synthesis has also been investigated for candidate memory storage mechanisms, such as LTP (for further detail see section 1.5.1). Intraventricular injection of inhibitors of translation (anisomycin, emetine, cycloheximide and puromycin), cause LTP in the dentate gyrus of freely moving or anaesthetised rats to return to pre-tetanus levels over the course of 3-6 hours, whilst LTP in non-injected animals can remain elevated for up to several days (Krug *et al.*, 1984; Otani & Abraham, 1989; Otani *et al.*, 1989) (see also section 1.5.1). These results suggest that LTP, like behavioural memory, consists of at least two phases, a protein synthesis independent early phase (E-LTP), followed by a late phase (L-LTP) that requires protein synthesis.

Pioneering work, exploring methods for prolonging the lifespan of hippocampal slice preparations has enabled this late-phase LTP to be studied *in vitro* (Reymann *et al.*, 1985; Thiemann *et al.*, 1986). Bath application of the translation inhibitor anisomycin, to rat hippocampal slices, causes population spike LTP in the stratum radiatum of the CA1 region to return to pre-tetanus levels over 8 hours (Frey *et al.*, 1988). Anisomycin produces a similar effect in mice, with LTP of the EPSP in the stratum radiatum returning to baseline levels within 3 hours (Nguyen & Kandel, 1997). In addition, L-LTP in the CA1 region of rat hippocampal slices is also blocked by application of the translation inhibitor emetine (Frey & Morris, 1997).

One important observation is that both *in vivo* and *in vitro*, a critical period of protein synthesis is required for the induction of L-LTP. This period occurs within the first 15-40 minutes following LTP induction. If translation inhibitors are applied after this

time period, then L-LTP induction occurs normally (Otani *et al.*, 1989; Frey & Morris, 1997).

In this chapter, the requirement for protein synthesis during LTP will be addressed. Two different inhibitors of translation (anisomycin and emetine), each with a different site of action, are examined.

4.2 THE SITE OF ACTION OF THE TRANSLATION INHIBITORS ANISOMYCIN AND EMETINE

During translation, polypeptide chain elongation occurs in three discrete steps. One of these steps is catalysed by the enzyme peptidyl transferase and involves the uncoupling of an amino acid from its corresponding tRNA complex and the formation of a peptide bond to the next amino acid in the chain. Anisomycin binds to the 60s ribosomal subunit and blocks this peptidyl transferase reaction, thereby blocking protein synthesis (Edwards and Machadevan, 1992).

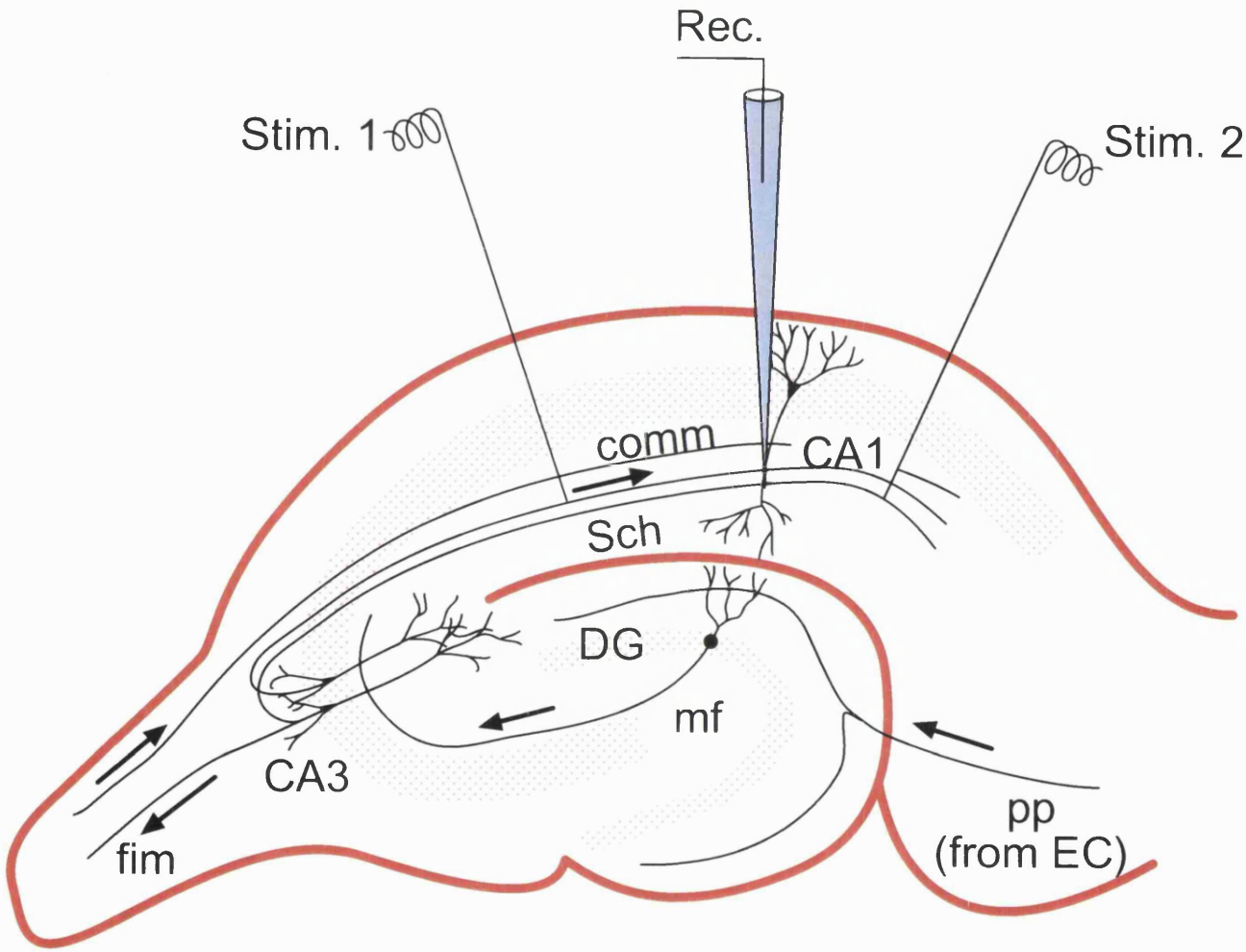
The protein synthesis inhibitor emetine also blocks polypeptide chain elongation. It acts by directly and irreversibly inactivating ribosomes. This is thought to result from its interaction with the tRNA binding site (the 'A site') on the 40s ribosomal subunit (Galasinski, 1996).

4.3 L-LTP INDUCTION IN THE PRESENCE OF THE TRANSLATION INHIBITOR ANISOMYCIN

4.3.1 Experimental Design

Electrode placement is illustrated in figure 4.1. Field EPSPs were recorded from two independent pathways in the stratum radiatum, in response to activation of the Schaffer collateral/commissural afferents. Test stimuli were delivered alternately to each pathway at 15 second intervals and responses were monitored for at least 8 hours. Following a 1 hour baseline, a tetanus (20 pulses @ 100Hz repeated 6 times

Figure 4.1 Schematic representation of electrode placement within the CA1 region of the hippocampal slice. Two stimulating electrodes (Stim.) are positioned within the stratum radiatum of the CA1 region, on either side of the recording electrode (Rec.). This maximises the chances of stimulating two populations of convergent but non-overlapping Schaffer collateral/commissural fibres, thereby ensuring the stimulation of independent synaptic populations. For key to abbreviations, see figure 2.1.


Rec.
Stim. 1
Stim. 2
comm
CA1
Sch
DG
mf
CA3
fim
pp
(from EC)

with an ITI of 3 seconds) was applied to one pathway whilst the other provided a non-tetanised control.

The translation inhibitor, anisomycin, was bath applied to the hippocampal slices at a range of concentrations (15μM-50μM). Anisomycin was applied between 30-60 minutes prior to tetanisation and remained on the slices for the duration of the experiment. In experiments in which D-AP5 (25μM) was used to allow for tetanisation in the absence of LTP, it was dissolved in ACSF containing anisomycin (30μM) and bath applied.

4.3.2 Results

In the majority of experiments, anisomycin application produced a drift in either one or both of the pathways. The drift in the non-tetanised pathway was usually larger than the 30% criterion, over the 8 hour test period (see section 3.4.2), thereby excluding the experiment from the data pool. Figures 4.2 and 4.3 show the results of one such experiment in which LTP was induced in the presence of anisomycin (50μM). The EPSP slope of the non-tetanised pathway gradually drifts upwards over the course of the experiment, from a baseline value of 3% immediately after the tetanus, reaching a value of 37% at the 8 hour time point (Fig. 4.3). The drift of the non-tetanised pathway can be clearly seen from the profile of individual EPSPs recorded from this pathway (Fig 4.2). EPSPs from the non-tetanised pathway increase in both slope and amplitude. However, the profile of the afferent volley of these potentials remains unaffected over the duration of the experiment, making changes produced by electrode movement an unlikely explanation for the EPSP drift.

In the experiment shown in figure 4.3, the tetanised pathway declines from an EPSP slope value of 168% immediately after the tetanus, reaching 44% at 8 hours. This value is equivalent to that seen in the upwardly drifting non-tetanised pathway at the same time point (37% at 8 hours) (Fig. 4.3). Is the upward drift of the EPSP slope masking the block of L-LTP induction by anisomycin? It is well established that the tetanised and non-tetanised pathways behave similarly in response to changes in the slice as a whole. It therefore seems reasonable to use subtraction as an indication of

Figure 4.2 Drifting of field EPSPs following LTP induction in response to bath application of the protein synthesis inhibitor anisomycin. Individual EPSPs from both the tetanised and non-tetanised pathways within the stratum radiatum, are shown at various time points prior to, and following the tetanus. Anisomycin (50μM) was added 1 hour prior to tetanisation, and remained present for the duration of the experiment. The dotted lines aid direct comparison of EPSP amplitude in the non-tetanised pathway. Potentials are taken from the experiment shown in figure 4.3.

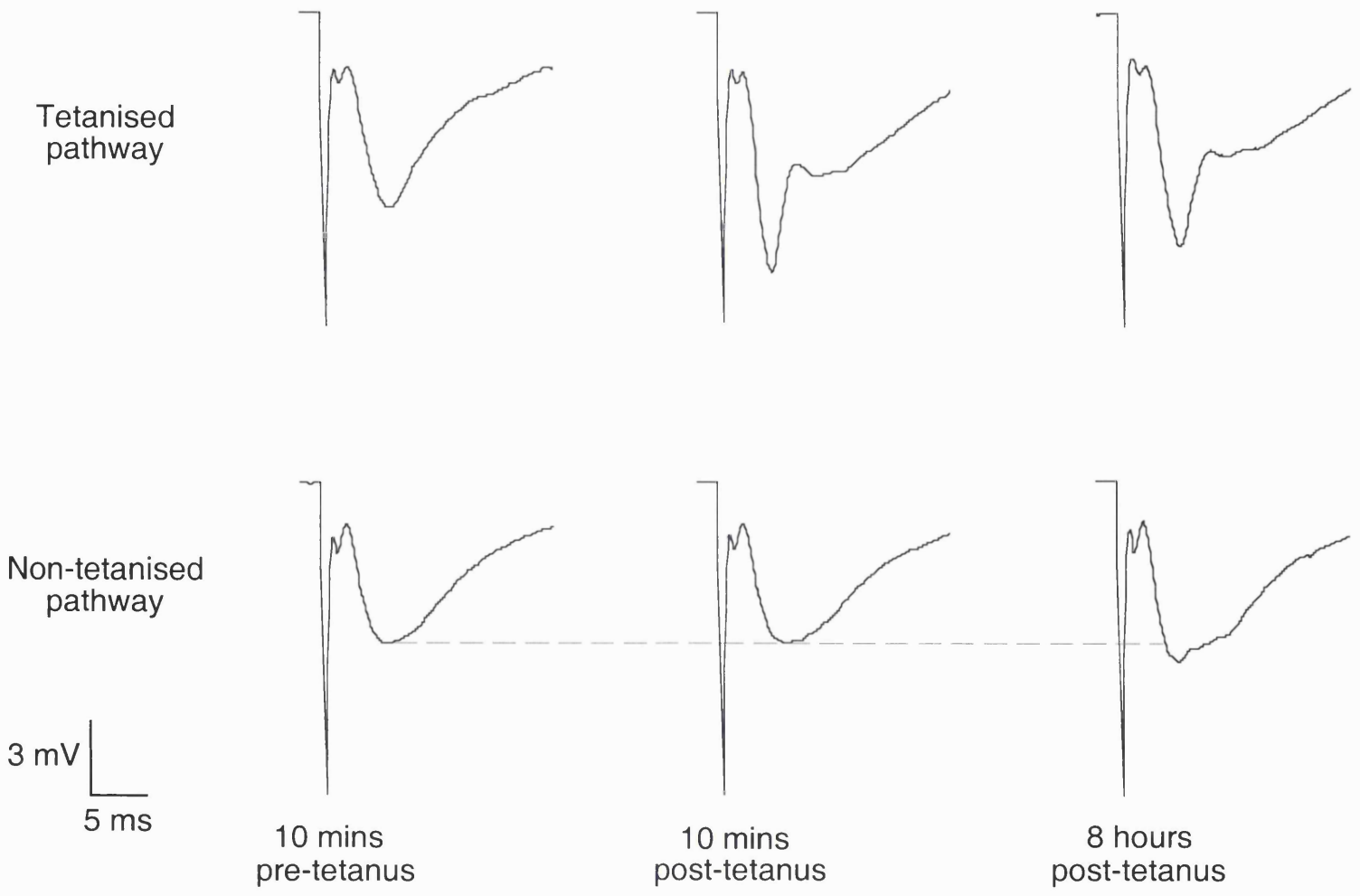
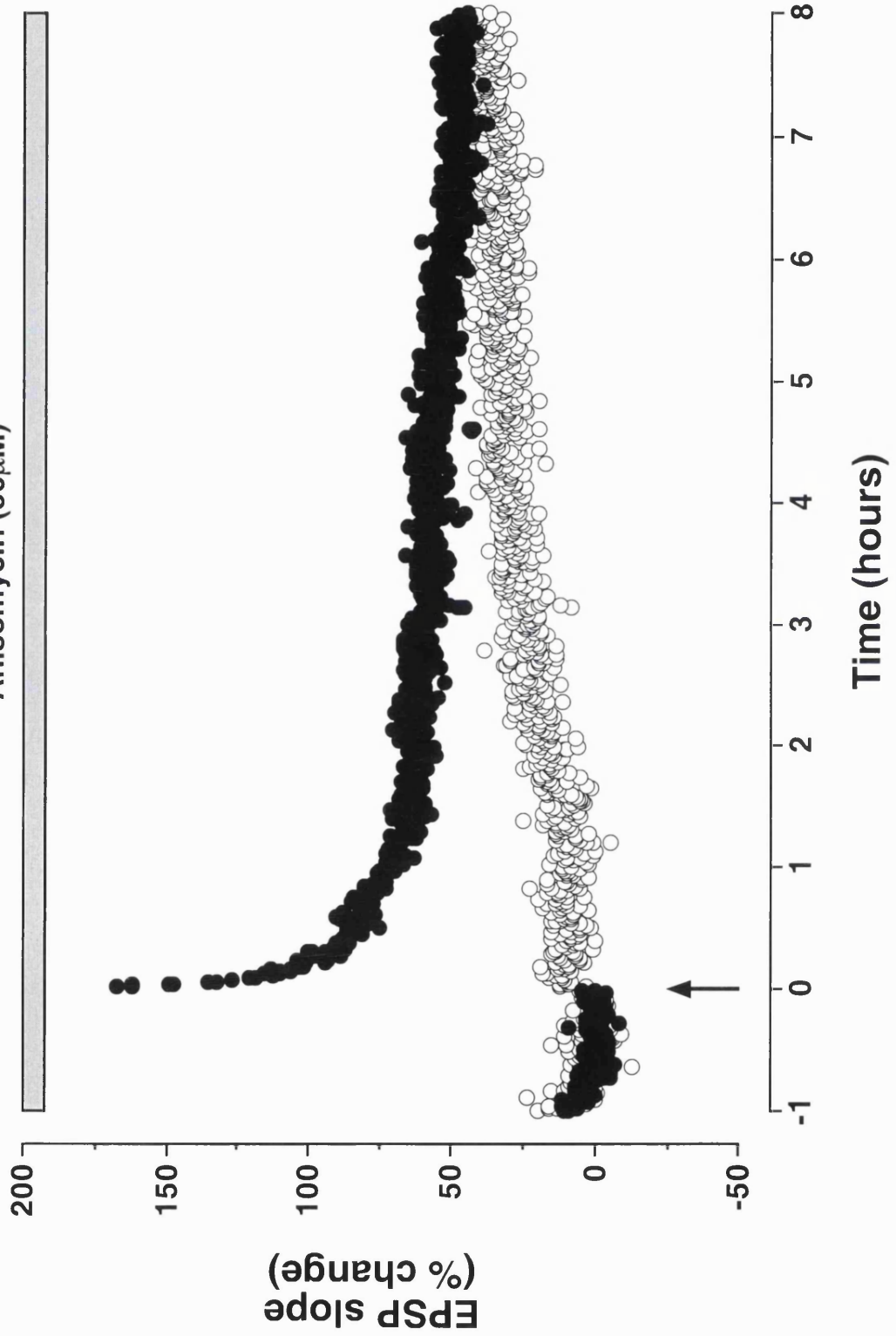

Tetanised pathway
Non-tetanised pathway
3 mV
5 ms
10 mins pre-tetanus
10 mins post-tetanus
8 hours post-tetanus

Figure 4.3 Individual example of drift during LTP in response to bath application of the protein synthesis inhibitor anisomycin. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates the duration of anisomycin application. Symbols: ● tetanised pathway; O non-tetanised pathway.


Anisomycin (50μM)
EPSP slope
(% change)
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

the response in the tetanised pathway. The results of arithmetically subtracting the EPSP slope of the non-tetanised pathway from that of the tetanised pathway are shown in figure 4.4a. From these results it would seem that anisomycin is having an effect on L-LTP induction. The level of potentiation at 8 hours is 15±8% and is not significantly different from the value 10 minutes prior to tetanisation (1±1%)(Fig. 4.4a). However, although these subtraction results are suggestive, the validity of the subtraction procedure is debatable.

In an attempt to understand the drift produced by anisomycin additional experiments were performed. For example, is anisomycin producing a general effect on the pyramidal cells in the CA1 region, or is its action linked to the induction of LTP? Baseline responses that were maintained for 8 hours in the presence of anisomycin (30μM) showed no drift over this time period (Fig. 4.4b). Similarly, a two pathway experiment, in which a tetanus was given to one pathway, during bath application of anisomycin (30μM) and the NMDA receptor antagonist AP5 (25μM), showed no drift in either pathway over 5 hours (Fig. 4.4c). The fact that anisomycin does not produce a drift during baseline recordings, or in response to a tetanus that does not produce LTP, suggests that the drift is a result of an interaction between LTP induction and anisomycin application.

Although the majority (n=17) of experiments showed drift (>30%) in the presence of anisomycin, one experiment did not. This experiment is shown in figures 4.5 and 4.6. The non-tetanised pathway remains stable for the duration of the experiment, showing a change from baseline of only 2% at 8 hours. In this experiment the tetanised pathway returns towards pre-tetanus levels over the course of 8 hours (13% at 8 hours). This result is revealing in that it shows a similar profile to that seen for the experiments that required arithmetical correction (Fig. 4.4a).

Despite some encouraging results, the problems associated with anisomycin led to the termination of its use. Instead, the translation inhibitor emetine was examined to explore its utility in the determination of the dependence of L-LTP on protein synthesis.

Figure 4.4 Stability of field EPSPs during bath application of the protein synthesis inhibitor anisomycin. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. **a**, Subtraction data. The EPSP slope percentage change for the non-tetanised pathway, was arithmetically subtracted from the EPSP slope percentage change for the tetanised pathway in the same experiment (n=6). Anisomycin was added either 30 or 60 minutes prior to tetanisation (indicated by dotted outline on shaded bar). Anisomycin concentration, ranges from 15μM-50μM (15μM n=3; 30μM n=2; 50μM n=1). **b**, Baseline values recorded in the presence of anisomycin. (n=6, from 3 slices). **c**, Individual experiment in which tetanisation occurred in the presence of anisomycin + AP5 (symbols: ● tetanised pathway; **O** non-tetanised pathway)(n=1). Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. In cases where no tetanus was given, zero percent corresponded to the mean of the values 10 minutes prior to drug application. Arrows indicate tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bars indicate the duration of drug application. Error bars indicate ±SEM.

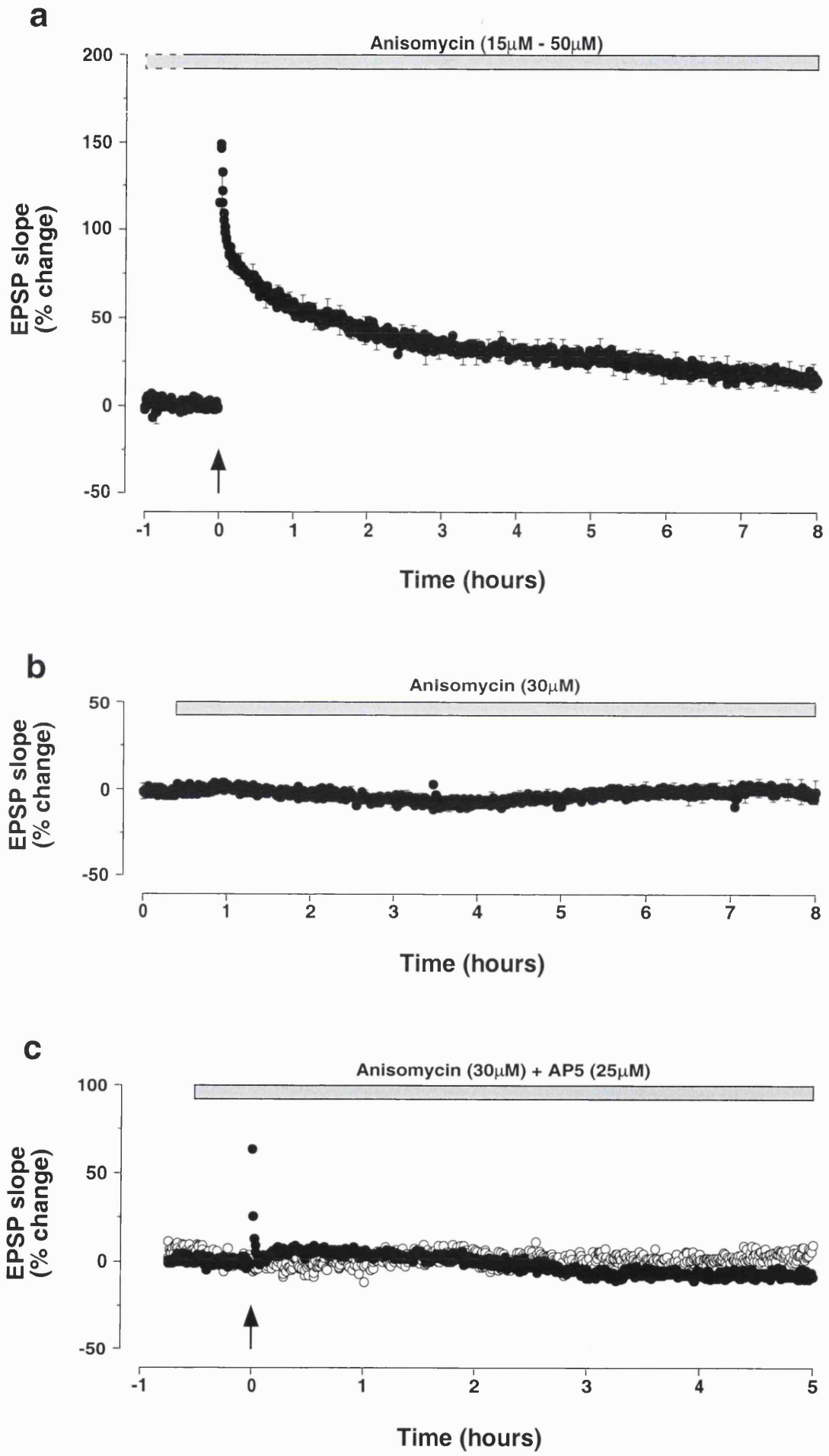

a
Anisomycin (15μM - 50μM)
EPSP slope (% change)
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)
b
Anisomycin (30μM)
EPSP slope (% change)
50
0
-50
0
1
2
3
4
5
6
7
8
Time (hours)
c
Anisomycin (30μM) + AP5 (25μM)
EPSP slope (% change)
100
50
0
-50
-1
0
1
2
3
4
5
Time (hours)

Figure 4.5 Field EPSPs taken from an individual LTP induction experiment during bath application of the protein synthesis inhibitor anisomycin. Individual EPSPs from both the tetanised and non-tetanised pathways within the stratum radiatum, are shown at various time points prior to, and following the tetanus. Anisomycin (50μM) was added 30 minutes prior to tetanisation and remained present for the duration of the experiment. Potentials are taken from the experiment shown in figure 4.6.

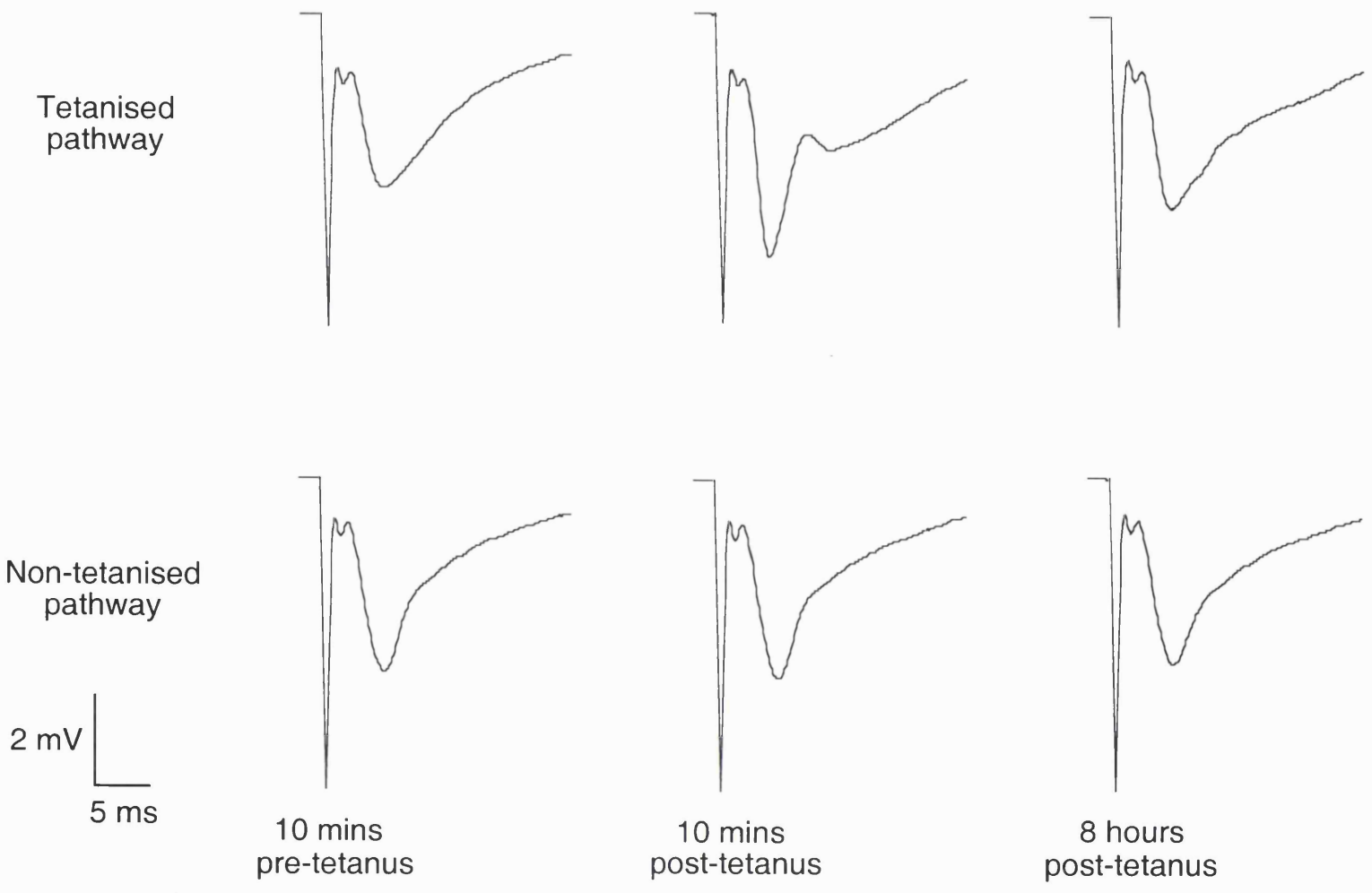
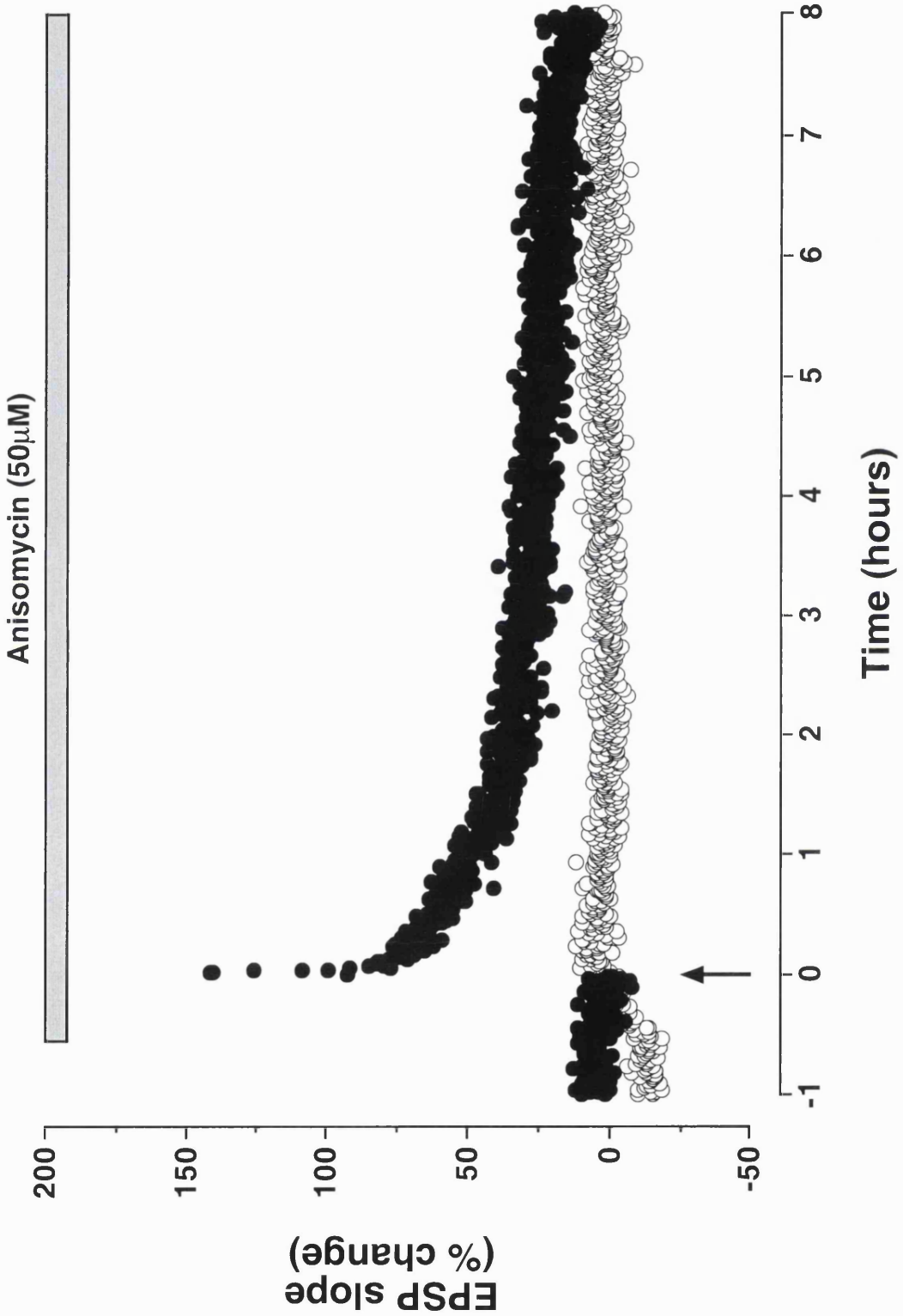

Tetanised pathway
Non-tetanised pathway
2 mV
5 ms
10 mins pre-tetanus
10 mins post-tetanus
8 hours post-tetanus

Figure 4.6 Individual example of L-LTP induction block during bath application of the protein synthesis inhibitor anisomycin. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates the duration of anisomycin application. Symbols: ● tetanised pathway; O non-tetanised pathway.


Anisomycin (50μM)
EPSP slope
(% change)
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

4.4 L-LTP INDUCTION IN THE PRESENCE OF THE TRANSLATION INHIBITOR EMETINE

4.4.1 Experimental Design

Electrodes were placed in order to stimulate two independent pathways in the stratum radiatum (Fig. 4.1). Test stimuli and tetanisation protocols were identical to those outlined in section 4.3.1. The translation inhibitor, emetine (100μM), was bath applied to the hippocampal slices 30 minutes prior to tetanisation and was perfused over the slices for the duration of the experiment.

Translational assays were conducted to assess the extent of protein synthesis block by emetine (100μM). This was achieved by comparing the amount of [^{3}H] valine incorporation into hippocampal slices over a 90 minute time period, in the presence and absence of the drug.

4.4.2 Results

Figure 4.7 and 4.8 show an individual LTP induction experiment in the presence of the translation inhibitor emetine (100μM). Following LTP induction, the tetanised pathway exhibits an initial potentiation of 168% that decays over the 8 hour time period to 22% of baseline. At the same 8 hour time point the non-tetanised control pathway reaches 9% of baseline (Fig.4.8). The changes in EPSP slope values seen in figure 4.8 are clearly seen in the individual EPSP traces over this time period (Fig. 4.7). Importantly, the afferent volley of both the tetanised and non-tetanised pathway remain unchanged throughout the experiment, making it unlikely that poor slice health or electrode movement generated the L-LTP block seen in the presence of emetine.

Pooled data for emetine application, and interleaved control experiments, are shown in figure 4.9. L-LTP induction is unaffected in the control, emetine-free experiments (Fig 4.9b). The difference at 8 hours between the tetanised (45±11%) and non-

Figure 4.7 Field EPSPs taken from an individual LTP induction experiment during bath application of the protein synthesis inhibitor emetine. Individual EPSPs from both the tetanised and non-tetanised pathways within the stratum radiatum, are shown at various time points prior to, and following the tetanus. Emetine (100μM) was added 30 minutes prior to tetanisation, and remained present for the duration of the experiment. Potentials are taken from the experiment shown in figure 4.8.

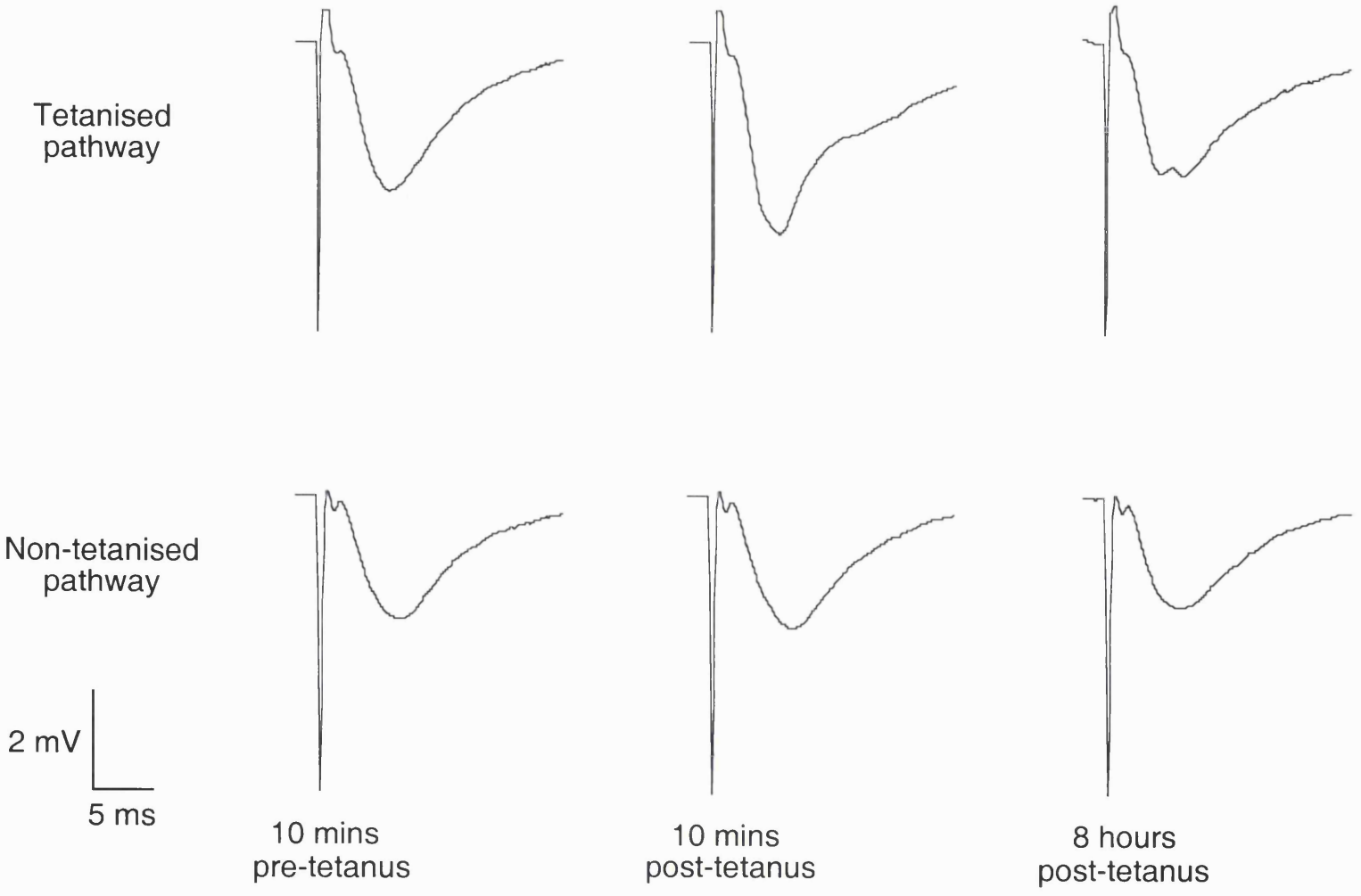
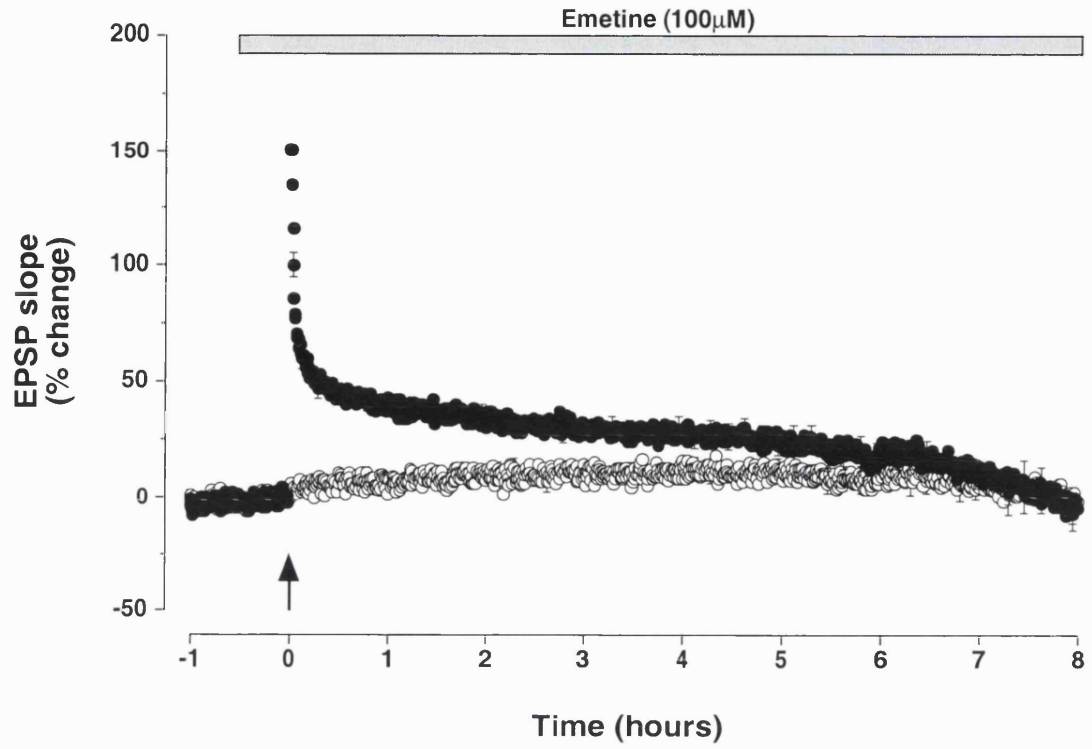

Tetanised pathway
Non-tetanised pathway
2 mV
5 ms
10 mins pre-tetanus
10 mins post-tetanus
8 hours post-tetanus

Figure 4.8 Individual example of L-LTP induction block during bath application of the protein synthesis inhibitor emetine. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates the duration of emetine application. Symbols: ● tetanised pathway; O non-tetanised pathway.

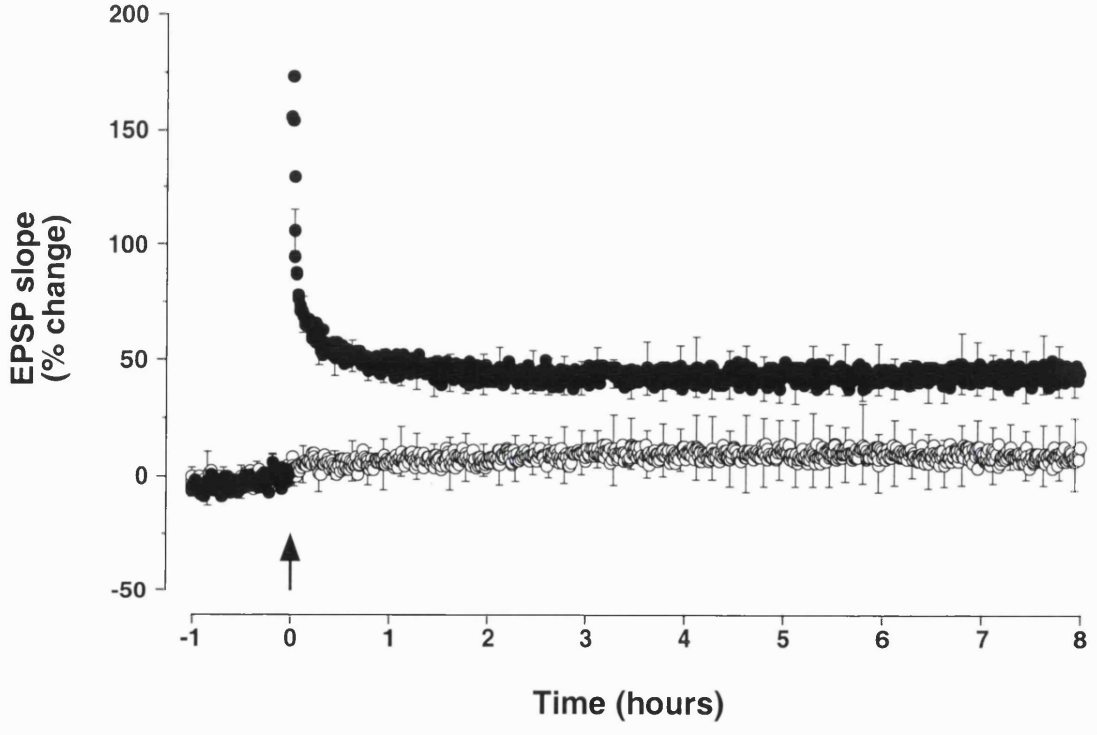
EPSP slope
(% change)
200
150
100
50
0
-50
Emetine (100μM)
-1
0
1
2
3
4
5
6
7
8
Time (hours)

Figure 4.9 Induction of L-LTP is blocked during bath application of the protein synthesis inhibitor emetine. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. **a**, L-LTP induction block in the presence of emetine (100μM), applied 30 minutes pre-tetanus (n=5, up to 6 hours; n=4, up to 8 hours). **b**, Interleaved control experiments, performed in the absence of emetine (n=6). Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates the duration of emetine application. Symbols: ● tetanised pathway; **O** non-tetanised pathway. Error bars indicate ±SEM.

a


Emetine (100μM)
200
150
100
50
0
-50
EPSP slope
(% change)
-1
0
1
2
3
4
5
6
7
8
Time (hours)

b


200
150
100
50
0
-50
EPSP slope
(% change)
-1
0
1
2
3
4
5
6
7
8
Time (hours)

tetanised (9±4%) pathways remains significant (t=3.1220, P≤0.0108)(Fig. 4.9b and Fig. 4.10b). In experiments where emetine (100μM) is present, no significant difference between the tetanised (-2±7%) and non-tetanised (-1±7%) pathways is apparent at 8 hours (Fig. 4.10a). It is important to note that the application of emetine appears to have no significant effect on the early stages of LTP. Similar levels of PTP and LTP are induced during the first 30 minutes post-tetanus, in both the control and drug experiments. However, at 8 hours, the difference between the level of potentiation seen in the drug (-2±7%), compared to the emetine-free control (45±11%) experiments is significant (t=3.44551, P≤0.0072; Fig. 4.10.c).

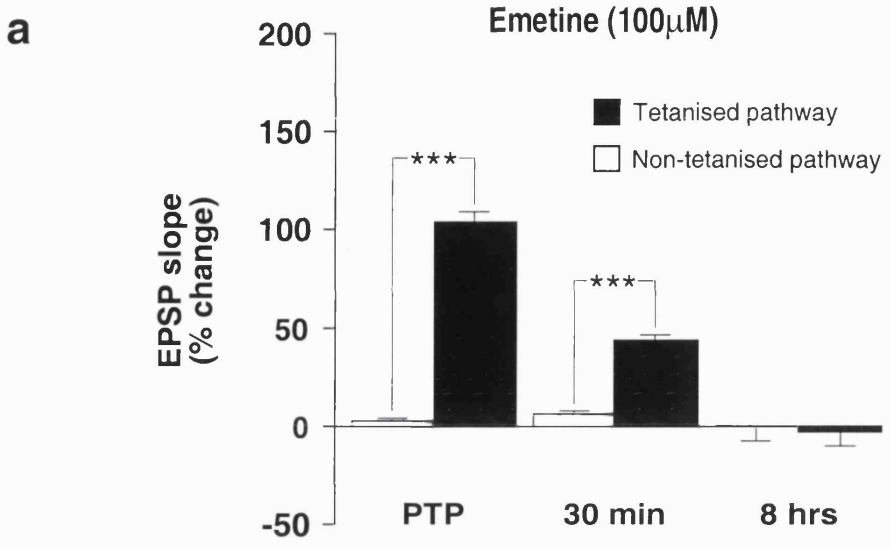
In addition to studying the ability of emetine to block L-LTP induction. The extent of the translation block caused by emetine (100μM) was assessed (Fig. 4.11). In all cases hippocampal slices that were incubated in the presence of [^{3}H] valine without emetine, showed a high degree of new protein synthesis over the course of the experiment (Fig. 4.11). However, [^{3}H] valine incorporation in slices incubated in the presence of emetine (100μM) was negligible, and in one case undetectable, suggesting that protein synthesis in these slices was almost completely abolished (Fig. 4.11). Therefore, bath application of emetine (100μM), to hippocampal slice preparations, results in a block of new protein synthesis, accompanied by a failure to induce L-LTP.

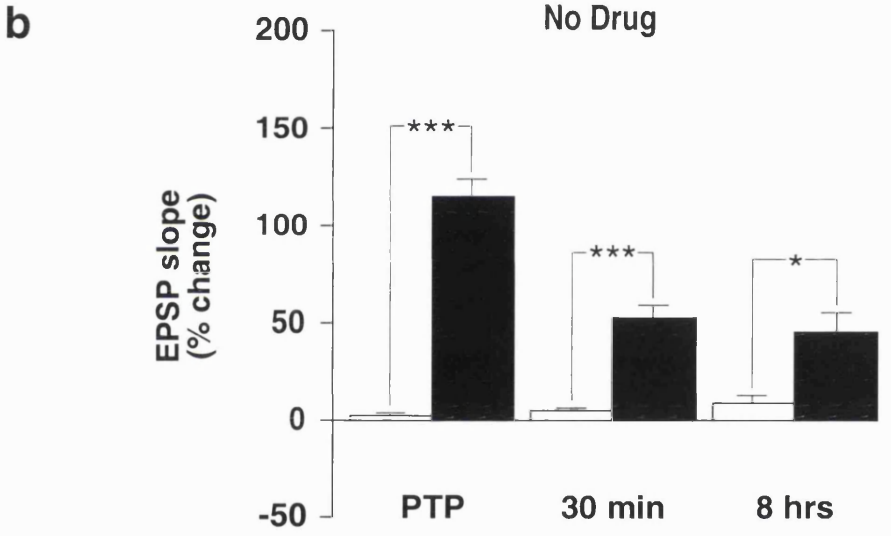
4.5 DISCUSSION

4.5.1 The Dependence of L-LTP on Protein Synthesis

The initial experiments designed to ascertain whether the LTP described in this study was dependent on protein synthesis proved problematic. The drug of choice for this type of study was the translation inhibitor anisomycin, an antibiotic that has been extensively used to successfully block the induction of L-LTP in both rats and mice (Frey *et al.*, 1988; Huang *et al.*, 1994; Nguyen & Kandel, 1996; Frey & Morris, 1997; Nguyen & Kandel, 1997). However, in this study, application of anisomycin to hippocampal slices, was found to cause an upward drift in the EPSP slope values in the non-tetanised pathway following LTP induction. The drift may be responsible for

Figure 4.10 Statistical comparisons during L-LTP induction block in the presence of the protein synthesis inhibitor emetine. a, L-LTP induction block in the presence of emetine (100μM), applied 30 minutes pre-tetanus (n=5, up to 6 hours; n=4, up to 8 hours). **b**, Interleaved control experiments, performed in the absence of emetine (n=6). **c**, Tetanised pathways from both drug and control experiments shown in a and b. In all cases **PTP** (post-tetanic potentiation) corresponds to the 5 minute time period immediately following tetanisation; **30 min** corresponds to the 10 minute time period, between 25-35 minutes post-tetanus; and **8 hrs** corresponds to the 10 minute time period, between 7 hours 50 minutes - 8 hours post-tetanus. Values represent the mean EPSP slope measured during each time period. ***P<0.001, **P<0.01, *P<0.05 (unpaired Student t test, two tailed). Error bars indicate ±SEM. Raw data sources are identical to those used for figure 4.9.


a
Emetine (100μM)
Tetanised pathway
Non-tetanised pathway
EPSP slope (% change)
200
150
100
50
0
-50
***
***
PTP
30 min
8 hrs


b
No Drug
EPSP slope (% change)
200
150
100
50
0
-50
***
***
*
PTP
30 min
8 hrs

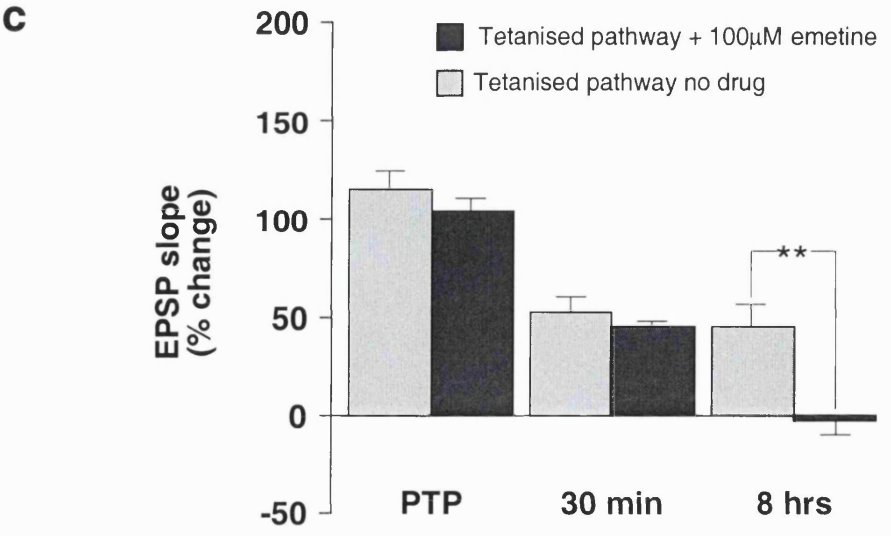
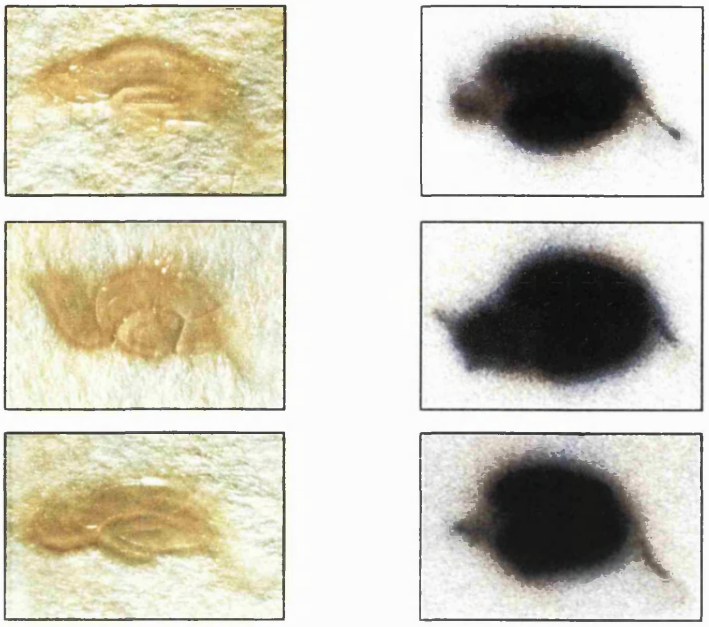

c
Tetanised pathway + 100μM emetine
Tetanised pathway no drug
EPSP slope (% change)
200
150
100
50
0
-50
**
PTP
30 min
8 hrs

Figure 4.11 Bath application of emetine blocks new protein synthesis in hippocampal slices. Data shows *in situ* autoradiography images (right) from corresponding hippocampal slices (left) incubated for 90 minutes in the presence of [^{3}H] valine. **a**, Control hippocampal slices, incubated in the absence of emetine. **b**, Hippocampal slices incubated during application of emetine (100μM).

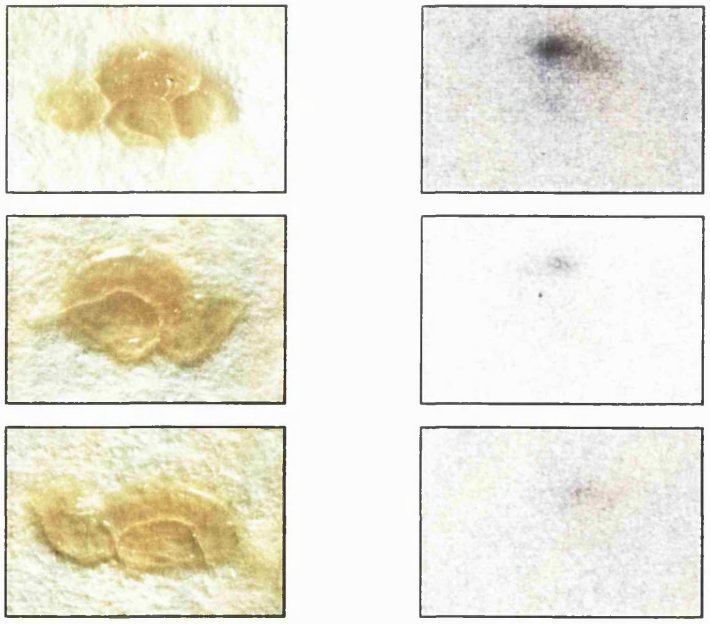
Control

a



Emetine

b



obscuring a block in L-LTP over 8 hours. This block became apparent when the data were arithmetically corrected for the drift. Drift occurred only when LTP was induced in slices in the presence of anisomycin and was not present under baseline conditions, or when tetanisation was applied but LTP was not induced. The reasons for this drift remain unclear. One possible explanation could be due to the fact that in addition to acting as a translation inhibitor, anisomycin also strongly activates a number of stress-activated MAP kinases, leading to the induction of several immediate early genes such as *c-fos* and *c-jun* (Edwards & Machadevan, 1992; Hazzalin *et al.*, 1998; Torocsik and Szeberenyi, 2000). It is therefore not unreasonable to suggest that the drift of the EPSPs during L-LTP induction in the presence of anisomycin, may be indicative of a convergent interaction between L-LTP induced MAP kinase activity and a stress-activated MAP kinase response, as a consequence of anisomycin application. Other translational inhibitors, such as emetine and puromycin have been shown to produce negligible stress responses (Edwards & Machadevan, 1992), whereas, even concentrations of anisomycin that do not affect translation, can still cause stress activated gene induction (Edwards & Machadevan, 1992). Taken together, these results suggest that the stress-induced response to anisomycin is not due to secondary effects brought on by translational arrest, but caused by chemical toxicity in the cells. It is this chemical toxicity combined with LTP induction that is suspected of producing the drift of the EPSPs seen in the slice experiments.

Due to the problems of EPSP slope drift associated with anisomycin, its use was discontinued. Instead, the requirement for protein synthesis in L-LTP was assessed using the translation inhibitor emetine. Emetine has been reported to block L-LTP induction, over a similar time scale to anisomycin, in the CA1 region of rat hippocampal slices (Frey & Morris, 1997). In this study, application of emetine was seen to block the induction of L-LTP in the CA1 region, causing potentials to return to pre-tetanus levels over 6-8 hours, a time scale similar to that reported in response to protein synthesis inhibition. (Frey *et al.*, 1988; Frey & Morris, 1997). Others have shown that the L-LTP block in the CA1 region, in response to application of translational inhibitors, has a more rapid decline of the potentials to pre-tetanus levels i.e. over 3-4 hours (Huang & Kandel, 1994; Nguyen *et al.*, 1994; Winder *et al.*, 1998). The difference in time course between these and my experiments may be due to

variations in the precise conditions under which the hippocampal slices are maintained.

In conclusion, the induction of L-LTP in this study has been shown to be dependent on the synthesis of new protein.

THE DEPENDENCE OF L-LTP ON TRANSCRIPTION

5.1 IS L-LTP DEPENDENT ON GENE INDUCTION?

The role of gene transcription has been examined in a number of early behavioural studies. One such study, involving hippocampal injection of the transcription inhibitor actinomycin-D in rats, suggested that long-term (>4 hours) but not short-term memory (<3 hours) was dependent on transcription (Daniels, 1971). However the majority of other similar studies could find no role for RNA synthesis over these time periods, following actinomycin-D application (Barondes & Jarvik, 1964; Cohen & Barondes, 1966; Squire & Barondes, 1970).

Studies looking at the involvement of transcription in LTP are equally confusing. Early work in which actinomycin-D was injected intraventricularly or focally into the dentate gyrus of rats, produced no effect on LTP of the population spike or EPSP slope for 6 hours following LTP induction; i.e. the period of study (Otani & Abraham, 1989)(see section 1.5.1). These results suggest that the proteins necessary for the maintenance of LTP in the dentate gyrus over this time period, are synthesised from pre-existing mRNA - a situation that is also thought to occur during LTD in the CA1 region of freely moving rats (Manahan-Vaughan *et al.*, 2000). Conflicting results have, however, been published that show a block of population spike L-LTP in the dentate gyrus of freely moving rats, following intrahippocampal or intraventricular injection with actinomycin-D (Frey *et al.*, 1996b).

Data obtained from similar experiments performed in the CA1 region in hippocampal slices are no clearer. In this preparation L-LTP induction has also been shown to be dependent on transcription. In both rat and mouse slices, EPSPs return to pre-tetanus levels over 3-4 hours in the presence of actinomycin-D or another transcription inhibitor DRB, (5,6-dichloro-1-β-D-ribofuranosyl benzimidazole), (Nguyen *et al.*, 1994; Nguyen & Kandel, 1997). However, in contrast to these findings Frey *et al.* (1996a) report that actinomycin-D produces only a slight attenuation of EPSP L-LTP

in the CA1 region of rats over 8 hours, although, induction of population spike L-LTP is blocked within this time period.

Consistencies however, can be found in experiments where the application of transcription inhibitors *does* effect the induction of L-LTP. These studies report a critical time window for transcription. The initial experiments in rat hippocampal slices showed this time window to be within 2 hours of tetanisation (Nguyen *et al.*, 1994); however, more recently this has been reduced to 20 minutes in rats (Frey *et al.*, 1996a), and 30 minutes in mice (Nguyen & Kandel, 1997). Application of transcription inhibitors outside the 'critical period' does not block the induction of L-LTP (Nguyen *et al.*, 1994; Frey *et al.*, 1996a; Nguyen & Kandel, 1997).

To date, the conflicting results from both the *in vivo* and *in vitro* data have made it difficult to provide a definitive answer as to whether gene transcription is necessary for the induction of L-LTP. Using transcription inhibitors with quite different modes of action (actinomycin-D and α-amanitin), I have attempted to address the role of mRNA synthesis in L-LTP.

5.2 THE SITE OF ACTION OF THE TRANSCRIPTION INHIBITORS ACTINOMYCIN-D AND α-AMANITIN

Actinomycin-D binds to DNA, blocking the movement of RNA polymerase I, II and III along DNA and consequently produces a complete inhibition of all RNA synthesis (Sorbell, 1973). α-amanitin functions by binding directly and preferentially to RNA polymerase II whilst having no effect on RNA polymerase I and a modest effect on RNA polymerase III at high concentrations (Lindell *et al.*, 1970). RNA polymerases I, II and III are responsible for the synthesis of ribosomal RNA (rRNA), messenger RNA (mRNA) and transfer RNA (tRNA) respectively. Therefore in experiments where actinomycin-D is used, it is difficult to attribute its effects exclusively to an mRNA block, without also considering its effect on both rRNA and tRNA levels in the cell. In contrast, the effects of α-amanitin, at least at low concentrations, can be attributed to a specific block of mRNA synthesis.

5.3 L-LTP INDUCTION IN THE PRESENCE OF THE TRANSCRIPTION INHIBITOR ACTINOMYCIN-D

5.3.1 Experimental Design

Electrode placement within the hippocampal slice is illustrated in figure 5.1. Field EPSPs were recorded from two independent pathways in the stratum radiatum, in response to stimulation via the Schaffer collateral/commissural system. Test stimuli were delivered alternately to each pathway at 15 second intervals and responses were monitored for in excess of 8 hours. Following a 1 hour baseline (1 hour 20 minutes in drug experiments), a tetanus (20 pulses @ 100Hz repeated 6 times with an ITI of 3 seconds) was applied to one pathway whilst the other provided a non-tetanised control.

The transcription inhibitor, actinomycin-D (25μM), was bath applied to the hippocampal slices 1 hour prior to tetanisation and remained on the slices for the duration of the experiment.

5.3.2 Results

Bath application of actinomycin-D (25μM) was found to block the induction of L-LTP in the stratum radiatum of the CA1 region. Data from a single representative experiment (Fig. 5.2 and 5.3) show an initial LTP of 188% in the tetanised pathway that declines slowly over the course of the experiment, reaching 1% by 8 hours post-tetanus (Fig. 5.3). Over the same time period the EPSP slope of the non-tetanised pathway remains relatively stable, decreasing only slightly to reach a value of -13% at 8 hours (Fig. 5.3). Individual EPSPs taken at different points throughout this experiment show little appreciable change in the profile of the afferent volley (Fig. 5.2).

The pooled data from a series of actinomycin-D experiments (Fig. 5.4a), show a profile similar to that of the individual experiment. EPSP potentiation at 8 hours in the tetanised pathway is 0±5% and is not significantly different from the EPSP slope

Figure 5.1 Schematic representation of electrode placement within the CA1 region of the hippocampal slice. Two stimulating electrodes (Stim.) are positioned within the stratum radiatum of the CA1 region, on either side of the recording electrode (Rec.). This maximises the chances of stimulating two populations of convergent but non-overlapping Schaffer collateral/commissural fibres, thereby ensuring the stimulation of independent synaptic populations. For key to abbreviations, see figure 2.1.

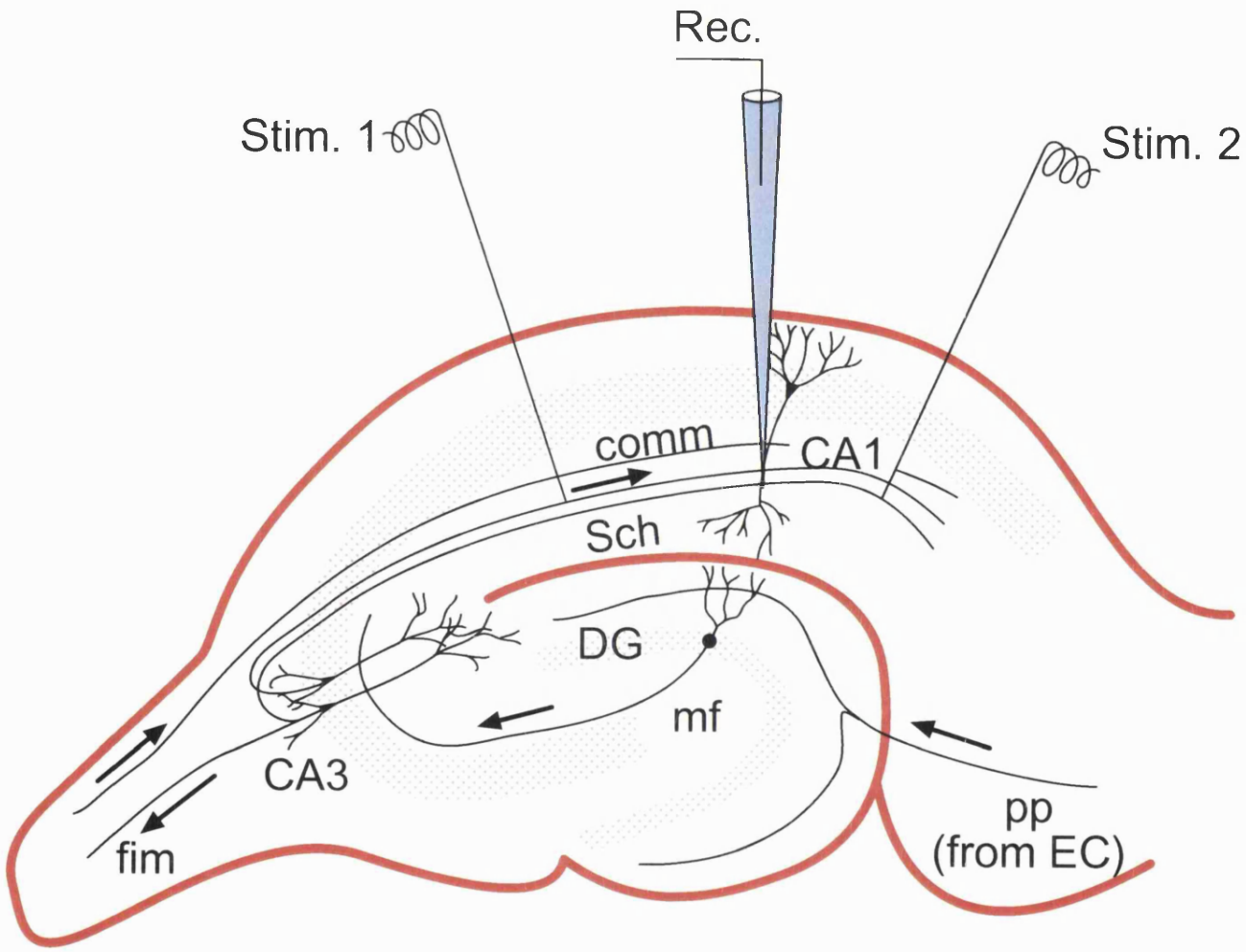

Rec.
Stim. 1
Stim. 2
comm
CA1
Sch
DG
mf
CA3
fim
pp
(from EC)

Figure 5.2 Field EPSPs taken from an individual LTP induction experiment during bath application of the RNA synthesis inhibitor actinomycin-D. Individual EPSPs from both the tetanised and non-tetanised pathways within the stratum radiatum, are shown at various time points prior to, and following the tetanus. Actinomycin-D (25μM) was added 1 hour prior to tetanisation and remained present for the duration of the experiment. Potentials are taken from the experiment shown in figure 5.3.

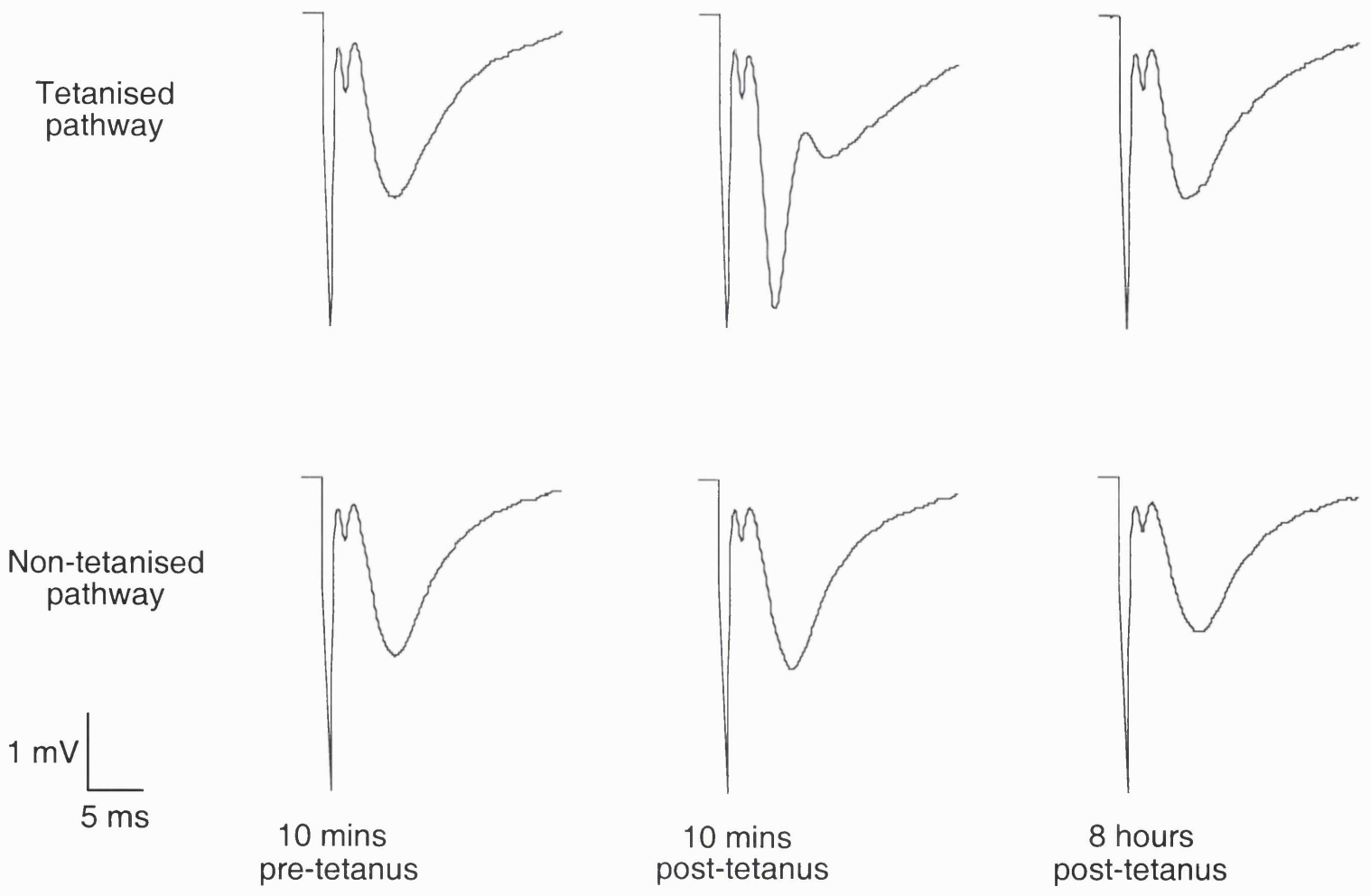
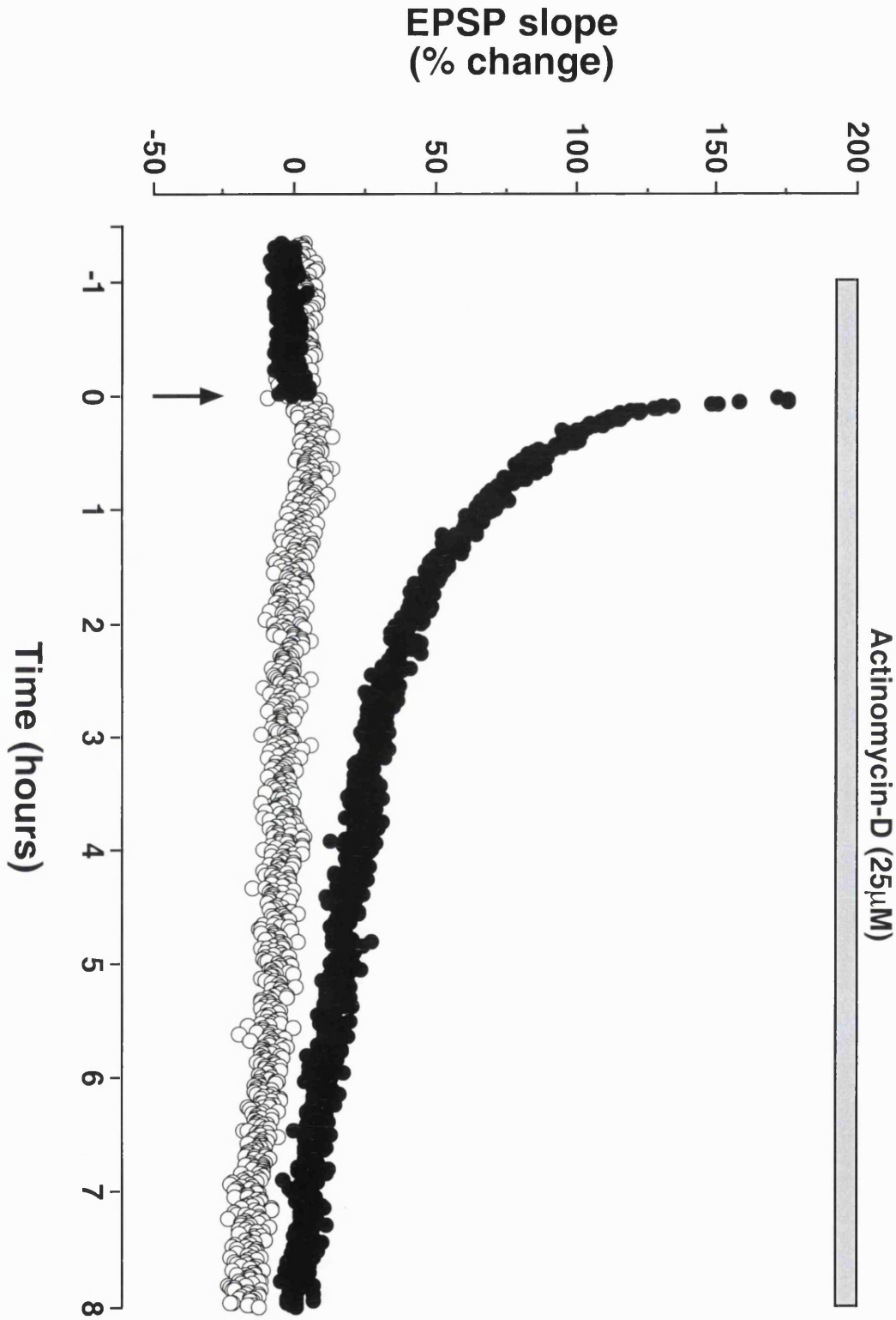

Tetanised pathway
Non-tetanised pathway
1 mV
5 ms
10 mins pre-tetanus
10 mins post-tetanus
8 hours post-tetanus

Figure 5.3 Individual example of L-LTP induction block during bath application of the RNA synthesis inhibitor actinomycin-D. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates the duration of actinomycin-D application. Symbols: ● tetanised pathway; **O** non-tetanised pathway.


EPSP slope
(% change)
200
150
100
50
0
-50
Actinomycin-D (25μM)
Time (hours)
-1
0
1
2
3
4
5
6
7
8

value seen for the non-tetanised pathway (-8±11%)(Fig. 5.5a). Potentiation in the 'interleaved controls' remains elevated above pre-tetanus levels at the 8 hour time point (50±6%) (Fig. 5.4b) and is significantly different from the non-tetanised pathway ($t=3.8477$, $P\leq0.0049$) (Fig. 5.5b), despite a slight drift in this pathway over the course of the experiment (17±6% at 8 hours). Application of actinomycin-D had no significant effect on the induction of LTP over the first 30 minutes following tetanisation (Fig. 5.5c), whilst at the 8 hour time point, the difference between the tetanised pathways exposed to the drug, compared to those of the interleaved controls is significant ($t=6.1387$, $P\leq0.0003$)(Fig. 5.5c).

Although these data show that the transcription inhibitor actinomycin-D blocks L-LTP, the range of results, as highlighted in section 5.1, raises questions about the possible side effects of actinomycin-D, due to its range of substrates. These results led me to ask whether the block of L-LTP induction seen in response to actinomycin-D, could in fact be attributed to an indirect effect on protein synthesis produced by the block of rRNA and tRNA synthesis (see section 5.2). I attempted to address this question using the drug α-amanitin, a potent and specific inhibitor of RNA polymerase II, and therefore a specific inhibitor of mRNA synthesis (see section 5.2).

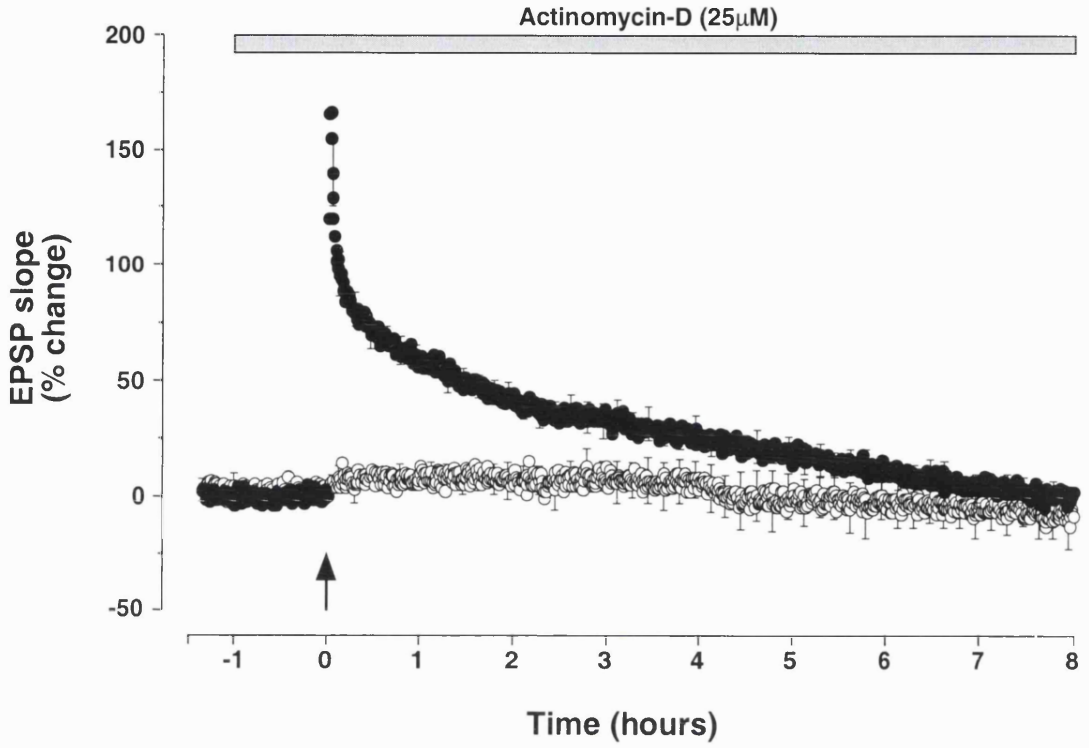
5.4 L-LTP INDUCTION IN THE PRESENCE OF THE SPECIFIC mRNA TRANSCRIPTION INHIBITOR α-AMANITIN

5.4.1 Experimental Design

Electrodes were placed to ensure the stimulation of two independent pathways in the stratum radiatum (Fig. 5.1). Test stimuli and tetanisation protocols were identical to those outlined in section 5.3.1. The specific mRNA transcription inhibitor, α-amanitin (2.5μM or 25μM), was bath applied to the hippocampal slices 1 hour prior to tetanisation and remained on the slices for the duration of the experiment. The concentrations of α-amanitin used in this investigation, are within the range reported to block mRNA synthesis in mouse embryos (Braude *et al.*, 1979; Schindler and Sherman, 1981).

Figure 5.4 Induction of L-LTP is blocked during bath application of the RNA synthesis inhibitor actinomycin-D. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. **a**, L-LTP induction block in the presence of actinomycin-D (25μM), applied 1 hour pre-tetanus (n=5). **b**, Interleaved control experiments, performed in the absence of actinomycin-D (n=5). Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates the duration of actinomycin-D application. Symbols: ● tetanised pathway; **O** non-tetanised pathway. Error bars indicate ±SEM.

a


Actinomycin-D (25μM)
EPSP slope
(% change)
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

b

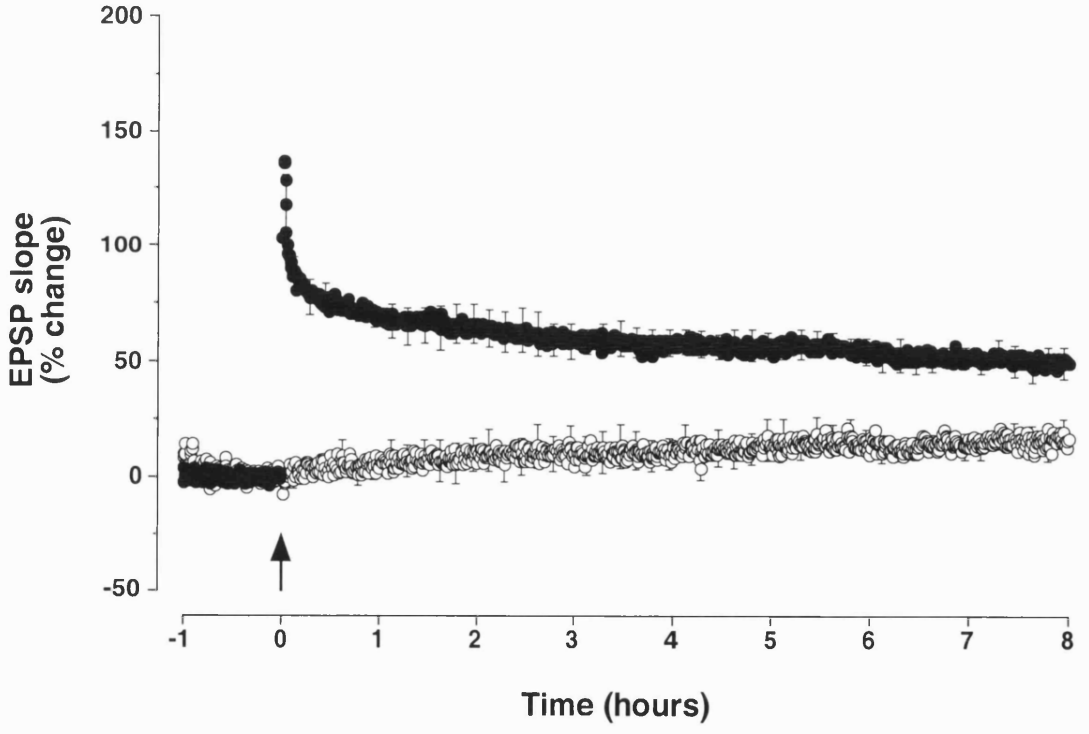
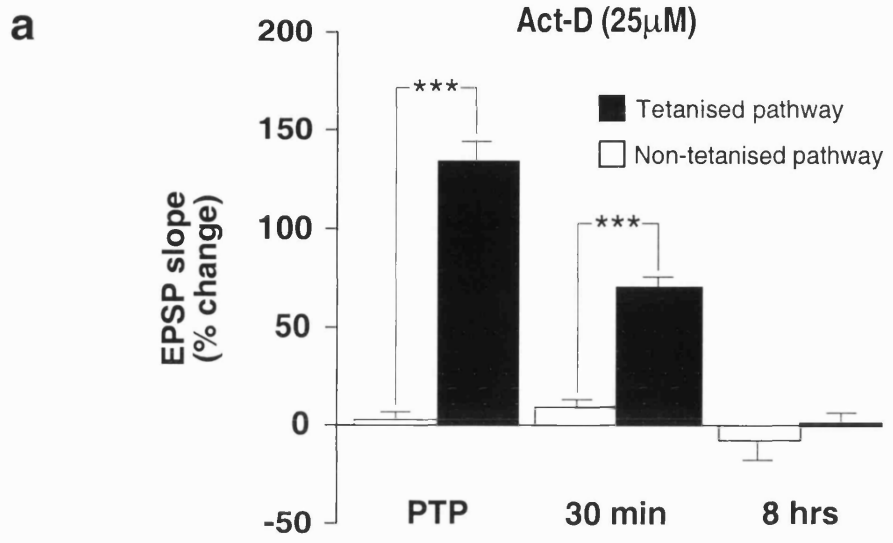
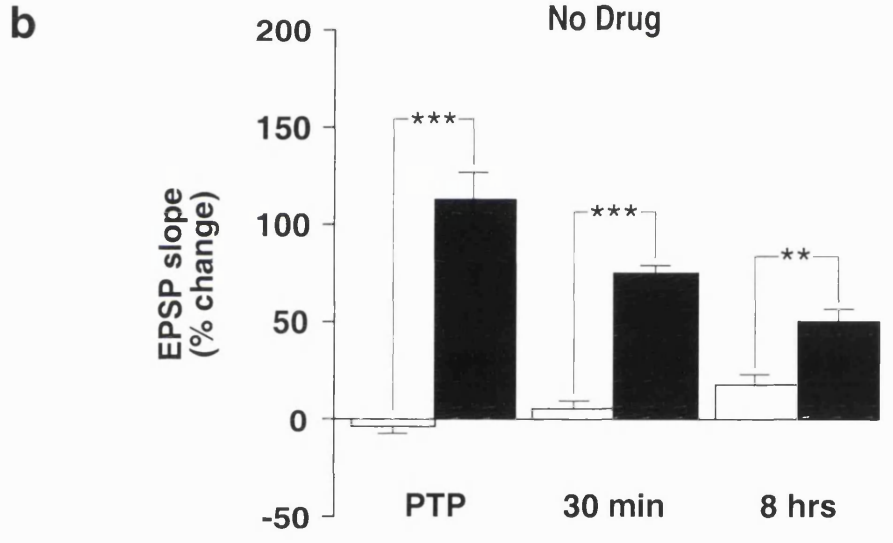
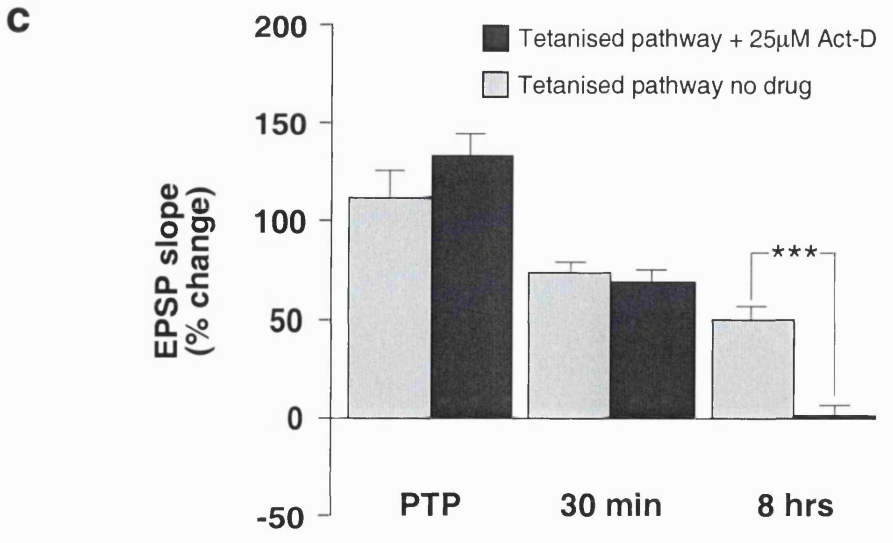

EPSP slope
(% change)
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

Figure 5.5 Statistical comparisons during L-LTP induction block in the presence of the RNA synthesis inhibitor actinomycin-D. a, L-LTP induction block in the presence of actinomycin-D (25μM), applied 1 hour pre-tetanus (n=5). **b**, Interleaved control experiments, performed in the absence of actinomycin-D (n=5). **c**, Tetanised pathways from both drug and control experiments shown in a and b. In all cases **PTP** (post-tetanic potentiation) corresponds to the 5 minute time period immediately following tetanisation; **30 min** corresponds to the 10 minute time period, between 25-35 minutes post-tetanus; and **8 hrs** corresponds to the 10 minute time period, between 7 hours 50 minutes - 8 hours post-tetanus. Values represent the mean EPSP slope measured during each time period. ***P<0.001, **P<0.01 (unpaired Student t test, two tailed). Error bars indicate ±SEM. Raw data sources are identical to those used for figure 5.4.


a
Act-D (25μM)
Tetanised pathway
Non-tetanised pathway
EPSP slope (% change)
200
150
100
50
0
-50
***
***
PTP
30 min
8 hrs


b
No Drug
EPSP slope (% change)
200
150
100
50
0
-50
***
***
**
PTP
30 min
8 hrs


c
Tetanised pathway + 25μM Act-D
Tetanised pathway no drug
EPSP slope (% change)
200
150
100
50
0
-50
***
PTP
30 min
8 hrs

During transcriptional assays, the extent of mRNA synthesis block by α-amanitin (2.5μM and 25μM) and actinomycin-D (25μM), was assessed by observing the amount of [^{3}H] uridine incorporation into hippocampal slices over a 2 hour time period, in the presence of drug.

5.4.2 Results

Following the bath application of α-amanitin (2.5μM), L-LTP induction is unaffected. In the presence of α-amanitin, individual EPSPs remain stable in the non-tetanised pathway, whilst the tetanised pathway shows a high level of potentiation that is still apparent in the EPSPs at 8 hours post-tetanus (Fig. 5.6). In addition, the profile of the afferent volleys in both the tetanised and non-tetanised pathways over this time period does not change (Fig. 5.6). The level of potentiation immediately after tetanisation is large (242%). After a decay period of 3-4 hours, potentiation stabilises; remaining 88% potentiated at the 8 hour time point (Fig. 5.7). Pooled data for α-amanitin application are shown in figure 5.8. Initial LTP is large in the tetanised pathway, and a high level of potentiation is maintained throughout the remainder of the experiment (63±17% at 7 hours). This is accompanied by stable EPSP slope values in the non-tetanised pathway over the same time period (17±4.9% at 7 hours)(Fig. 5.8).

Although these results appear to suggest that the inhibition of mRNA synthesis has no effect on L-LTP induction, it was necessary to quantify the extent of the inhibition in the presence of α-amanitin (2.5μM) by an independent measure. Using the extent of [^{3}H] uridine incorporation in RNA purified by targeting the poly A tail, as an assay of mRNA synthesis, it was found that the α-amanitin (2.5μM) block was far from complete (25±29%)(Fig. 5.9). This was in marked contrast to the actinomycin-D (25μM) block of 88±9%, a level known to block L-LTP induction (Fig. 5.9). These results suggested that the concentration of α-amanitin (2.5μM) applied during the L-LTP experiments (Figs. 5.6-5.8) was not producing a significant block of mRNA synthesis in the slices. This could account for the failure of the drug to block L-LTP induction. In an attempt to overcome this problem, the concentration of α-amanitin was increased to 25μM. However, at this concentration, in one experiment,

Figure 5.6 Field EPSPs taken from an individual LTP induction experiment during bath application of the specific mRNA synthesis inhibitor α-amanitin. Individual EPSPs from both the tetanised and non-tetanised pathways within the stratum radiatum, are shown at various time points prior to, and following the tetanus. α-amanitin (2.5μM) was added 1 hour prior to tetanisation, and remained present for the duration of the experiment. Potentials are taken from the experiment shown in figure 5.7.

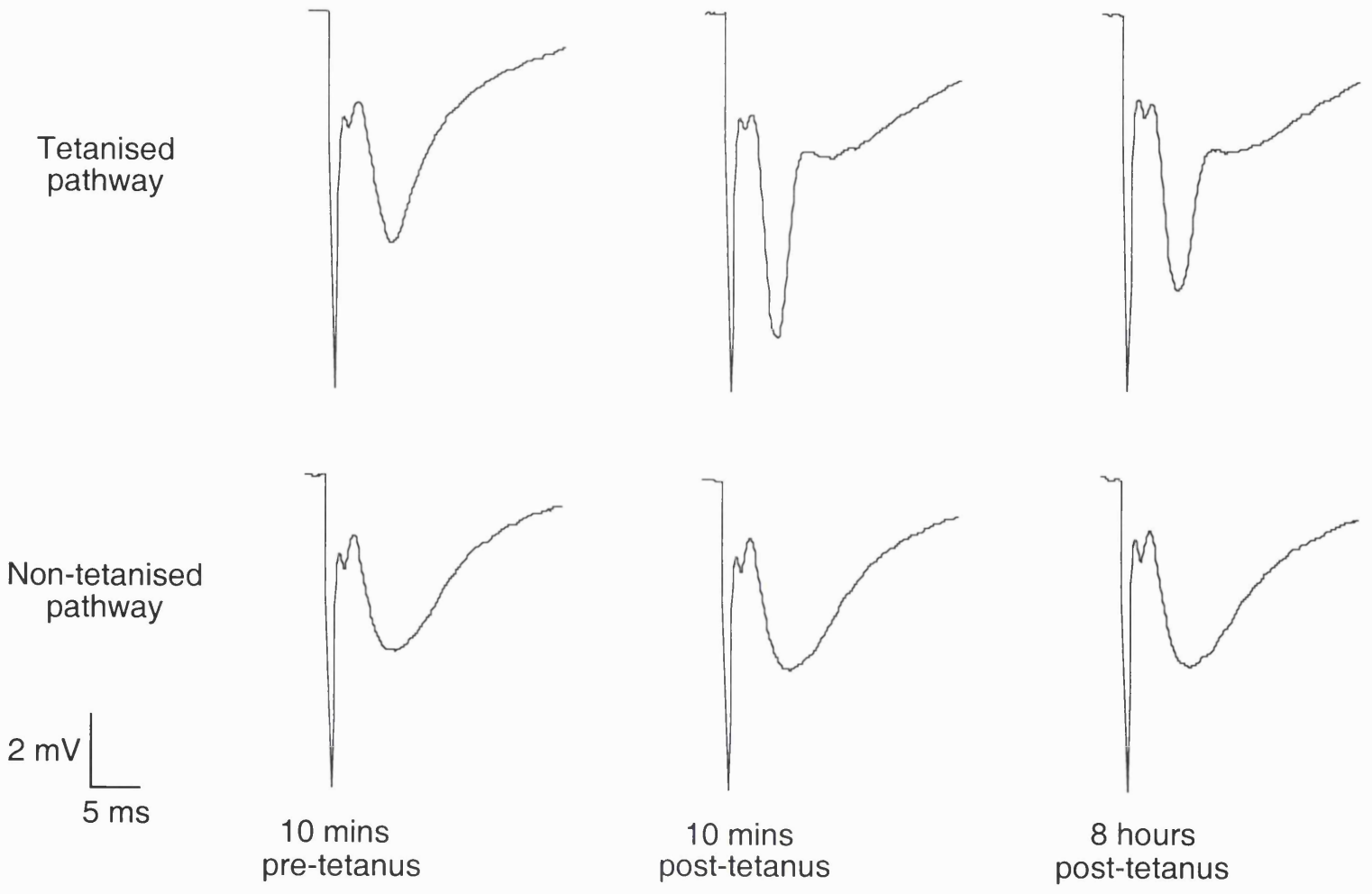

Tetanised pathway
Non-tetanised pathway
2 mV
5 ms
10 mins pre-tetanus
10 mins post-tetanus
8 hours post-tetanus

Figure 5.7 Individual example of L-LTP induction during bath application of the specific mRNA synthesis inhibitor α-amanitin. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates the duration of α-amanitin application. Symbols: ● tetanised pathway; **O** non-tetanised pathway.

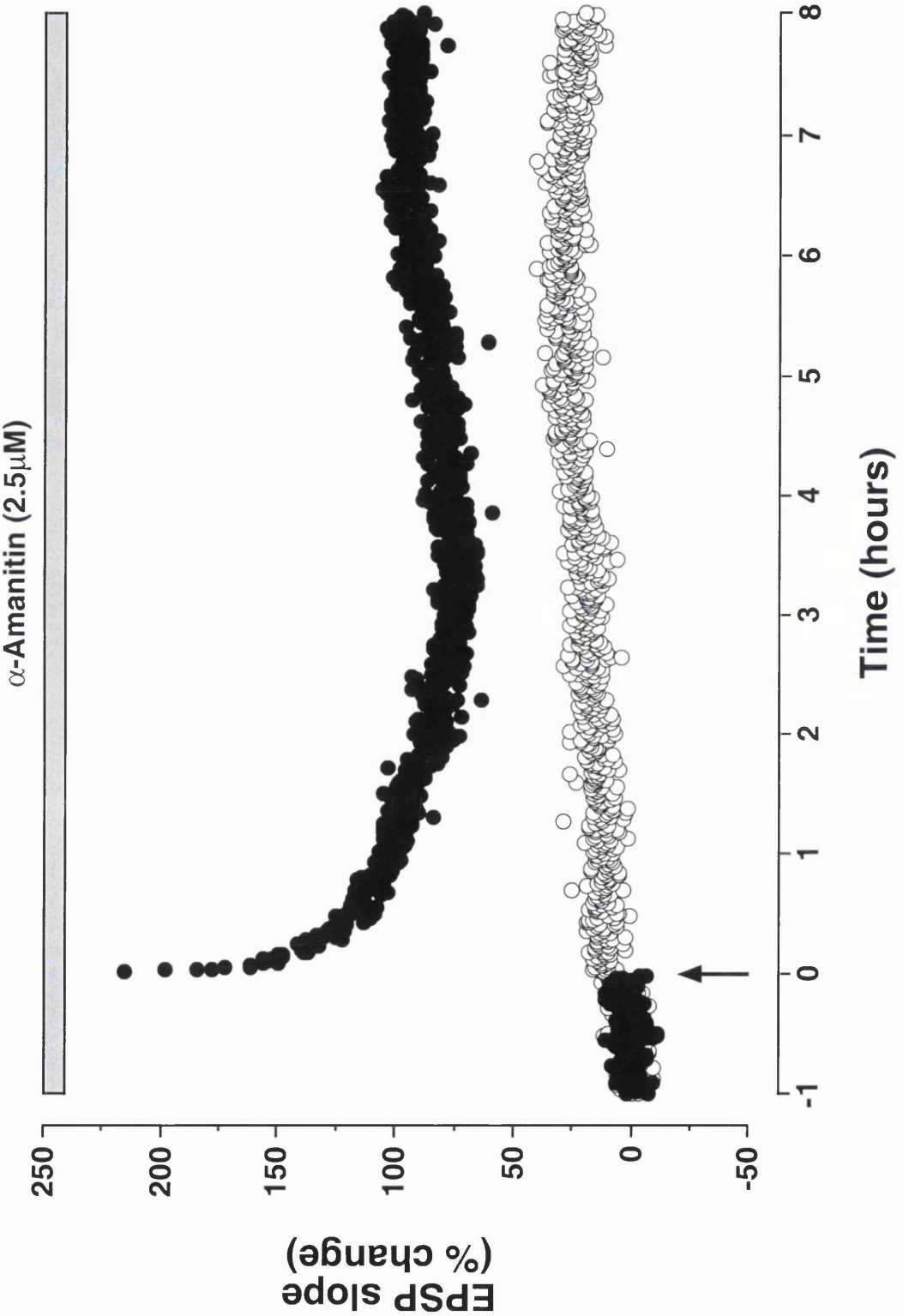
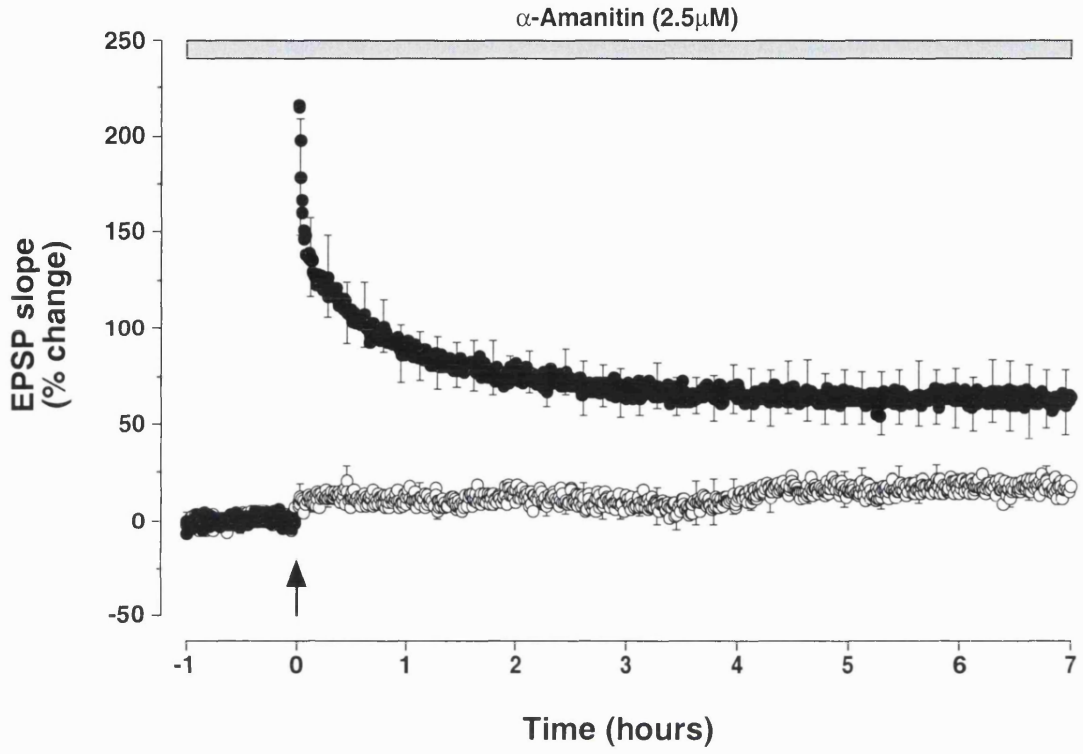

α-Amanitin (2.5μM)
EPSP slope
(% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

Figure 5.8 Induction of L-LTP appears unaffected during bath application of the specific mRNA synthesis inhibitor α-amanitin. a, Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. Data shows L-LTP induction in the presence of α-amanitin (2.5μM), applied 1 hour pre-tetanus (n=3). Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates the duration of α-amanitin application. Symbols: ● tetanised pathway; **O** non-tetanised pathway. Error bars indicate ±SEM. **b,** Statistical comparison of tetanised and non-tetanised pathways following L-LTP induction in the presence of α-amanitin (2.5μM)(n=3). Raw data sources are identical to those used for a. **PTP** (post-tetanic potentiation) corresponds to the 5 minute time period immediately following tetanisation; **30 min** corresponds to the 10 minute time period, between 25-35 minutes post-tetanus; and **7 hrs** corresponds to the 10 minute time period, between 6 hours 50 minutes - 7 hours post-tetanus. Values represent the mean EPSP slope measured during each time period. **P<0.01 (unpaired Student t test, two tailed). Error bars indicate ±SEM.

a


α-Amanitin (2.5μM)
EPSP slope (% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
Time (hours)

b

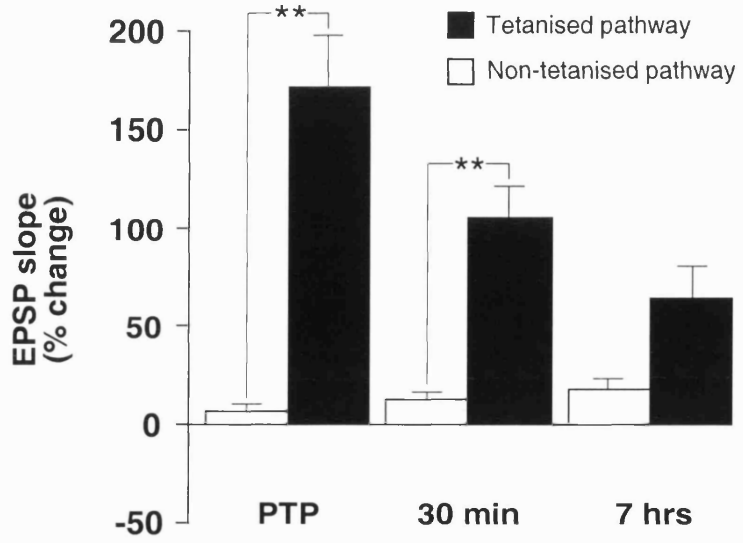
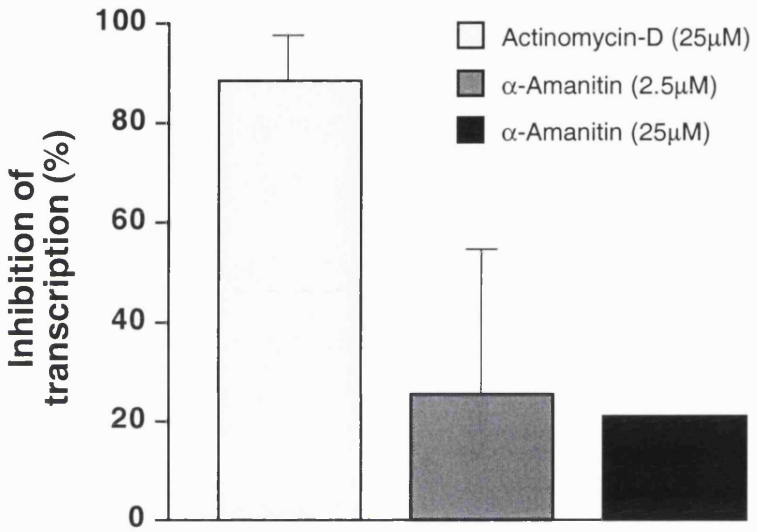

Tetanised pathway
Non-tetanised pathway
EPSP slope (% change)
200
150
100
50
0
-50
**
**
PTP
30 min
7 hrs

Figure 5.9 The extent of mRNA synthesis block during incubation with the transcription inhibitors actinomycin-D and α-amanitin. Data corresponds to inhibition of [^{3}H] uridine incorporation within hippocampal slices over a 2 hour time period, in the presence of each drug. Percentage inhibition was determined by calculating the ratio of [^{3}H] uridine incorporation, between control and drug samples. 25μM actinomycin-D (n=4), 2.5μM α-amanitin (n=3), 25μM α-amanitin (n=1). Error bars indicate ±SEM.

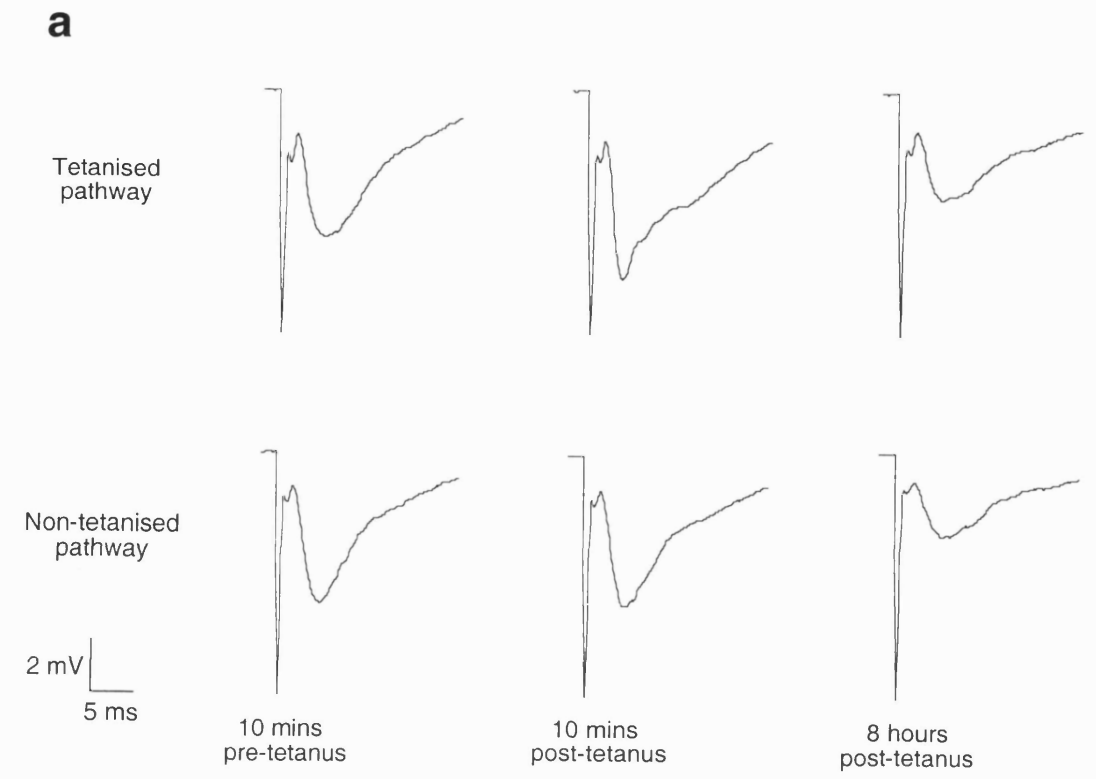

Inhibition of transcription (%)
100
80
60
40
20
0
Actinomycin-D (25μM)
α-Amanitin (2.5μM)
α-Amanitin (25μM)

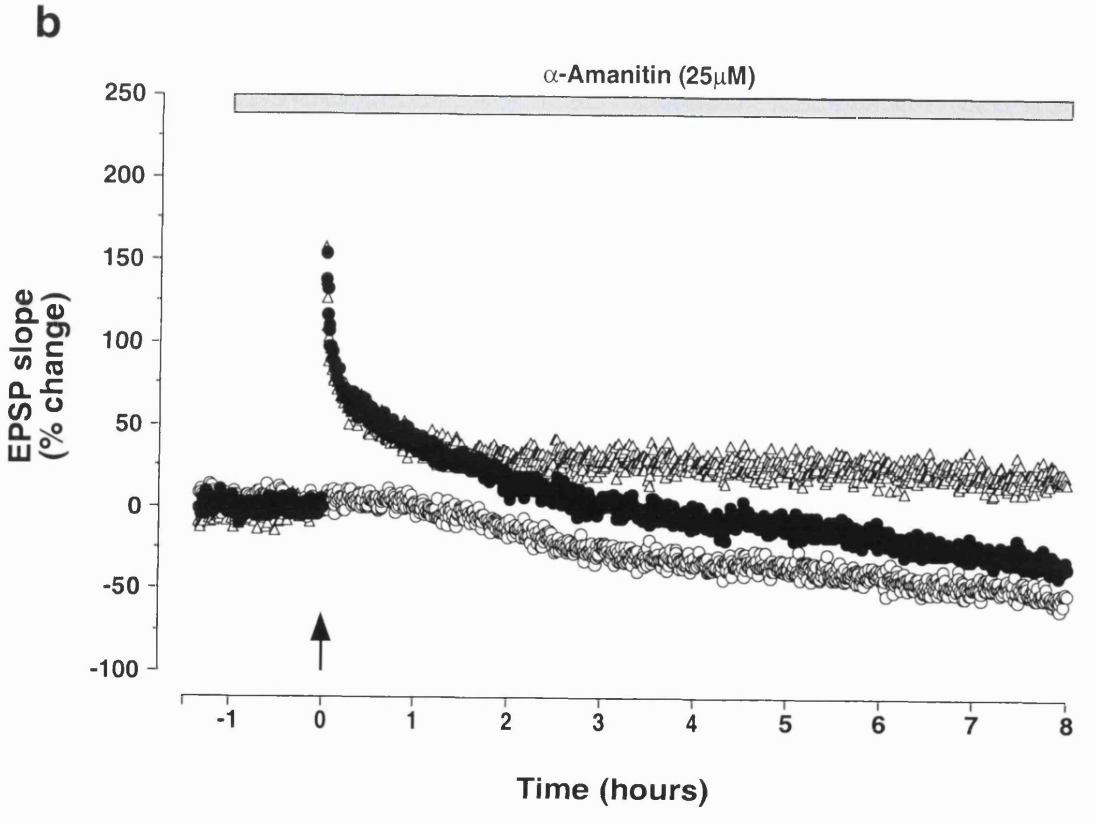
α-amanitin was only able to produce a 21% block of mRNA synthesis (Fig. 5.9), a level that was still not comparable to that seen by actinomycin-D (25μM). Furthermore it appeared to be no greater than that seen in response to 2.5μM α-amanitin (Fig. 5.9).

At this increased concentration, the specificity of α-amanitin as an RNA polymerase II inhibitor and consequently as an mRNA synthesis inhibitor cannot be assured and non-specific effects of the drug on RNA polymerase III or other pathways in the cell cannot be ruled out. Electrophysiological experiments from hippocampal slices, following application of 25μM α-amanitin, were found to show deterioration in slice health around 2 hours after application of the drug (Fig. 5.10). This deterioration was manifested as a decrease in EPSP slope and amplitude, in both the tetanised and non-tetanised pathways, coupled with changes in the profile of the afferent volley in each (Fig. 5.10a). The individual example shown in figure 5.10b shows a decrease in the EPSP slope of the tetanised pathway, from a PTP of 155% immediately after tetanisation to -36% after 8 hours. This decrease is mirrored by the non-tetanised pathway, which reaches an EPSP slope value of -52% at the 8 hour time point. Interestingly, if the non-tetanised pathway is arithmetically subtracted from the tetanised pathway (as described in section 4.3.2) then an underlying LTP becomes visible, that is stable (although at a lower level than that normally seen) over the course of the experiment and shows a potentiation of 16% at 8 hours. It therefore appears that even at a concentration of α-amanitin (25μM) that is toxic to the slices, an underlying L-LTP still remains.

Data from both the electrophysiological and transcriptional block experiments appear to provide contradictory results. They state that at a dose far in excess of the effective concentration, the transcription inhibitor α-amanitin causes cell death, but does not produce a significant block of mRNA synthesis. A suitable alternative assay to assess mRNA synthesis could not be devised and this line of study was discontinued.

Figure 5.10 Deterioration of hippocampal slice health in response to increased doses of the specific mRNA synthesis inhibitor α-amanitin. **a**, Individual field EPSPs taken from a representative experiment, during bath application of α-amanitin (25μM). EPSPs from both the tetanised and non-tetanised pathways within the stratum radiatum, are shown at various time points prior to, and following L-LTP induction. Potentials are taken from the experiment shown in b. **b**, A representative example of LTP induction, during bath application of α-amanitin. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates the duration of α-amanitin application. Symbols: ● tetanised pathway; **o** non-tetanised pathway; Δ subtraction data (tetanised pathway minus non-tetanised pathway).


a
Tetanised pathway
Non-tetanised pathway
2 mV
5 ms
10 mins pre-tetanus
10 mins post-tetanus
8 hours post-tetanus


b
α-Amanitin (25μM)
EPSP slope (% change)
250
200
150
100
50
0
-50
-100
-1
0
1
2
3
4
5
6
7
8
Time (hours)

5.5 THE LOCUS OF TRANSCRIPTION DURING L-LTP

The dependence of L-LTP on transcription and translation has been examined extensively and discussed in sections 1.5.1, 4.1 and 5.1. However, the *locus* of transcription and translation is less well studied. The locus of translation in L-LTP is investigated in detail in chapter 6 and will not be discussed here. However, the locus of transcription in L-LTP, is addressed in the remainder of this chapter. For these experiments the hippocampal CA1 mini-slice preparation was utilised (Lynch *et al.*, 1982). Mini-slices are formed by the excision of the CA3 region (Fig. 5.11) and therefore do not contain the cell bodies of the presynaptic Schaffer collateral/commissural projections. As a result, CA1 mini-slices provide a system in which to address whether presynaptic or postsynaptic gene activation is involved in the induction of L-LTP.

5.6 L-LTP INDUCTION IN THE CA1 MINI-SLICE PREPARATION

5.6.1 Experimental Design

The CA3 region was excised from mouse hippocampal slices, along the CA3-CA1 border. The resulting CA1 mini-slices were then subjected to electrophysiological investigation. Electrode placement is illustrated in figure 5.11. Field EPSPs were recorded from two independent pathways in the stratum radiatum, in response to activation of the Schaffer collateral/commissural afferents. Test stimuli were delivered alternately to each pathway at 15 second intervals and responses were monitored for in excess of 8 hours. Following a 20 minute baseline, a tetanus (20 pulses @100Hz repeated 6 times with an ITI of 3 seconds) was applied to one pathway whilst the other remained as the non-tetanised control.

5.6.2 Results

CA1 mini-slice preparations show L-LTP that is maintained for at least 8 hours. An individual experiment is shown in figure 5.13. This experiment exhibits an initial

Figure 5.11 Schematic representation of electrode placement within the hippocampal CA1 mini-slice. Two stimulating electrodes (Stim.) are positioned within the stratum radiatum of the CA1 region, on either side of the recording electrode (Rec.). This maximises the chances of stimulating two populations of convergent but non-overlapping Schaffer collateral/commissural fibres, thereby ensuring the stimulation of independent synaptic populations. The dotted line indicates the approximate location of the cut made to excise the CA3 region. For key to abbreviations, see figure 2.1.

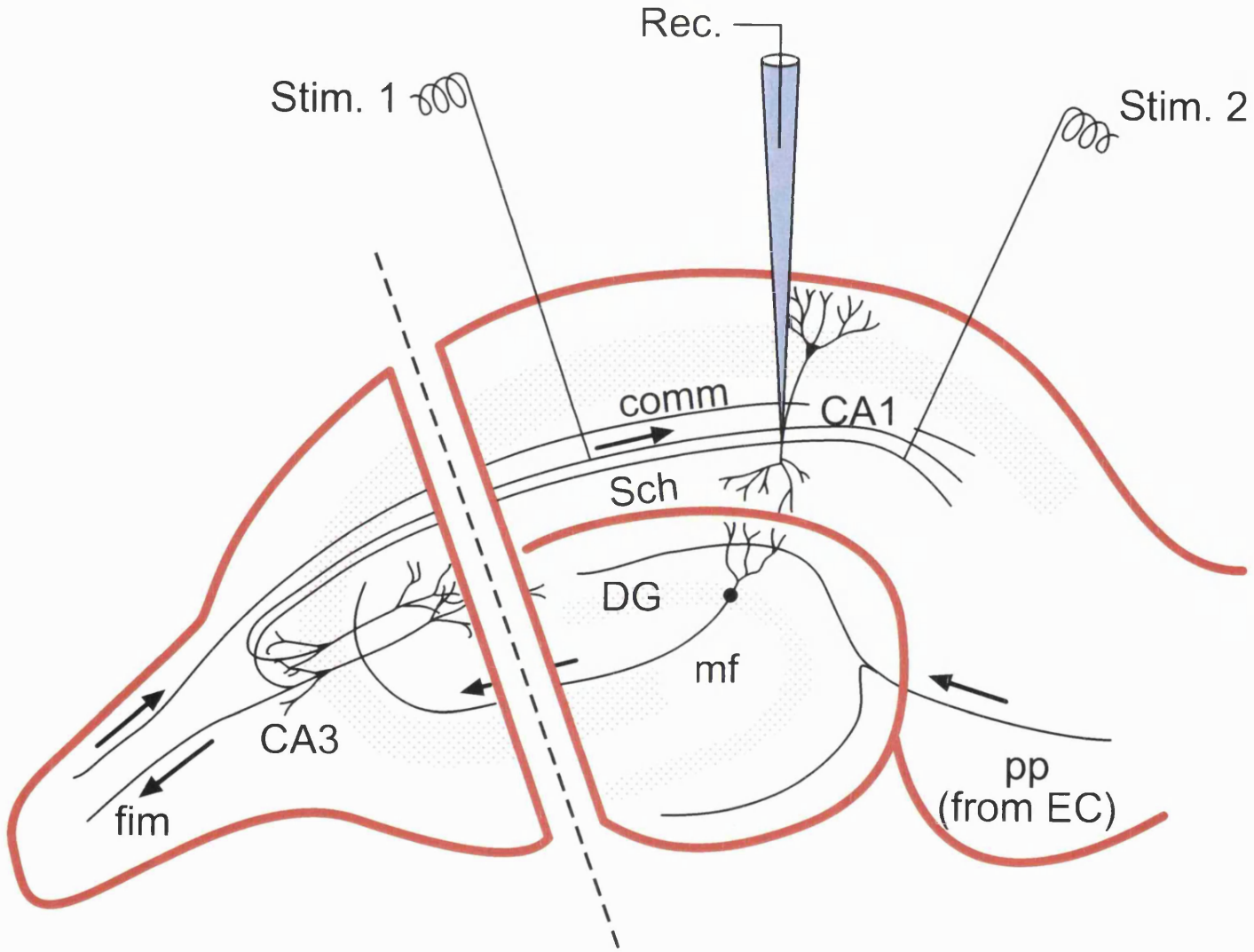

Rec.
Stim. 1
Stim. 2
comm
CA1
Sch
DG
mf
CA3
fim
pp
(from EC)

Figure 5.12 Field EPSPs taken from an individual LTP induction experiment in the stratum radiatum region of a hippocampal CA1 mini-slice. Individual EPSPs from both the tetanised and non-tetanised pathways, are shown at various time points prior to, and following the tetanus. Potentials are taken from the experiment shown in figure 5.13.

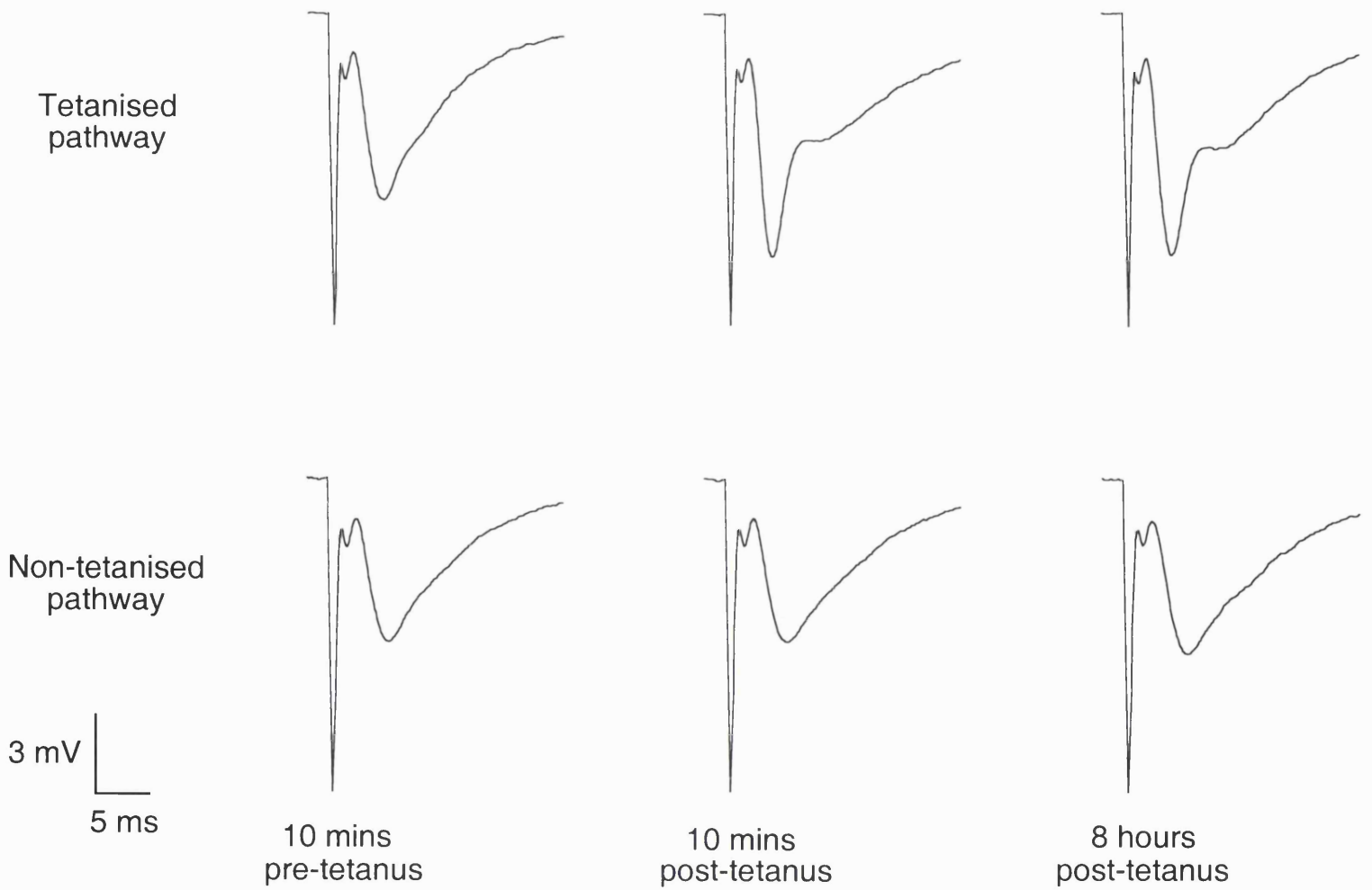

Tetanised pathway
Non-tetanised pathway
3 mV
5 ms
10 mins pre-tetanus
10 mins post-tetanus
8 hours post-tetanus

Figure 5.13 Individual example of L-LTP induction in the stratum radiatum region of a hippocampal CA1 mini-slice. Field EPSP slope, expressed as the percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Symbols: ● tetanised pathway; O non-tetanised pathway.

EPSP slope
(% change)
-50
0
50
100
150
200
-1
0
1
2
3
4
5
6
7
8
Time (hours)

potentiation of 127% immediately after tetanisation, that declines over 3 hours to 44%, and then remains at this level for 8 hours (47%). There is also a stable baseline in the non-tetanised pathway for the same time period (-7% at 8 hours; Fig 5.13). Individual responses to test stimuli in both the tetanised and non-tetanised pathways are displayed in figure 5.12, and show no change in the profile of the afferent volley over the time course of the experiment.

Data summarising the mean of six experiments, show robust LTP induction (PTP is 128±14%), that declines to a stable potentiated level over 3 hours (Fig. 5.14a). The potentiation is maintained up to the 8 hour time point (54±6%), at a level that is significantly different from the non-tetanised control pathway (13±9%)($t=3.6737$, $P\leq0.0043$)(Fig. 5.14). Furthermore, the level of L-LTP seen in CA1 mini-slices at 8 hours (54±6%) is not significantly different from that seen in hippocampal slices in which the CA3 region remains intact (47±6% at 8 hours)(see Figs. 3.4 and 5.14).

5.7 DISCUSSION

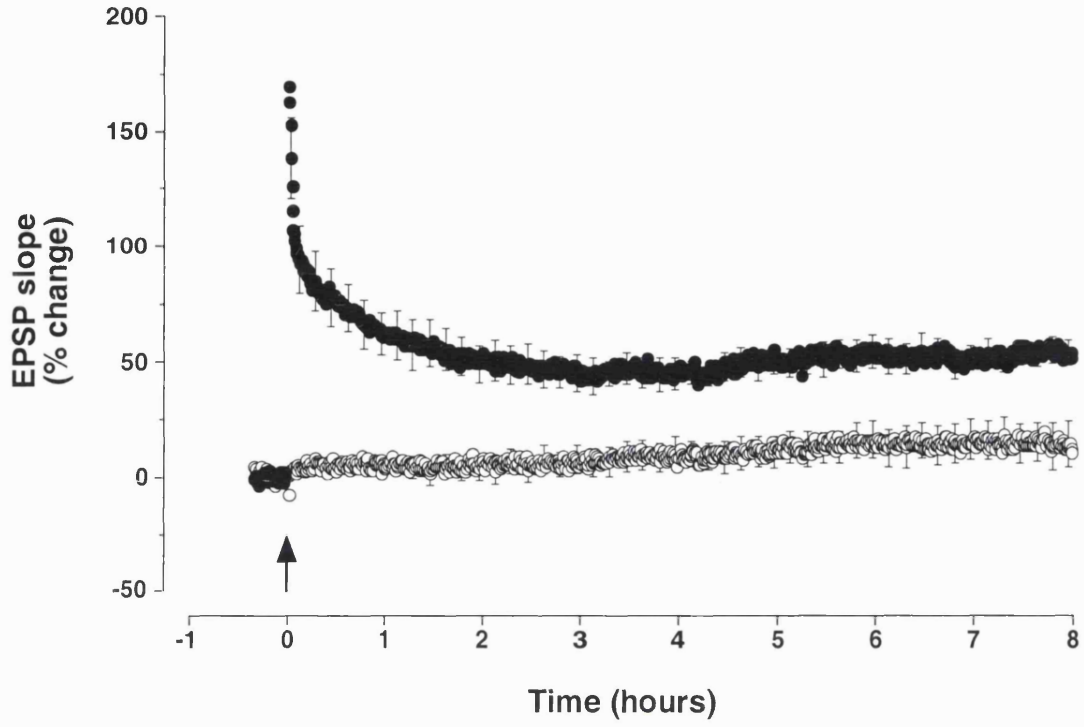
5.7.1 The Dependence of L-LTP on mRNA Synthesis

In this study, L-LTP induction in the CA1 region of hippocampal slices was blocked by application of the broad-spectrum transcription inhibitor actinomycin-D. EPSP slope returned to baseline levels between 7-8 hours post-tetanus. In hippocampal slices, L-LTP block by actinomycin-D has been shown to occur in both rats and mice (Nguyen *et al.*, 1994; Nguyen & Kandel, 1997), although, controversy still surrounds the result (Frey *et al.*, 1996b). Furthermore, conflicting reports as to the effectiveness of actinomycin–D as an inhibitor in both the behavioural and *in vivo* LTP literature (discussed in sections 1.5.1 and 5.1), has placed a question mark over the reliability of this drug as an indicator of the dependence of either long-term memory and/or L-LTP on transcription.

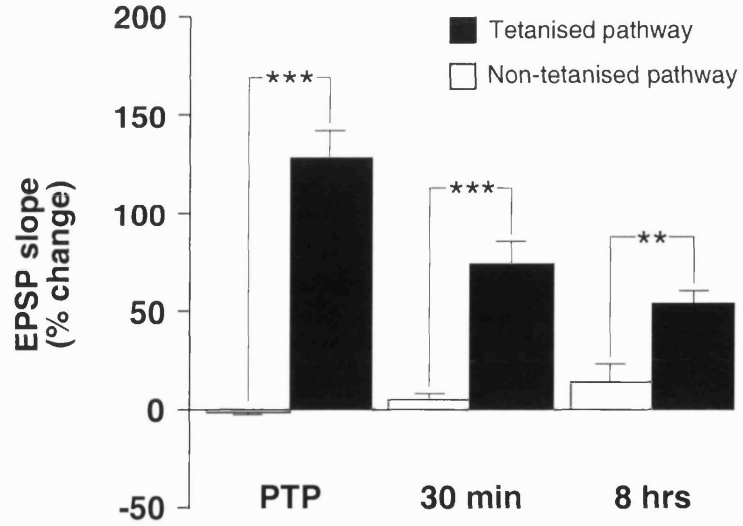
Actinomycin-D blocks mRNA synthesis in a non-specific manner; rRNA and tRNA are also inhibited. Therefore differing results seen within the field, may be explained by actinomycin-D's block of these other RNA species. Both rRNA and/or tRNA are

Figure 5.14 Induction of L-LTP is unaffected in the stratum radiatum region of the hippocampal CA1 mini-slice. a, Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as the percentage change over time is shown. Data shows L-LTP induction in CA1 mini-slices (n=6). Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Symbols: ● tetanised pathway; O non-tetanised pathway. Error bars indicate ±SEM. **b**, Statistical comparison of tetanised and non-tetanised pathways following L-LTP in CA1 mini-slices (n=6). Raw data sources are identical to those used for a. **PTP** (post-tetanic potentiation) corresponds to the 5 minute time period immediately following tetanisation; **30 min** corresponds to the 10 minute time period, between 25-35 minutes post-tetanus; and **8 hrs** corresponds to the 10 minute time period, between 7 hours 50 minutes - 8 hours post-tetanus. Values represent the mean EPSP slope measured during each time period. ***P<0.001, **P<0.01 (unpaired Student t test, two tailed). Error bars indicate ±SEM.

a


200
150
100
50
0
-50
EPSP slope
(% change)
-1
0
1
2
3
4
5
6
7
8
Time (hours)

b


200
150
100
50
0
-50
EPSP slope
(% change)
Tetanised pathway
Non-tetanised pathway
***
***
**
PTP
30 min
8 hrs

required for protein synthesis via translation of mRNA: rRNA is essential for ribosomal function and tRNA is required for the delivery of amino acids required for polypeptide chain elongation. It is unlikely that a block of tRNA and rRNA synthesis by actinomycin-D application will effect the basal levels of these molecules in the cells over the time course of the experiments in this study. Indeed both tRNA and rRNA are extremely abundant in the cell, whilst cytoplasmic ribosomes (of which rRNA is a part) have been shown to turn over with a half-life of about 6 days in rat brain (Stoykova *et al.*, 1983). However, following stimulus conditions suitable to induce L-LTP, the increased demand for proteins may be accompanied by a increased requirement for both ribosomal and/or transfer RNA. A block of new rRNA and tRNA synthesis may result in the demand for new proteins out weighing supply, resulting in a failure to induce L-LTP. This situation may occur both *in vivo* and *in vitro*, during L-LTP experiments in which the number of synaptic connections potentiated is presumably larger than would occur during learning. This would place a huge demand on the cell for new proteins, a situation that is not thought to occur under normal physiological conditions. Therefore the varying levels of L-LTP block seen between research groups in response to actinomycin-D could be depend on the extent of the L-LTP induced and the ability of the cells to synthesise enough proteins to maintain it. These results could therefore be interpreted as an apparent block of mRNA synthesis, when they are in fact due to an indirect effect on protein synthesis, brought about by a lack of some of the necessary components involved in translation.

The controversy over the role of mRNA synthesis in L-LTP and the non-specific effects of actinomycin-D led me to apply α-amanitin, a highly selective mRNA synthesis inhibitor. α-amanitin applied at concentrations known to block mRNA synthesis in mouse embryos (Braude *et al.*, 1979; Schindler & Sherman, 1981), was found to have no effect on L-LTP induction. These initial results suggested that newly synthesised mRNA is not required for either the induction or maintenance of L-LTP over these time periods. They also provided weight to the argument that the block of L-LTP in response to actinomycin-D, may be due to non-specific effects on rRNA and tRNA synthesis. Unfortunately, the assay employed to measure the block of mRNA synthesis in response to α-amanitin (2.5μM), suggested that the levels of block seen with this drug were low when compared to the levels seen in the presence

of actinomycin-D (25μM), a drug already shown to block L-LTP in these slices. Even following increases in the concentration of α-amanitin (25μM) the level of mRNA synthesis block was not increased. Furthermore, at this higher concentration the hippocampal slices exhibited a deterioration in health (although an underlying L-LTP was still seen, following subtraction of the non-tetanised pathway from the tetanised pathway). The observation that α-amanitin was causing cell death at levels below those required to block mRNA synthesis seems unlikely. These apparently contradictory results are likely to reflect some failure in the reliability of the transcription assay. This assay was dependent on a commercially available kit that purified mRNA from the total RNA pool by targeting the poly A tail. This kit is only intended for the enrichment of mRNA and is not an absolute mRNA purification procedure. Therefore contamination of the final mRNA samples with the more abundant tRNA or rRNA (~15% and ~80% respectively, of the total RNA within cells) could produce misleading results concerning the level of block of mRNA synthesis produced by α-amanitin. The fact that actinomycin-D blocks the synthesis of all RNA species means that at an effective concentration, the apparent mRNA block for this drug would always remain high, irrespective of the sample purity. Unfortunately, a suitable replacement assay could not be devised, to provide more accurate results.

The uncertainties surrounding the results from the transcription assay do not permit firm conclusions to be drawn from the α-amanitin data. However, the controversy surrounding the actinomycin-D data, coupled with the preliminary α-amanitin data, at least raises the possibility that L-LTP induction could occur without the need for new mRNA synthesis. Even if this is the case, however, it does not rule out a role for new mRNA synthesis in the maintenance of L-LTP over periods exceeding 8 hours.

5.7.2 The Locus of mRNA Synthesis in L-LTP

The locus of LTP expression is a topic that has been intensely debated over the last 15 years and has still to be resolved (see also section 1.4.2). In addition to a large number of pre-existing proteins that have been implicated in the early expression of LTP, a requirement for transcription and translation has been suggested for the maintenance

of LTP into the late phase (>3-6 hours). In this chapter the locus of transcription was addressed using the hippocampal CA1 mini-slice preparation. These slices do not contain the cell bodies of the presynaptic CA3 cells and so provide a system in which to address whether L-LTP requires presynaptic gene induction. L-LTP induction in these preparations was found to be completely unaffected, remaining stable for at least 8 hours, at levels comparable to those seen in intact hippocampal slices. These results show that the transcription of presynaptic genes is not required for the induction of L-LTP in mice. This finding has been recently verified by Nayak *et al.* (1998) who showed that L-LTP in rat CA1 mini-slices could be maintained for at least 5 hours. Both the results of this thesis and those of Nayak *et al.* (1998), strongly suggest that if gene transcription occurs in L-LTP, then it must occur in the nucleus of the postsynaptic cell bodies. Indeed, removal of the postsynaptic cell bodies has been shown to block L-LTP in the CA1 region of rat hippocampal slices (Frey *et al.*, 1989). However, clearly this finding is not sufficient evidence to assert that postsynaptic gene transcription is necessary for L-LTP induction.

In conclusion, the L-LTP induced in this study has been shown to exhibit an apparent requirement for new mRNA synthesis, although these data need to be interpreted cautiously. Finally, a requirement for presynaptic mRNA synthesis for L-LTP has been ruled out.

THE LOCUS OF TRANSLATION IN L-LTP

6.1 IS L-LTP DEPENDENT ON LOCAL PROTEIN SYNTHESIS?

The results presented in this study, coupled with previously published data, provide evidence that the induction of L-LTP is critically dependent on protein synthesis. Traditionally, translation has been thought of a process that takes place in the soma of each neuron, some distance from the site of potentiation in the dendritically located synapses. How then does the cell ensure that specific synapses that have undergone potentiation, receive newly synthesised proteins required for the maintenance of L-LTP? Three hypotheses have been proposed to explain how protein targeting in L-LTP could be achieved: the *mail* hypothesis, the *synaptic tag* hypothesis and the *local synthesis* hypothesis (discussed in detail in section 1.6). Both the *mail* hypothesis and to a certain extent, the *synaptic tag* hypothesis, have been used to explain the delivery of somatically synthesised proteins to specific synapses, for the subsequent stabilisation of L-LTP (see section 1.6.1 and 1.6.2 for a full explanation). The *local synthesis* hypothesis however, assumes that the proteins required for L-LTP induction are not synthesised in the soma, but instead are produced locally, utilising mRNA and translational machinery present in the dendrites (see section 1.6.3 for more detail). Indeed, *in situ* hybridisation has identified a small number of dendritically localised mRNA species, including mRNA for αCaMKII, *Arc* and NMDAR1 (Burgin *et al.*, 1990; Link *et al.*, 1995; Lyford *et al.*, 1995; Gazzaley *et al.*, 1997). In addition, PCR based amplification techniques, suggest the existence of a number of others (Miyashiro *et al.*, 1994; Crino & Eberwine, 1996)(section 1.7.1).

The fact that only a small number of mRNA species show dendritic localisation, coupled with the high levels of some of these mRNA species in distal dendrites, suggests that active transport mechanisms rather than simple diffusion are responsible for their localisation. Consistent with this, the expression levels and dendritic distribution of a number of mRNA species in both hippocampal and *Aplysia* neurons, have been shown to increase in response to either synaptic growth or synaptic activity (Thomas *et al.*, 1994; Tongiorgi *et al.*, 1997; Muslimov *et al.*, 1998; Steward *et al.*,

1998; Schacher *et al.*, 1999; Zhang *et al.*, 1999)(see section 1.7.2). Attempts to visualise dendritic mRNA transport in real time, have identified mRNA-containing granules that undergo bi-directional microtubule-dependent transport within the dendrites of cultured neurons (Knowles *et al.*, 1996; Knowles & Kosik, 1997). Although a number of protein families have been identified that display RNA binding properties (see section 1.7.3), it is not yet clear whether these granules represent protein complexes responsible for the dendritic transport of mRNA.

In addition to dendritically localised mRNA, elements of the translational machinery such as polyribosomes, tRNAs, elongation factors and elements of the cotranslational recognition system have all been found throughout the dendrites of hippocampal neurons (Steward & Levy, 1982; Tiedge & Brosius, 1996). Furthermore, endoplasmic reticulum and golgi apparatus have also been shown to localise in the proximal and middle dendrites of cultured hippocampal neurons (Torre & Steward, 1996). This dendritic translational machinery appears to be functional. Protein synthesis occurs in hippocampal neurons isolated from their cell bodies (Torre & Steward, 1992), whilst transfection of isolated dendrites with mRNA, results in the appearance of the corresponding proteins a few hours later (Crino & Eberwine, 1996; Crino *et al.*, 1998). In addition, dendritic protein synthesis may be regulated by synaptic activity. Synaptosomal, polyribosomal aggregates increase in size in response to K^+ depolarisation or glutamate application (Weiler & Greenough, 1993), whilst application of the cholinergic agonist, carbachol, increases dendritic [^{3}H] leucine incorporation three fold in the CA1 region of hippocampal slices (Feig & Lipton, 1993)(see also section 1.7.4). Furthermore, the levels of CaMKII protein thought to be due to dendritic protein synthesis, have been seen to increase in response to NMDA receptor activation or tetanic stimulation (Ouyang *et al.*, 1997; Ouyang *et al.*, 1999; Scheetz *et al.*, 2000). Recently, a number of molecules have been identified that may play a role in the regulation of dendritic translation. These include the cytoplasmic polyadenylation element binding protein (CPEB), eukaryotic elongation factor (eEF2) and the fragile X mental retardation protein (FMRP)(see section 1.7.5). However, regulation of translation by any of these molecules has yet to be demonstrated in dendrites.

Despite considerable data suggesting the occurrence of local protein synthesis from dendritically localised mRNA, there is still no evidence that this process is required in the induction and maintenance of L-LTP. However, local protein synthesis has been shown to be involved in a number of other types of synaptic plasticity (see also section 1.7.6). Neurotrophin-induced, protein synthesis dependent, synaptic enhancement in the CA1 region of hippocampal slices was unaffected by the removal of both the pre and postsynaptic cell bodies. This suggests that the protein synthesis required to sustain this synaptic enhancement was dendritic in origin (Kang & Schuman, 1996). Similarly, protein synthesis dependent (mRNA synthesis independent) LTD, induced by the application of the mGluR agonist DHPG, was found to occur in the dendrites of CA1 neurons that had been separated from their cell bodies (Huber *et al.*, 2000). Although such results are suggestive, perhaps the best evidence for a role of dendritic protein synthesis in synaptic plasticity comes from experiments in *Aplysia* (see section 1.7.6 for further detail). In these experiments, 5 pulses of serotonin applied to a single sensory motor synapse induced a synapse specific long-term facilitation (LTF) that lasted >24 hours. This LTF was found to be blocked by the local application of the protein synthesis inhibitor emetine, exclusively to the synapse (Martin *et al.*, 1997a). In addition, local protein synthesis could occur in neurites that had been separated from their cell bodies, increasing three fold in response to the application of serotonin (Martin *et al.*, 1997a).

Although recent evidence suggests that local protein synthesis plays an important role in a number of different forms of synaptic plasticity, a role for dendritic translation in hippocampal L-LTP has yet to been shown. In this chapter, experiments are described that have allowed the role of local dendritic protein synthesis in the induction and maintenance of L-LTP to be addressed for the first time.

6.2 L-LTP INDUCTION IN THE STRATUM RADIATUM AND STRATUM ORIENS PATHWAYS

6.2.1 Experimental Design

Pyramidal cells in the CA1 region of the hippocampus possess two spatial distinct dendritic trees: the apical dendrites that project into the stratum radiatum region, and the basal dendrites that project into the stratum oriens region. Electrodes were positioned within each of these two distinct regions and EPSPs were recorded in response to activation of the Schaffer collateral/commissural afferents (Fig. 6.1). The spatial separation of areas radiatum and oriens ensured that not only were the two pathways independent, but the somata of the sampled cells were positioned between the two pathways (Fig. 6.1). It is also important to note that the recording electrodes were positioned opposite each other (one in the stratum radiatum and one in the stratum oriens), thus ensuring that the two pathways would have a large overlap with respect to the CA1 pyramidal cell populations that were sampled (Fig. 6.1). The high degree of overlap between the cell populations sampled by each pathway, means that either pathway can act as a control for the other. If L-LTP is reliably induced in both pathways and maintained for at least 8 hours then this experimental configuration will be useful in addressing a number of questions concerning the role of local dendritic protein synthesis in L-LTP.

Test stimuli were delivered alternately to each pathway at 15 second intervals and responses were monitored for in excess of 8 hours. Following a 30 minute baseline, a tetanus (20 pulses @ 100Hz repeated 6 times with an ITI of 3 seconds) was applied to the stratum radiatum. Five minutes later an identical tetanus was applied to the stratum oriens pathway.

6.2.2 Results

Figures 6.2 and 6.3 show an experiment in which both the stratum radiatum and stratum oriens pathways in a single hippocampal slice display L-LTP in response to tetani applied to each pathway. The EPSP response post-tetanus, in the stratum

Figure 6.1 Schematic representation of electrode placement within the CA1 region of the hippocampal slice. In both the stratum radiatum (sr) and stratum oriens (so) regions, a single stimulating electrode (Stim.) is placed in the trajectory of the Schaffer collateral/commissural projections, whilst a single recording electrode (Rec.) is positioned in the dendritic region of the CA1 pyramidal cells. The recording electrodes are positioned opposite each other so as to maximise the chances of recording from two spatially separated dendritic regions (and thus independent synaptic populations) in the same population of CA1 pyramidal cells. For key to abbreviations, see figure 2.1.

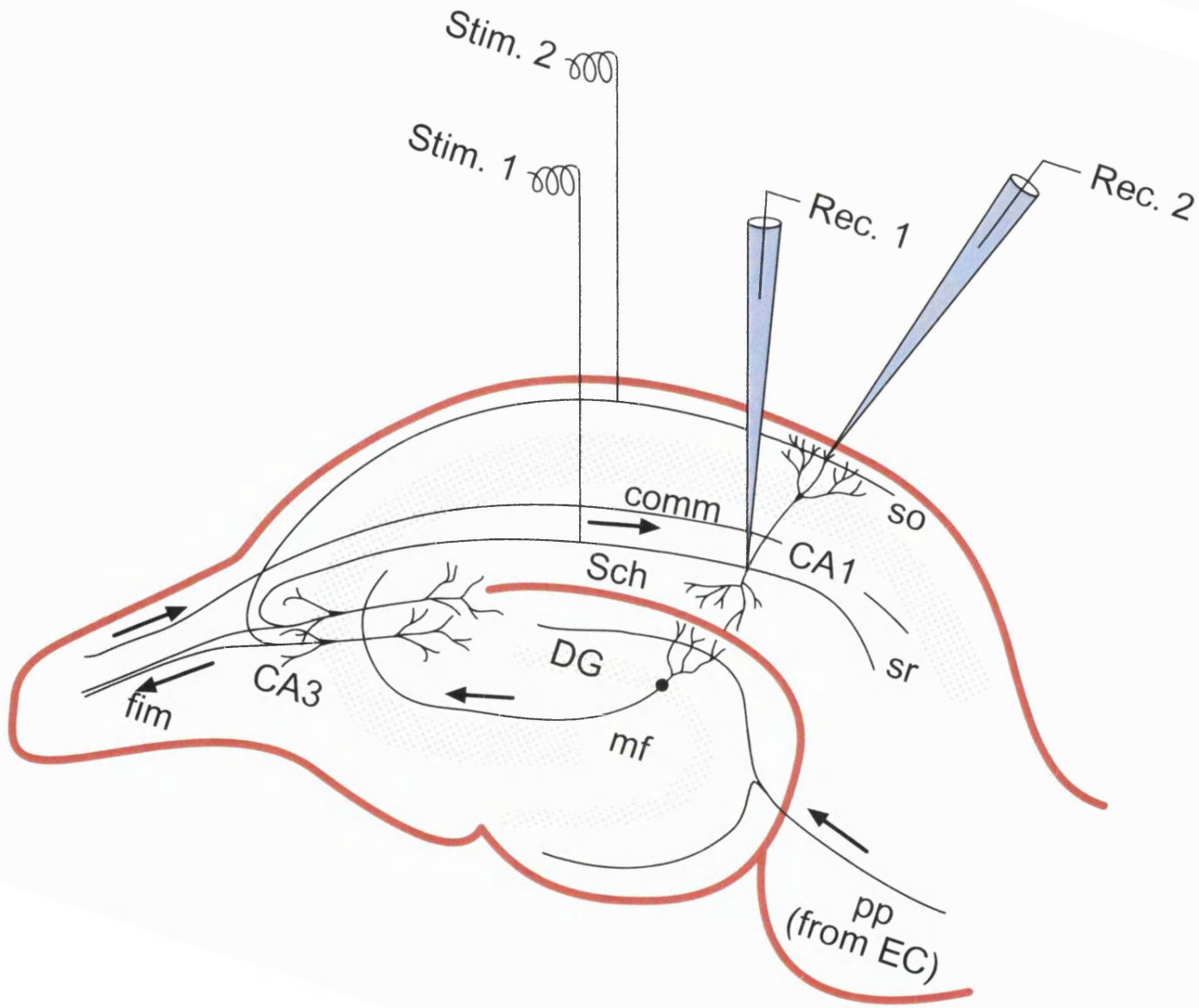

Stim. 2
Stim. 1
Rec. 1
Rec. 2
comm
so
Sch
CA1
sr
DG
CA3
fim
mf
pp
(from EC)

Figure 6.2 Field EPSPs taken from an individual L-LTP induction experiment in both the stratum radiatum and stratum oriens of the CA1 region. Individual EPSPs from both the stratum radiatum and stratum oriens are shown at various time points, prior to and following, a tetanus given to each pathway. Potentials are taken from the experiment shown in figure 6.3.

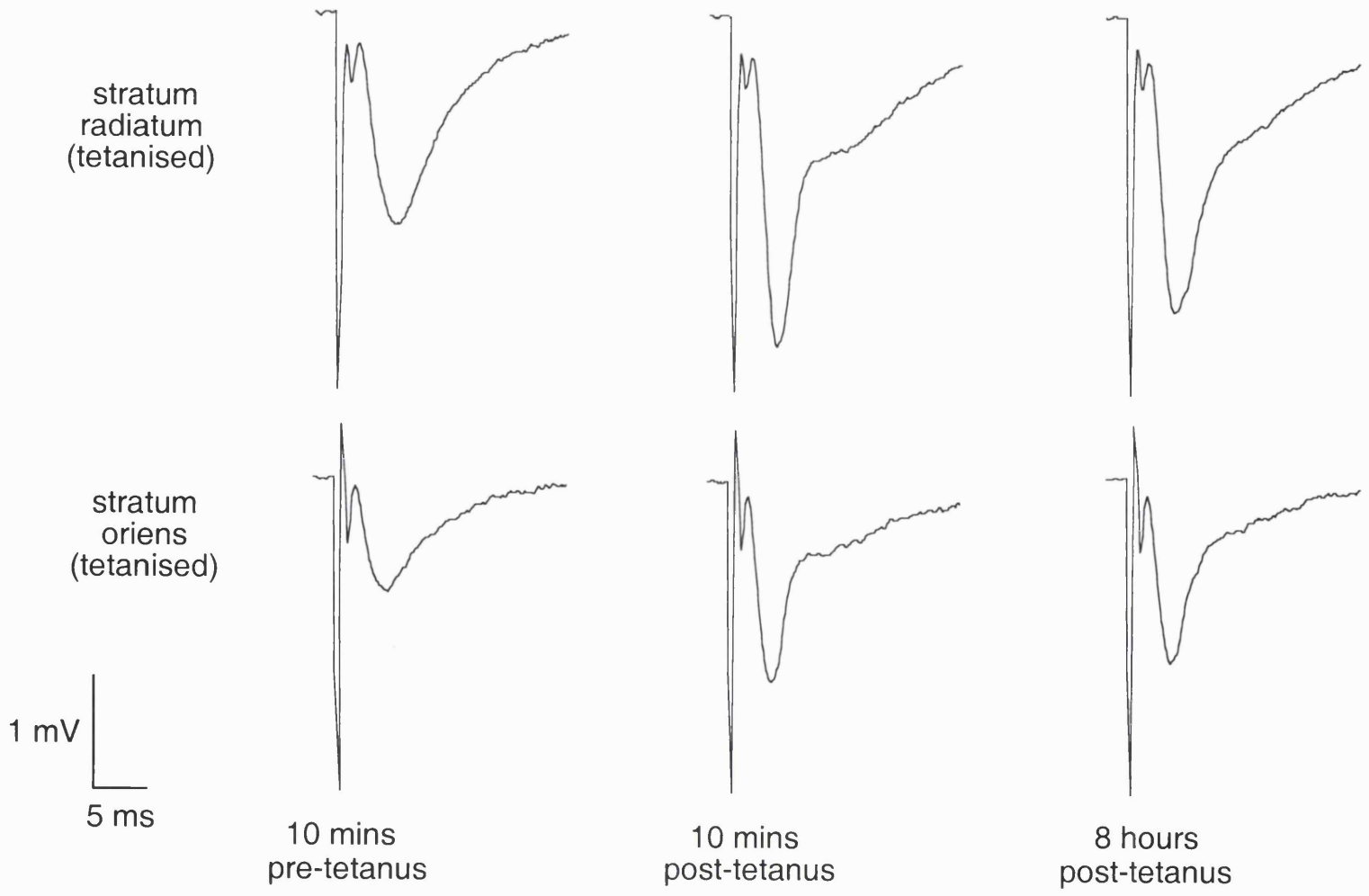
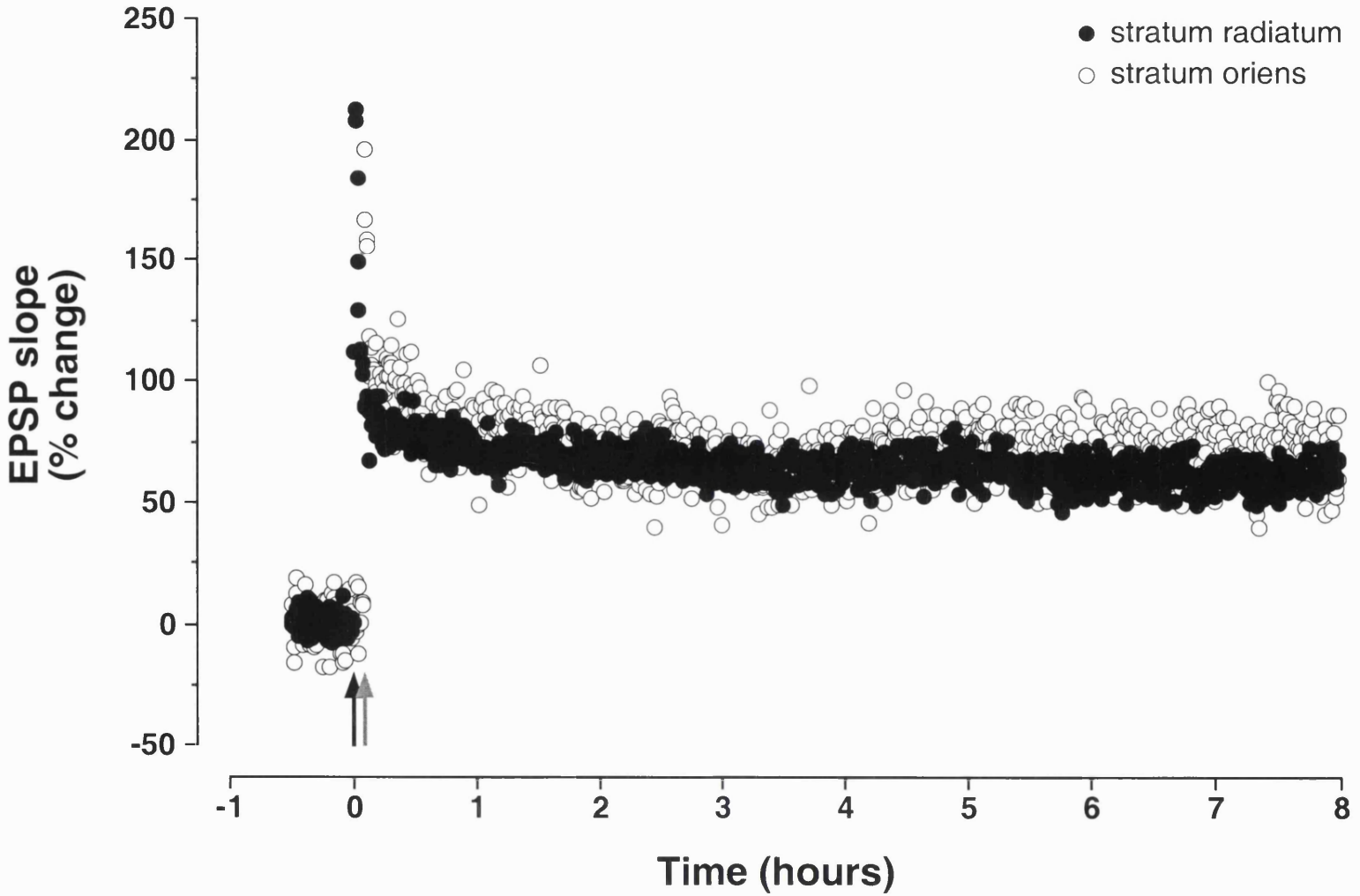

stratum
radiatum
(tetanised)
stratum
oriens
(tetanised)
1 mV
5 ms
10 mins
pre-tetanus
10 mins
post-tetanus
8 hours
post-tetanus

Figure 6.3 Individual example of L-LTP induction in both the stratum radiatum and stratum oriens of the CA1 region. Electrodes were positioned as shown in figure 6.1. Field EPSP slope, expressed as percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to the first tetanus. Black arrow indicates tetanus applied to the stratum radiatum pathway (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs); grey arrow indicates tetanus applied to the stratum oriens pathway.


stratum radiatum
stratum oriens
EPSP slope
(% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

radiatum and stratum oriens pathways shows comparable levels of PTP (213% and 196% respectively). EPSP slope also declines at a similar rate in both pathways and plateaus at around 3-4 hours post-tetanus. For the remaining 4 hours the EPSP slope potentiation in the two pathways is very similar, with stratum radiatum displaying a 60% potentiation at the 8 hour time point compared to 69% in the stratum oriens (Fig 6.3). Individual potentials taken from this experiment are illustrated in Fig 6.2; note the lack of any appreciable change in the afferent volley profile in either pathway.

The results seen in the individual example are reflected in the group data; L-LTP in the stratum oriens displays a similar decay profile and magnitude to that seen in the stratum radiatum (51±8% and 56±6% respectively, at 8 hours) (Fig. 6.4a). Although EPSP slope for both PTP and L-LTP after 8 hours, are not significantly different between pathways, an apparent difference over the first hour following tetanisation does exist (Fig 6.4a). Indeed, at 30 minutes post-tetanus, LTP in the stratum oriens pathway (82±5%) is significantly larger ($t=2.3676$, $P\leq0.0231$) than in the stratum radiatum pathway (66±4%)(Fig. 6.4b).

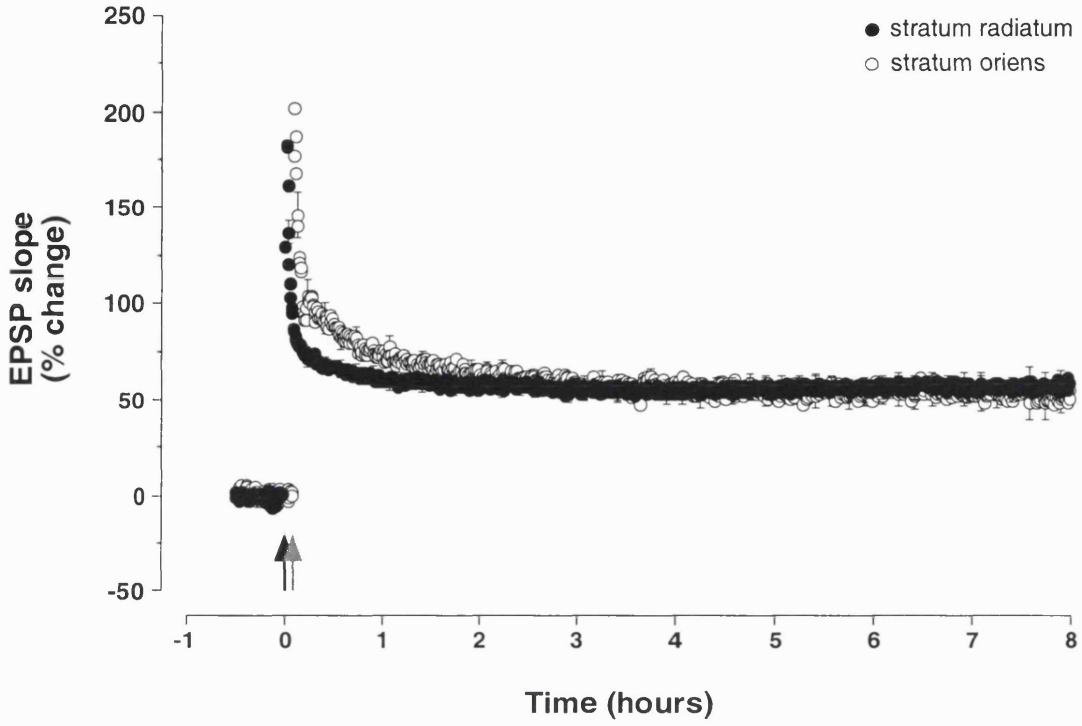
6.3 L-LTP INDUCTION DURING FOCAL APPLICATION OF EMETINE TO THE STRATUM RADIATUM PATHWAY

6.3.1 Experimental Design

Electrodes were positioned within the stratum radiatum and the stratum oriens, two spatially distinct areas of the CA1 region (Fig. 6.5). This ensured the sampling of two independent pathways within a largely common cell population (see section 6.2.1 for more detail). Using this experimental configuration, the requirement for local dendritic protein synthesis during L-LTP induction in the stratum radiatum, was addressed by focally applying the translation inhibitor emetine, specifically to this pathway. Because either pathway can act as a control for the other (see section 6.2.1), the stratum oriens can be used to demonstrate that the cell population has retained the ability to produce L-LTP, even when it is absent at the drug-applied region of the stratum radiatum pathway.

Figure 6.4 L-LTP induced in the stratum oriens is similar to that seen in the stratum radiatum. Electrodes were positioned as shown in figure 6.1. Field EPSP slope, expressed as percentage change over time is shown. **a**, L-LTP induction in both the stratum radiatum and stratum oriens pathways (n=20). Zero percent was taken as the mean of the values recorded 10 minutes prior to the first tetanus. Black arrow indicates tetanus applied to the stratum radiatum pathway (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs); grey arrow indicates tetanus applied to the stratum oriens pathway. Error bars indicate ±SEM. **b**, Statistical comparison of stratum radiatum and stratum oriens pathways following L-LTP induction (n=20). Raw data sources are identical to those used for a. In each pathway, **PTP** (post-tetanic potentiation) corresponds to the 5 minute time period immediately following tetanisation; **30 min** corresponds to the 10 minute time period between 25-35 minutes post-tetanus; and **8 hrs** corresponds to the 10 minute time period between 7 hours 50 minutes - 8 hours post-tetanus. Values represent the mean EPSP slope measured during each time period. *$P<0.05$ (unpaired Student t test, two tailed). Error bars indicate ±SEM.

a


stratum radiatum
stratum oriens
EPSP slope
(% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

b

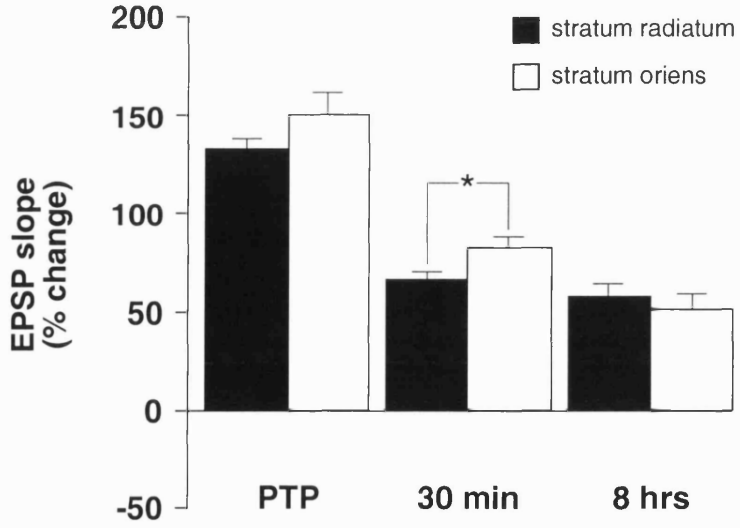
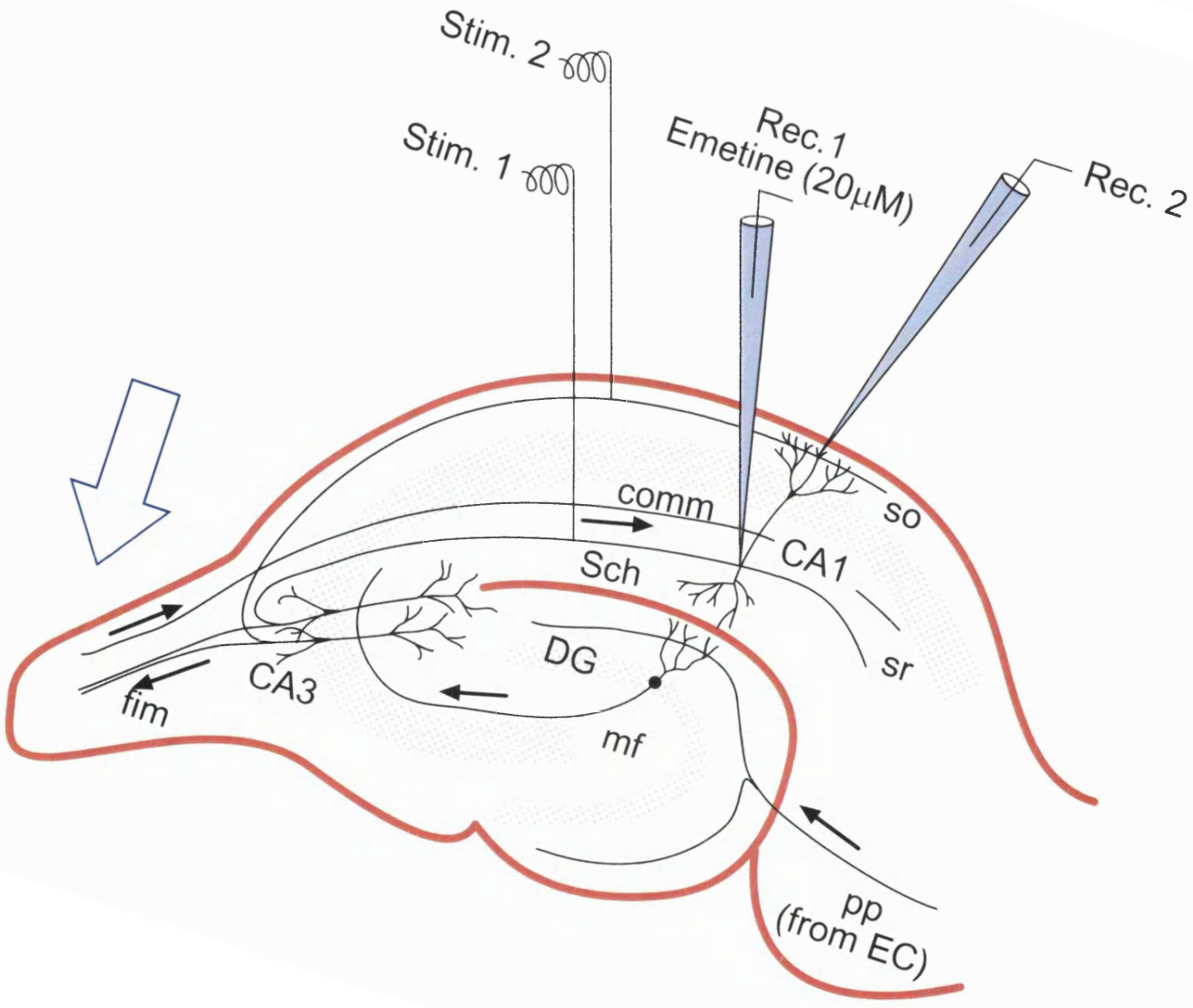

stratum radiatum
stratum oriens
EPSP slope
(% change)
200
150
100
50
0
-50
*
PTP
30 min
8 hrs

Figure 6.5 Schematic representation of electrode placement within the hippocampal slice during dendritic application of emetine to the stratum radiatum region. In both the stratum radiatum (sr) and stratum oriens (so) regions, a single stimulating electrode (Stim.) is placed in the trajectory of the Schaffer collateral/commissural projections, whilst a single recording electrode (Rec.) is positioned in the dendritic region of the CA1 pyramidal cells. The recording electrodes are positioned opposite each other so as to maximise the chances of recording from two spatially separated dendritic regions (and thus independent synaptic populations) in the same population of CA1 pyramidal cells. In addition, the recording electrode in the stratum radiatum (Rec.1) was filled with ACSF, containing emetine (20μM). Open arrow indicates the direction of ACSF flow across the slice. For key to abbreviations, see figure 2.1.


Stim. 2
Stim. 1
Rec. 1
Emetine (20μM)
Rec. 2
comm
so
Sch
CA1
sr
DG
CA3
fim
mf
pp
(from EC)

Test stimuli and tetanisation protocols were identical to those outlined in section 5.1.1. The irreversible translation inhibitor, emetine (20μM), was focally applied to the dendritic region of the stratum radiatum via the glass recording microelectrode, at least 30 minutes prior to tetanisation (see section 2.1.3 and Fig. 6.5). The emetine filled recording electrode remained present for the duration of the experiment. Slices were oriented so that both the cell body and stratum oriens region were upstream of the drug-containing electrode with respect to the flow of ACSF in the chamber, thereby minimising the spread of emetine to non-target regions (see Fig. 6.5, open arrow). The concentration of emetine used for the focal application experiments (20μM) was reduced, compared to levels used in previous bath application experiments (100μM). This reduced concentration was used, in an attempt to ensure that the effective concentration for translation block was only present in the area to which emetine was focally applied. In addition, focal application of emetine to specific areas deep within the tissue, does not require the higher doses used for bath application experiments, in which drug delivery is dependent on diffusion from the slice surface into the tissue.

In the translational assays, the extent of protein synthesis block by emetine (20μM), was assessed by observing the amount of [^{3}H] valine incorporation into hippocampal slices over a 90 minute time period, in the presence of the drug.

6.3.2 Results

In the individual experiment shown in figure 6.7, focal application of emetine (20μM) to pyramidal cell dendrites in the stratum radiatum region blocked the induction of L-LTP in this pathway. Following LTP induction, the PTP in both the stratum radiatum and the stratum oriens is comparable (192% and 223% respectively); however, at around 3 hours post-tetanus, a difference between the EPSP slope values of the two pathways becomes apparent. The stratum radiatum pathway (tetanised in the presence of 20μM emetine) declines to a level of 22%, less than half the level of potentiation seen in the drug-free stratum oriens pathway at the same time point (51%). At 8 hours the difference between the two pathways is considerable. The stratum radiatum pathway has completely returned to pre-tetanus levels (3%),

Figure 6.6 Field EPSPs taken from an individual LTP induction experiment during focal application of emetine to the stratum radiatum pathway. Individual EPSPs from both the stratum radiatum and stratum oriens are shown at various time points, prior to and following, a tetanus given to each pathway. Emetine (20μM) was focally applied to the stratum radiatum via the recording electrode, at least 30 minutes prior to tetanisation. The emetine filled recording electrode remained present for the duration of the experiment. Potentials are taken from the experiment shown in figure 6.7.

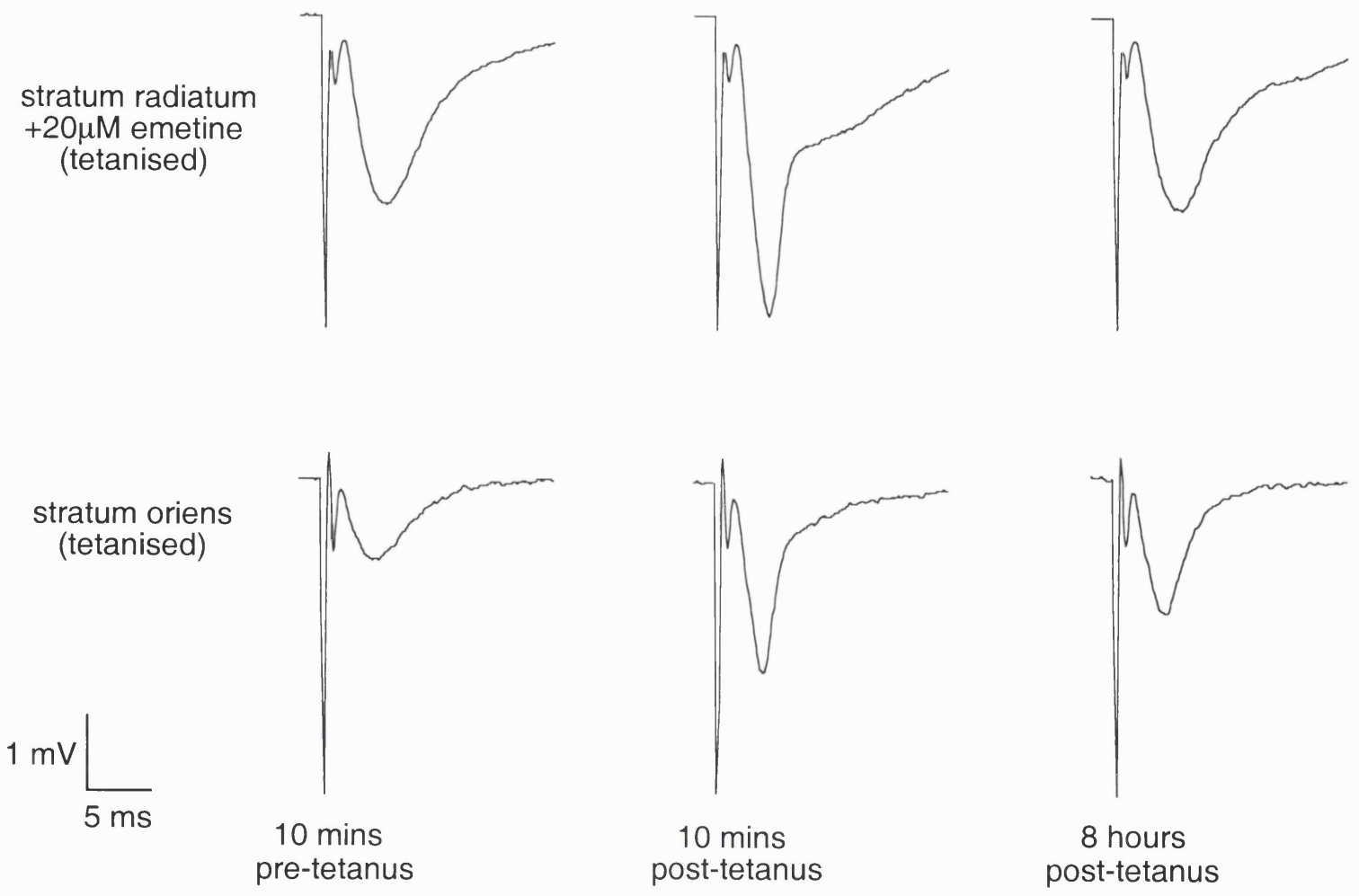
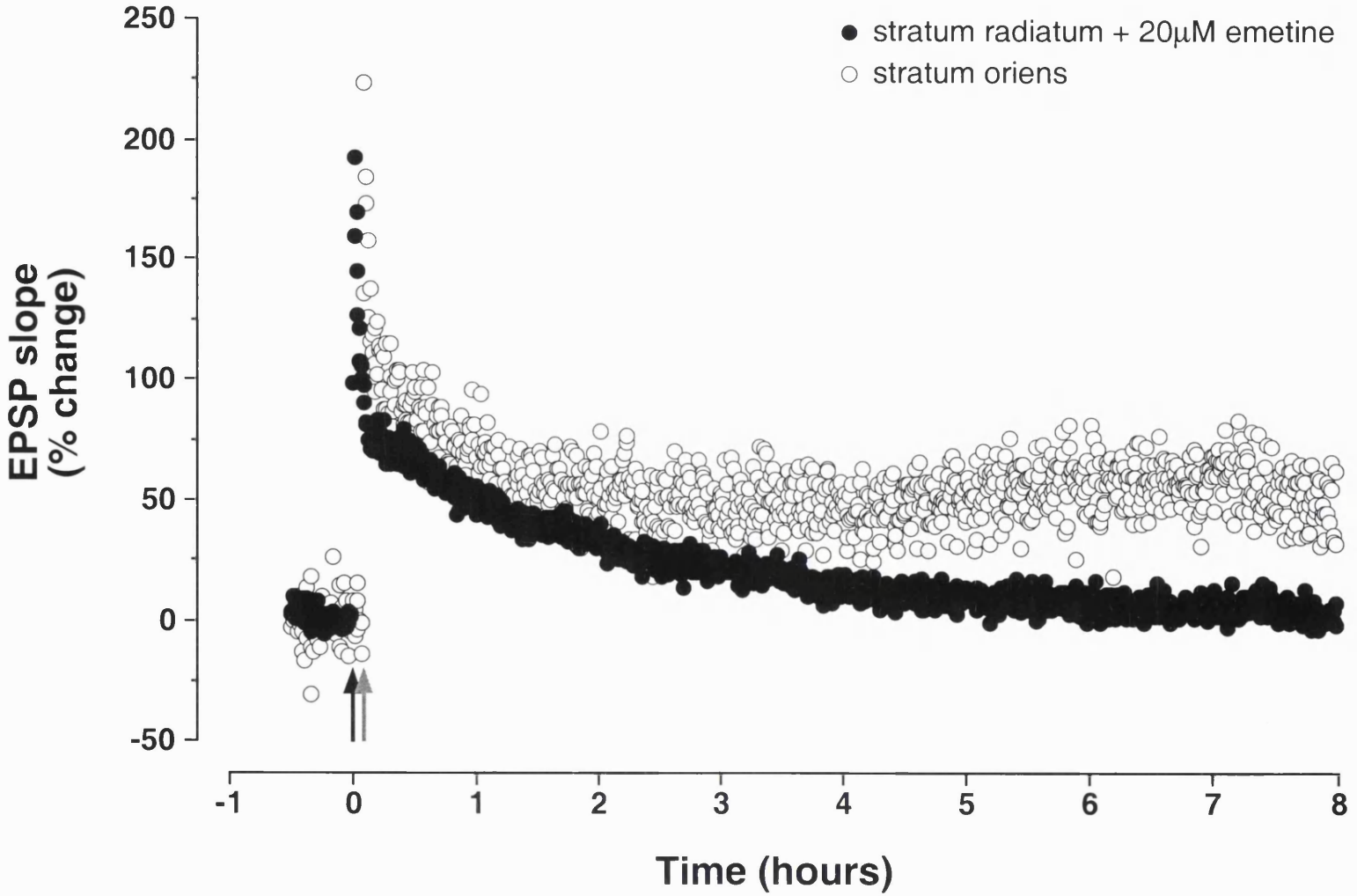

stratum radiatum
+20μM emetine
(tetanised)
stratum oriens
(tetanised)
1 mV
5 ms
10 mins
pre-tetanus
10 mins
post-tetanus
8 hours
post-tetanus

Figure 6.7 Individual example of L-LTP induction block in the stratum radiatum during focal application of emetine. Electrodes were positioned as shown in figure 6.5. Field EPSP slope, expressed as percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to the first tetanus. Black arrow indicates tetanus applied to the stratum radiatum pathway (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs); grey arrow indicates tetanus applied to the stratum oriens pathway. Emetine (20μM) was focally applied to the stratum radiatum via the recording electrode, at least 30 minutes prior to tetanisation. The emetine filled recording electrode remained present for the duration of the experiment.


stratum radiatum + 20μM emetine
stratum oriens
EPSP slope
(% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

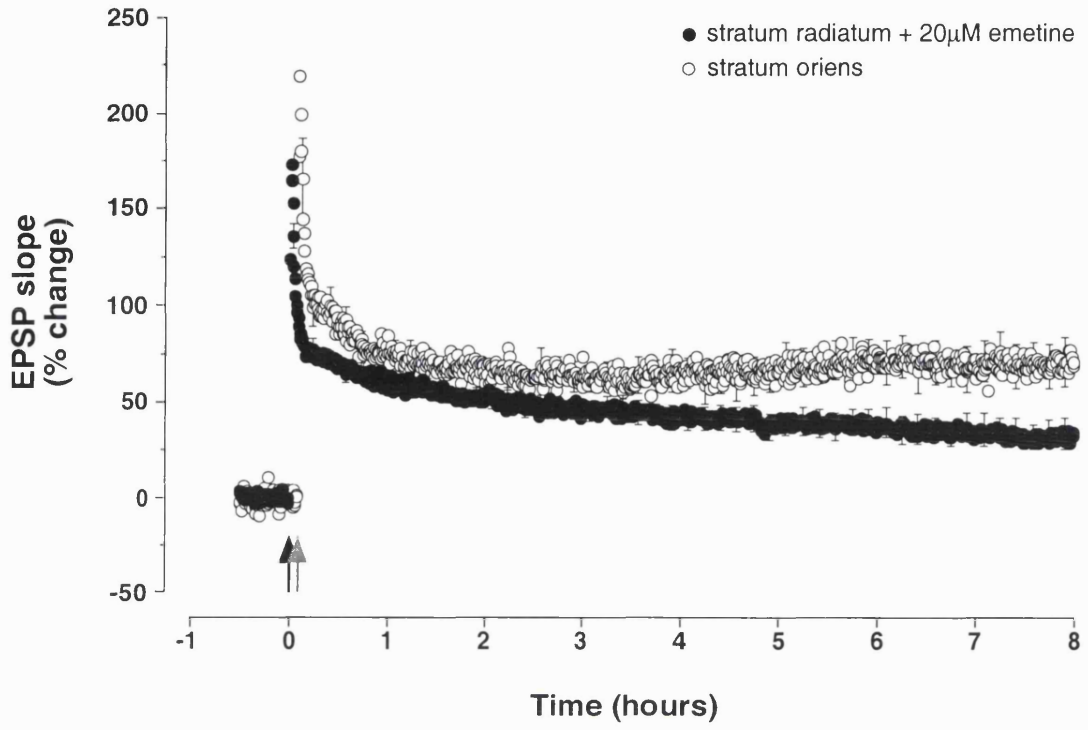
representing a complete block in L-LTP induction, whilst the stratum oriens maintains a stable, robust potentiation (49%)(Fig. 6.7). Once again the individual EPSPs do not reflect a notable change in the profile of the afferent volleys of either pathway, ruling out poor slice health as a contributing factor in L-LTP induction block (Fig. 6.6).

Although the pooled data for these experiments does not show quite the dramatic results seen in the example highlighted above, a significant difference between the two pathways at the 8 hour time point is seen ($t=2.5991$, $P\leq0.0210$)(Fig. 6.8a and Fig. 6.9a). LTP in both pathways remains comparable over the first 2 hours, however by 8 hours the level of potentiation in the stratum radiatum pathway (33±8%) is less than half the value of the potentiation seen in the stratum oriens (69±19%)(Fig. 6.8a). Interleaved control experiments exhibit a robust L-LTP that lasts for at least 8 hours and is not significantly different between the stratum radiatum and stratum oriens pathways (Fig. 6.8b and Fig. 6.9b). In addition, comparisons of the stratum radiatum from drug experiments, with the same pathway in the interleaved controls, also yields a significant difference at 8 hours post-tetanus ($t=2.1269$, $P\leq0.0475$)(Fig. 6.9c). The extent of the translation block caused by emetine when applied to the whole slice at a concentration of 20μM was also assessed (Fig. 6.10). The incorporation of [^{3}H] valine, in slices incubated in the presence of emetine (20μM) is negligible, compared to the high levels of incorporation seen in the control slices (Fig. 6.10). These results suggest that protein synthesis occurring in hippocampal slices, is almost completely abolished by the application of emetine (20μM).

Taken together, the results presented above, suggest that the focal application of the translation inhibitor emetine (at a concentration shown to block protein synthesis) to the stratum radiatum dendritic region, results in a significant reduction in the magnitude of L-LTP in that region.

Figure 6.8 The magnitude of L-LTP in the stratum radiatum is reduced during focal application of emetine. Electrodes were positioned as shown in figure 6.5. Field EPSP slope, expressed as percentage change over time is shown. **a**, Reduction in L-LTP magnitude in the stratum radiatum pathway, in response to focal application of emetine (20μM) to this pathway, at least 30 minutes prior to tetanisation (n=8). **b**, Interleaved control experiments, performed in the absence of emetine (n=13, up to 6.5 hours; n=12, up to 8 hours). Zero percent was taken as the mean of the values recorded 10 minutes prior to the first tetanus. Black arrow indicates tetanus applied to the stratum radiatum pathway (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs); grey arrow indicates tetanus applied to the stratum oriens pathway. Error bars indicate ±SEM.

a


stratum radiatum + 20μM emetine
stratum oriens
EPSP slope (% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

b

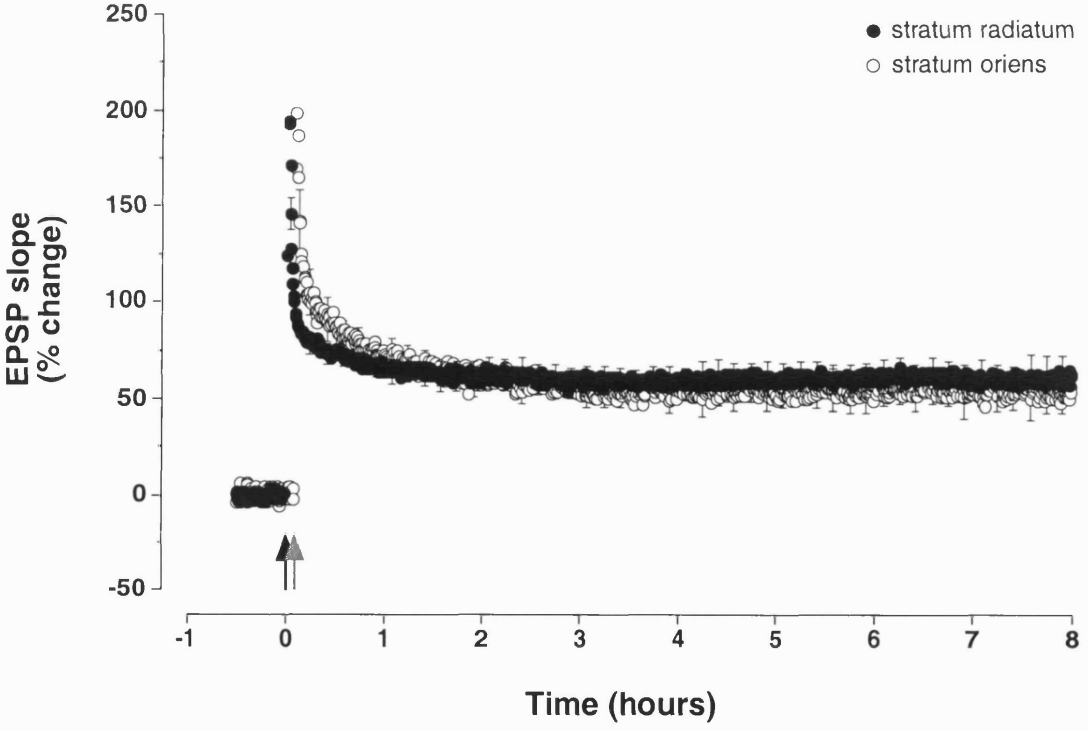
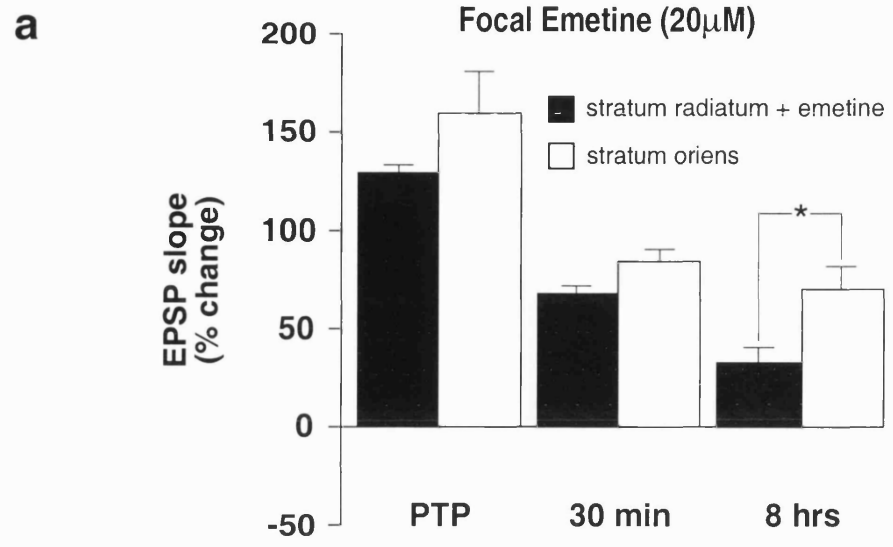
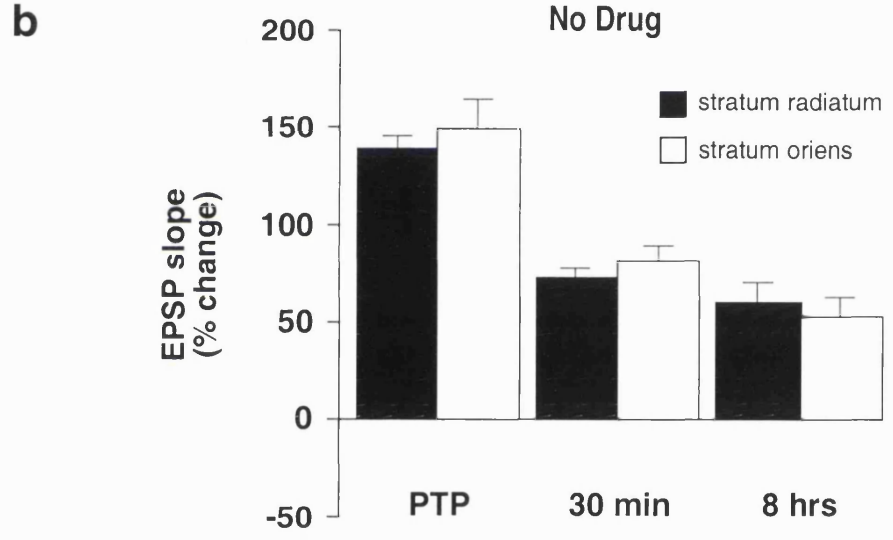

stratum radiatum
stratum oriens
EPSP slope (% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

Figure 6.9 Statistical comparisons during the reduction of stratum radiatum L-LTP in the presence of focally applied emetine. a, Reduction in L-LTP magnitude in the stratum radiatum pathway, during focal application of emetine (20μM) to this pathway, at least 30 minutes prior to tetanisation (n=8). **b**, Interleaved control experiments, performed in the absence of emetine (n=13, up to 6.5 hours; n=12, up to 8 hours). **c**, Stratum radiatum pathways taken from drug and control experiments shown in a and b. In all cases **PTP** (post-tetanic potentiation) corresponds to the 5 minute time period immediately following tetanisation; **30 min** corresponds to the 10 minute time period between 25-35 minutes post-tetanus; and **8 hrs** corresponds to the 10 minute time period between 7 hours 50 minutes - 8 hours post-tetanus. Values represent the mean EPSP slope measured during each time period. *$P<0.05$ (unpaired Student t test, two tailed). Error bars indicate ±SEM. Raw data sources are identical to those used for figure 6.8.


a
Focal Emetine (20μM)
stratum radiatum + emetine
stratum oriens
EPSP slope (% change)
200
150
100
50
0
-50
*
PTP
30 min
8 hrs


b
No Drug
stratum radiatum
stratum oriens
EPSP slope (% change)
200
150
100
50
0
-50
PTP
30 min
8 hrs

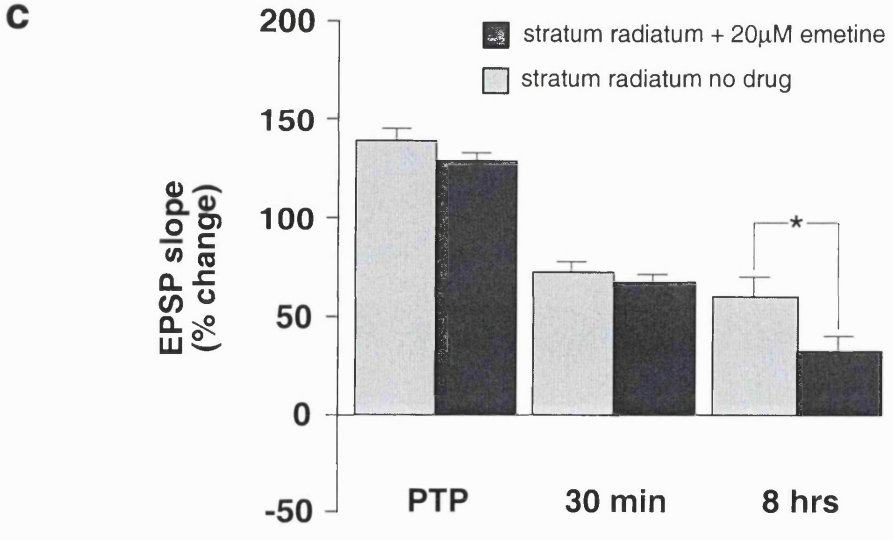
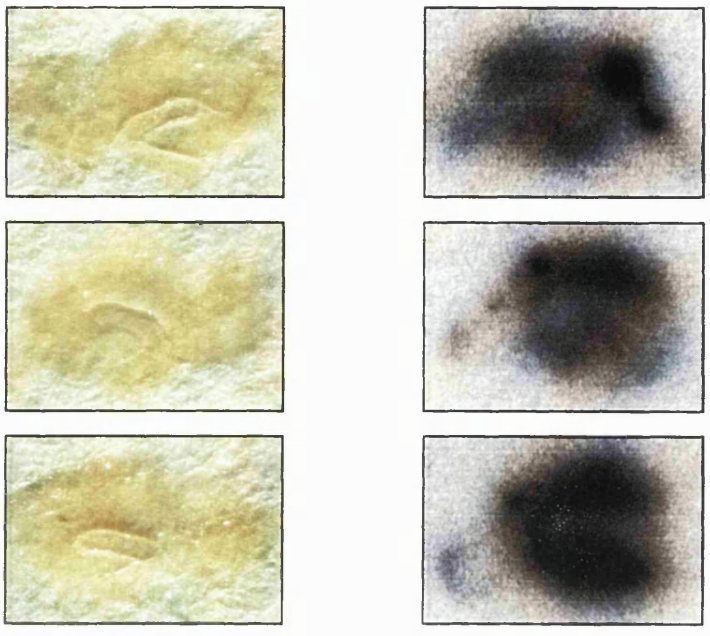

c
stratum radiatum + 20μM emetine
stratum radiatum no drug
EPSP slope (% change)
200
150
100
50
0
-50
*
PTP
30 min
8 hrs

Figure 6.10 Bath application of emetine (20μM) blocks new protein synthesis in hippocampal slices. Data shows *in situ* autoradiography images (right) from corresponding hippocampal slices (left) incubated for 90 minutes in the presence of [^{3}H] valine. **a**, Control hippocampal slices, incubated in the absence of emetine. **b**, Hippocampal slices incubated during application of emetine (20μM).

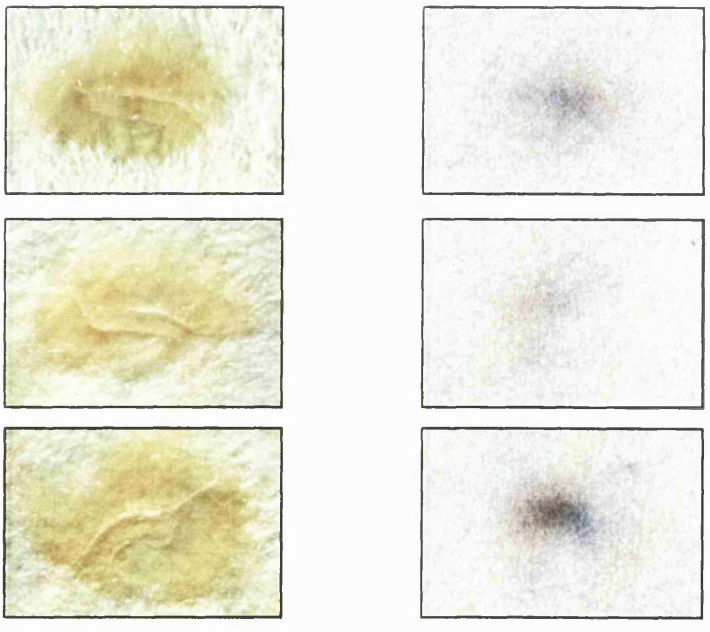
Control

a



Emetine

b



6.4 L-LTP INDUCTION DURING FOCAL APPLICATION OF EMETINE TO THE CELL BODIES IN THE CA1 REGION

6.4.1 Experimental Design

Electrodes were positioned within the stratum radiatum and the stratum oriens, two spatially distinct areas of the CA1 region (Fig. 6.11). This ensured the sampling of two independent pathways within a largely common cell population (see section 6.2.1 and 6.3.1 for more detail). Using this experimental configuration, the requirement for somatic protein synthesis during L-LTP induction in both the stratum radiatum and stratum oriens, was addressed, by focal application of the translation inhibitor emetine to the cell bodies of the sampled neuronal population.

Test stimuli and tetanisation protocols were identical to those outlined in section 6.2.1. The irreversible translation inhibitor, emetine (20μM), was focally applied to the somatic region of neurons within the sampled population. Drug application was achieved via a glass microelectrode, at least 30 minutes prior to tetanisation (see section 2.1.3 and Fig. 6.11). The emetine filled recording electrode remained present for the duration of the experiment.

6.4.2 Results

L-LTP induction in both the stratum radiatum and stratum oriens dendritic regions is unaffected, following focal application of the translation inhibitor emetine to their associated cell bodies. In the individual experiment highlighted in figure 6.13, both the stratum radiatum and the stratum oriens pathways shows comparable levels of PTP (184% and 179% respectively). Although the stratum radiatum pathway is smaller in magnitude following PTP, its profile over the course of the experiment is comparable to that seen in the stratum oriens. At the 8 hour time point, both the stratum radiatum and the stratum oriens display robust levels of potentiation (38% and 60% respectively). In the presence of somatic emetine, individual EPSPs in both

Figure 6.11 Schematic representation of electrode placement within the hippocampal slice during somatic application of emetine. In both the stratum radiatum (sr) and stratum oriens (so) regions, a single stimulating electrode (Stim.) is placed in the trajectory of the Schaffer collateral/commissural projections, whilst a single recording electrode (Rec.) is positioned in the dendritic region of the CA1 pyramidal cells. The recording electrodes are positioned opposite each other so as to maximise the chances of recording from two spatially separated dendritic regions (and thus independent synaptic populations) in the same population of CA1 pyramidal cells. In addition, a glass microelectrode filled with ACSF containing emetine (20μM), was positioned in the cell body region, directly between the two recording electrodes. For key to abbreviations, see figure 2.1.

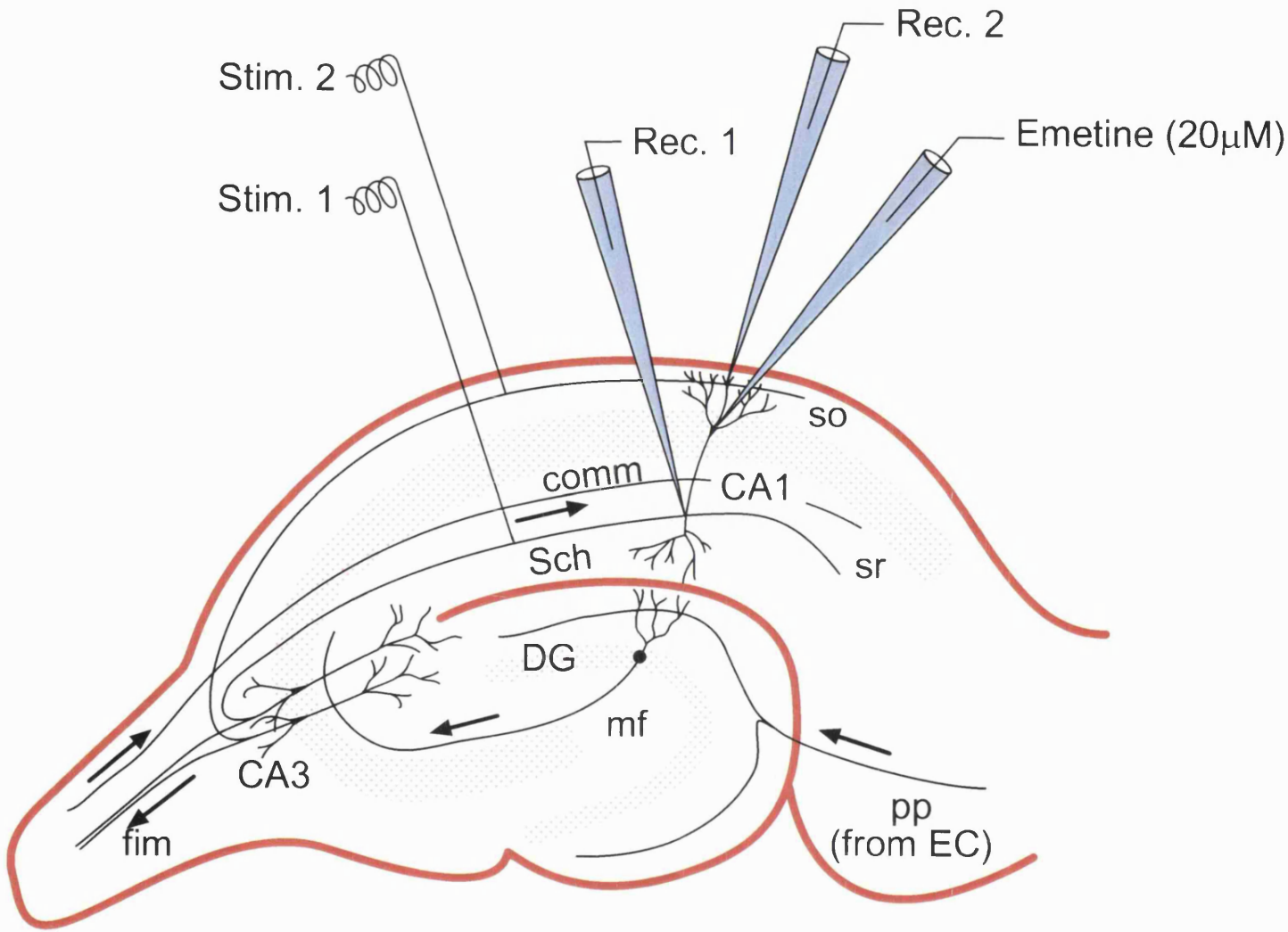

Rec. 2
Stim. 2
Rec. 1
Emetine (20μM)
Stim. 1
so
comm
CA1
Sch
sr
DG
mf
CA3
fim
pp
(from EC)

Figure 6.12 Field EPSPs taken from an individual LTP induction experiment during focal application of emetine to the cell body region. Individual EPSPs from both the stratum radiatum and stratum oriens are shown at various time points, prior to and following, a tetanus given to each pathway. Emetine (20μM) was focally applied to the cell bodies via a glass microelectrode, at least 30 minutes prior to tetanisation. The emetine filled microelectrode remained present for the duration of the experiment. Potentials are taken from the experiment shown in figure 6.13.

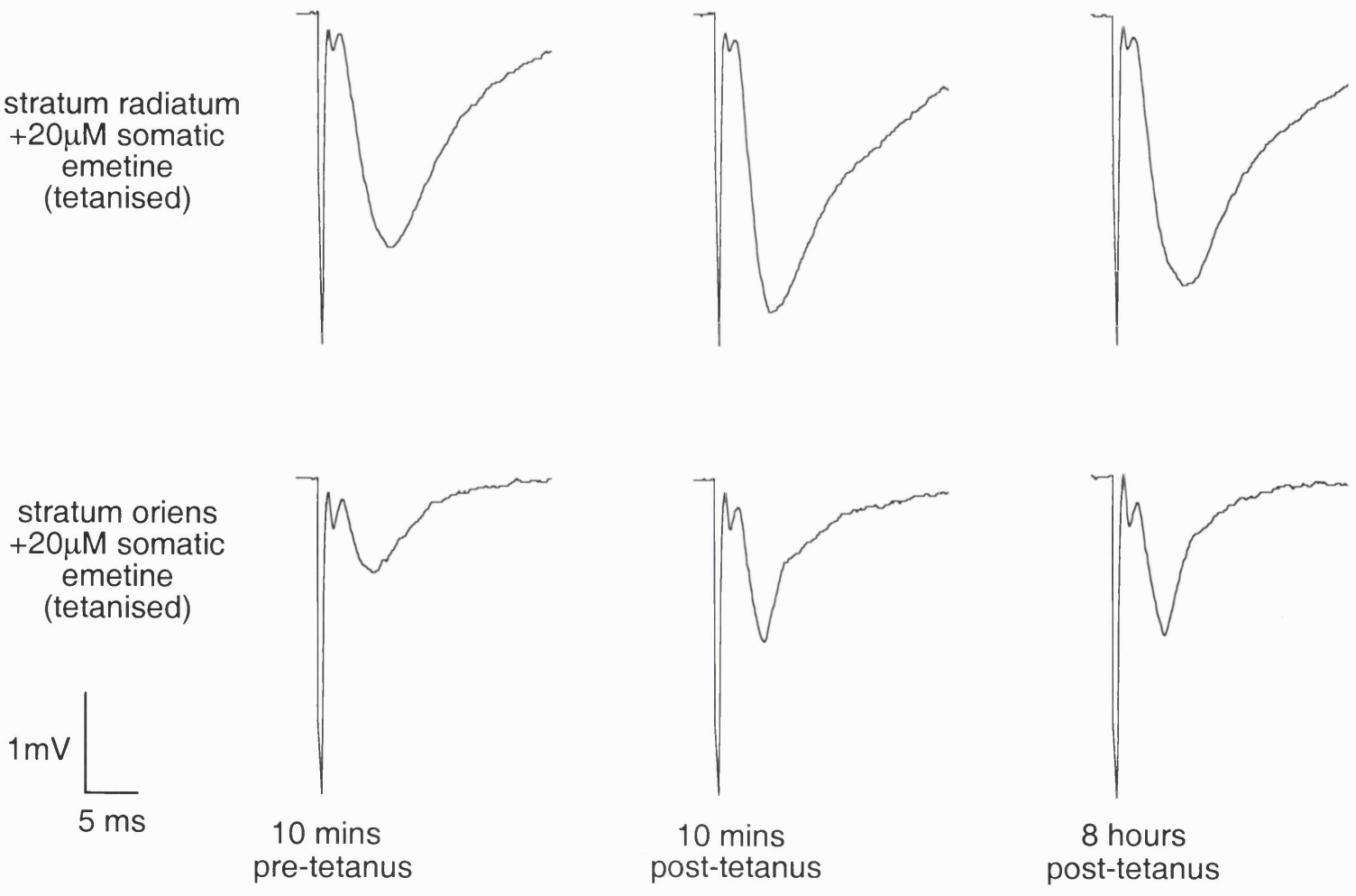
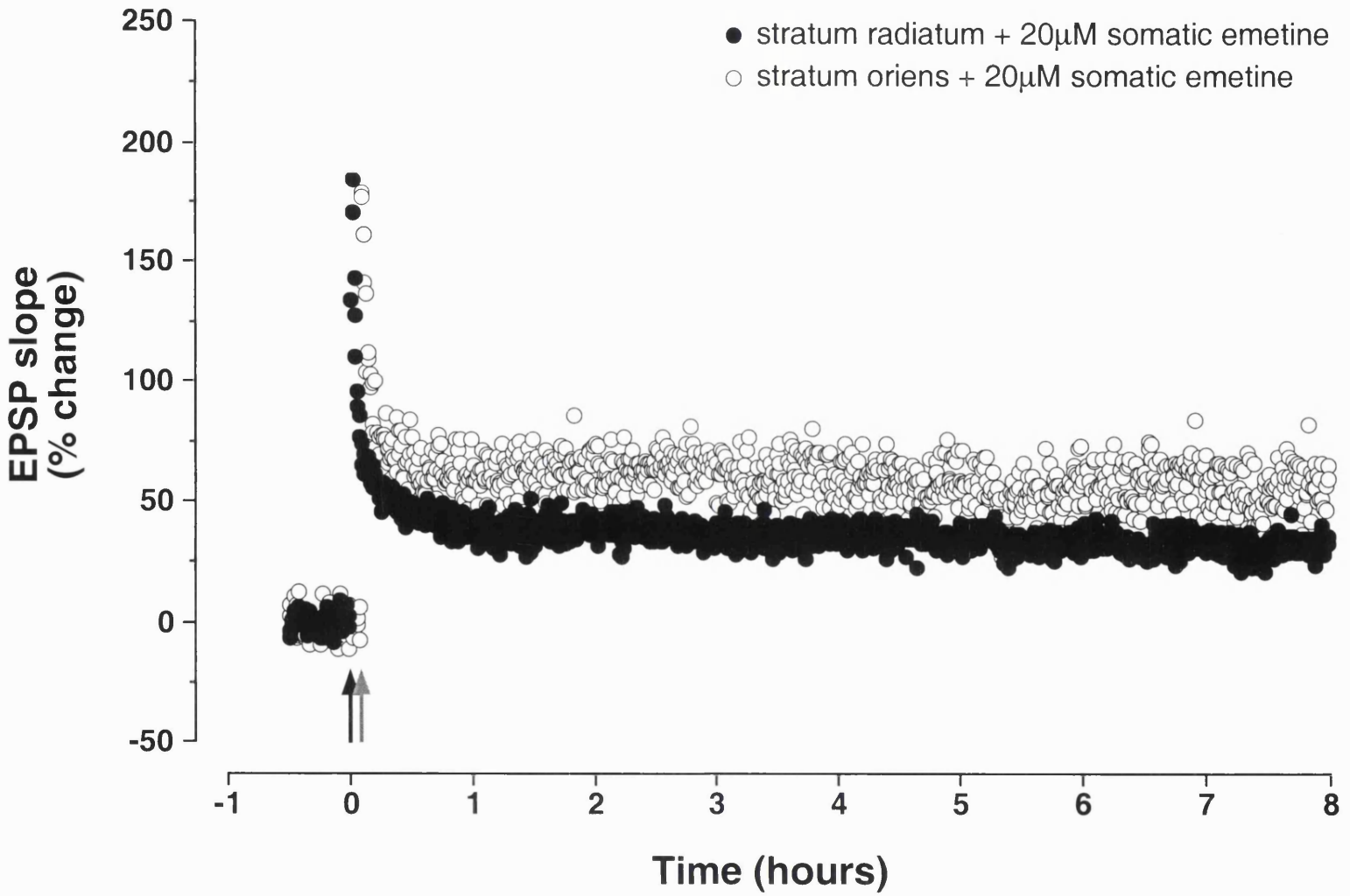

stratum radiatum
+20μM somatic
emetine
(tetanised)
stratum oriens
+20μM somatic
emetine
(tetanised)
1mV
5 ms
10 mins
pre-tetanus
10 mins
post-tetanus
8 hours
post-tetanus

Figure 6.13 Individual example of L-LTP induction in both the stratum radiatum and stratum oriens during focal application of emetine to the cell body region. Electrodes were positioned as shown in figure 6.11. Field EPSP slope, expressed as percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to the first tetanus. Black arrow indicates tetanus applied to the stratum radiatum pathway (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs); grey arrow indicates tetanus applied to the stratum oriens pathway. Emetine (20μM) was focally applied to the cell bodies via a glass microelectrode, at least 30 minutes prior to tetanisation. The emetine filled microelectrode remained present for the duration of the experiment.


stratum radiatum + 20μM somatic emetine
stratum oriens + 20μM somatic emetine
EPSP slope
(% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

pathways exhibit stable afferent volley profiles for at least 8 hours, suggesting good slice health throughout the experiment (Fig. 6.12).

A difference in the magnitude of potentiation between the two pathways, apparent in the individual experiment is not evident in the mean data (Fig. 6.13). In the presence of somatic emetine, EPSP slope values at 8 hours for both the stratum radiatum (36±12%) and stratum oriens (32±15%) are not significantly different (Fig. 6.14a and Fig. 6.15a). Interleaved control experiments also exhibit a robust L-LTP that lasts for the duration of the experiment and is not significantly different between the stratum radiatum and stratum oriens pathways at the 8 hour time point (Fig. 6.14b and Fig. 6.15b). Although the profile of the L-LTP in the drug experiments shows a slight decrease in magnitude at 8 hours when compared to the interleaved controls, this is not significant in either the stratum radiatum or stratum oriens pathways (Fig. 6.15c).

These results suggest that the somatic application of the translation inhibitor emetine, at a concentration known to block protein synthesis, does not effect L-LTP in either the stratum radiatum or stratum oriens over 8 hours.

6.5 L-LTP INDUCTION DURING FOCAL APPLICATION OF EMETINE TO THE STRATUM ORIENS PATHWAY

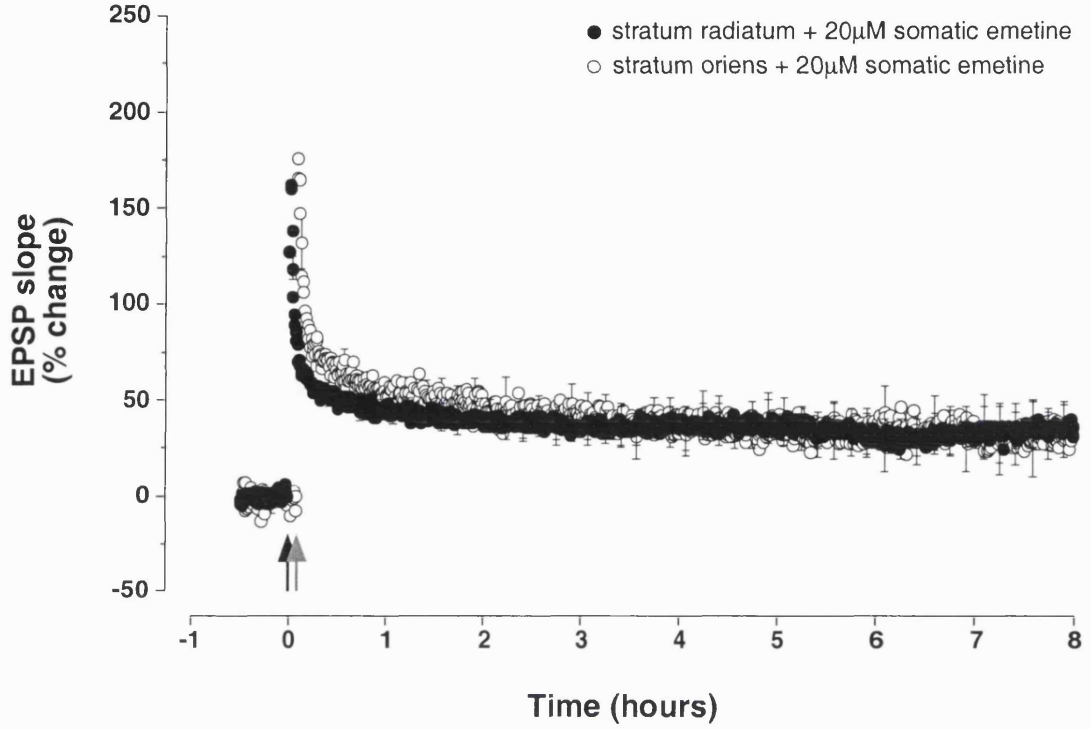
6.5.1 Experimental Design

Electrodes were positioned within the stratum radiatum and the stratum oriens, two spatially distinct areas of the CA1 region (Fig. 6.16). This ensured the sampling of two independent pathways within a largely common cell population (see section 6.2.1 and 6.3.1 for more detail). Using this experimental configuration, the requirement for local dendritic protein synthesis during L-LTP induction in the stratum oriens, was addressed by focally applying the translation inhibitor emetine, specifically to this pathway.

Test stimuli and tetanisation protocols were identical to those outlined in section 6.2.1. The irreversible translation inhibitor, emetine (20μM or 100μM), was focally

Figure 6.14 Induction of L-LTP in the stratum radiatum and stratum oriens is unaffected during focal application of emetine to the cell body region. Electrodes were positioned as shown in figure 6.11. Field EPSP slope, expressed as percentage change over time is shown. **a**, L-LTP induction in both the stratum radiatum and stratum oriens pathways during focal application of emetine (20μM) to the cell body region, at least 30 minutes prior to tetanisation (n=4). **b**, Interleaved control experiments, performed in the absence of emetine (n=5). Zero percent was taken as the mean of the values recorded 10 minutes prior to the first tetanus. Black arrow indicates tetanus applied to the stratum radiatum pathway (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs); grey arrow indicates tetanus applied to the stratum oriens pathway. Error bars indicate ±SEM.

a


● stratum radiatum + 20μM somatic emetine
○ stratum oriens + 20μM somatic emetine
EPSP slope (% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

b

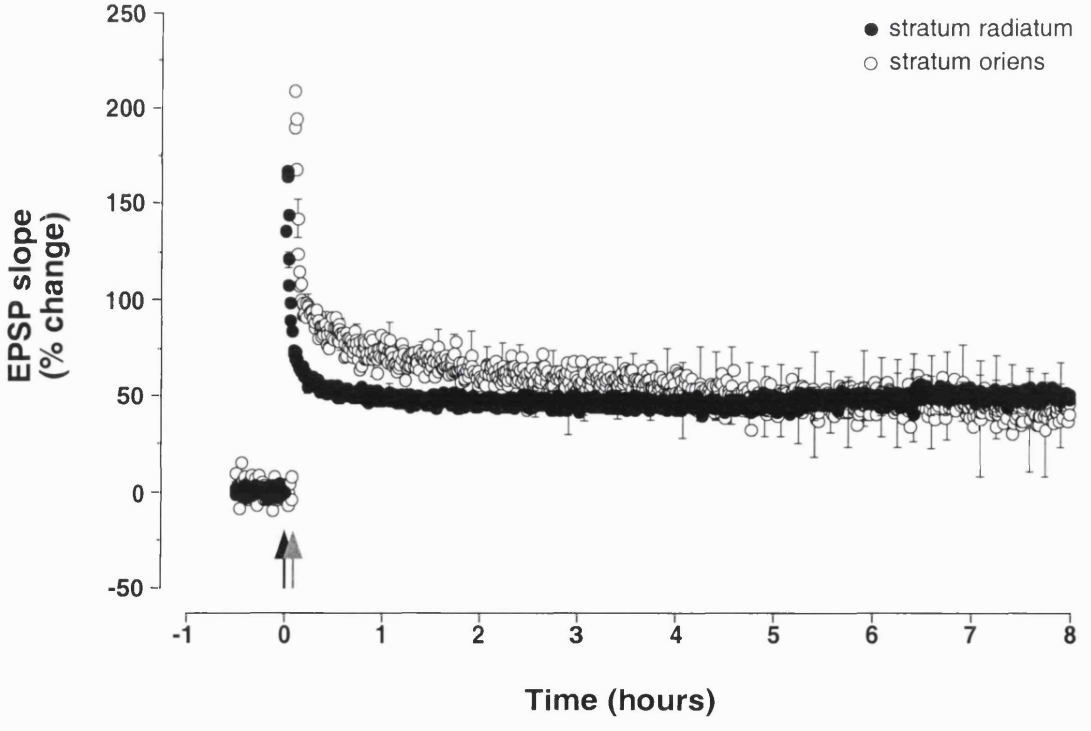
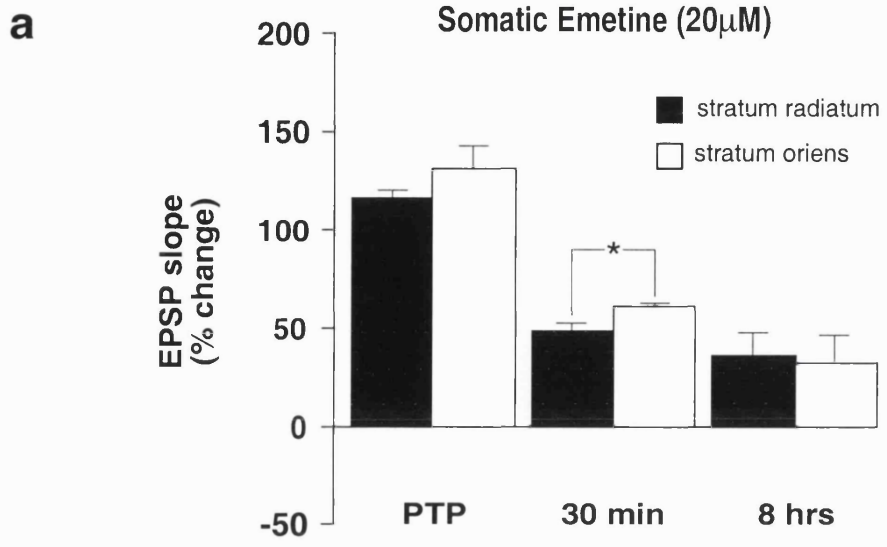
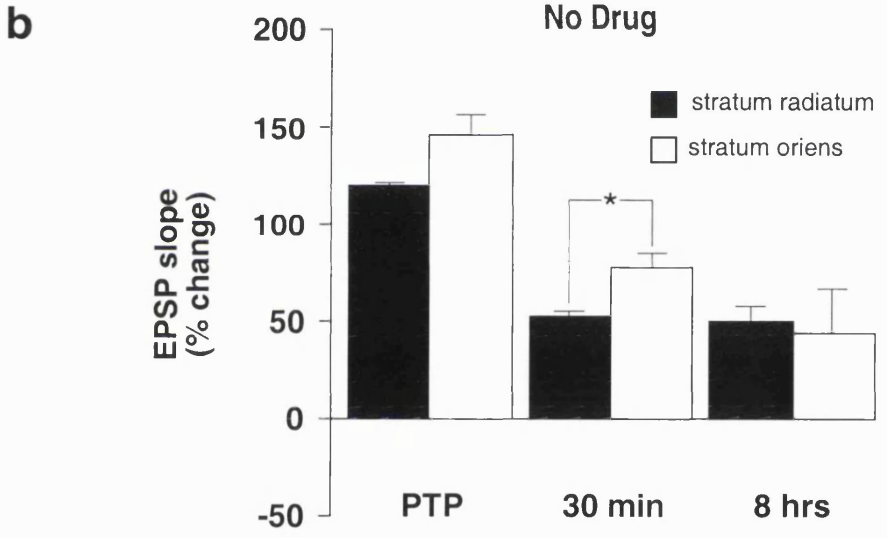

● stratum radiatum
○ stratum oriens
EPSP slope (% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

Figure 6.15 Statistical comparisons during L-LTP induction in both the stratum radiatum and stratum oriens in the presence of emetine applied focally to the cell bodies. a, L-LTP induction in both the stratum radiatum and stratum oriens pathways during focal application of emetine (20μM) to the cell body region, at least 30 minutes prior to tetanisation (n=4). **b**, Interleaved control experiments, performed in the absence of emetine (n=5). **c**, Stratum radiatum and stratum oriens pathways, taken from drug and control experiments shown in a and b. In all cases **PTP** (post-tetanic potentiation) corresponds to the 5 minute time period immediately following tetanisation; **30 min** corresponds to the 10 minute time period between 25-35 minutes post-tetanus; and **8 hrs** corresponds to the 10 minute time period between 7 hours 50 minutes - 8 hours post-tetanus. Values represent the mean EPSP slope measured during each time period. *P<0.05 (unpaired Student t test, two tailed). Error bars indicate ±SEM. Raw data sources are identical to those used for figure 6.14.


a
Somatic Emetine (20μM)
stratum radiatum
stratum oriens
EPSP slope (% change)
200
150
100
50
0
-50
*
PTP
30 min
8 hrs


b
No Drug
stratum radiatum
stratum oriens
EPSP slope (% change)
200
150
100
50
0
-50
*
PTP
30 min
8 hrs

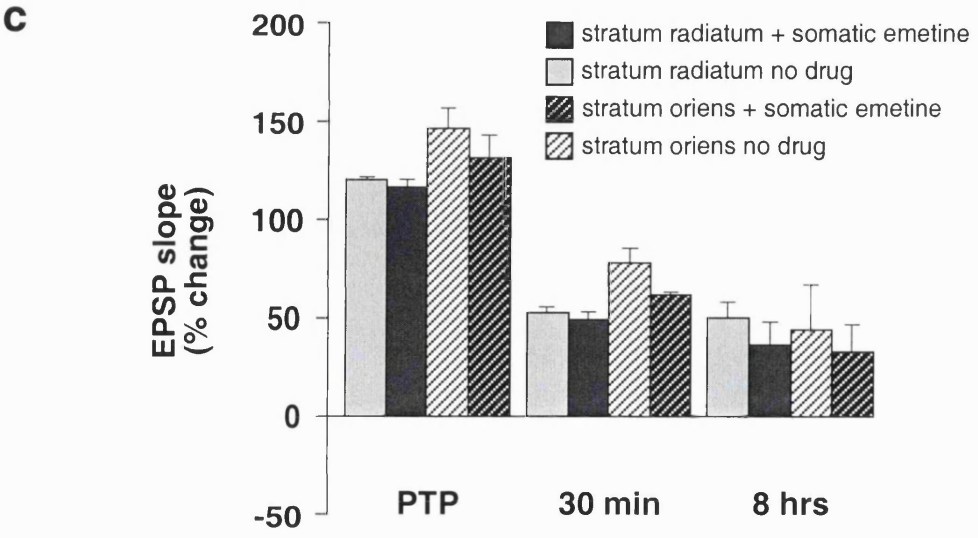
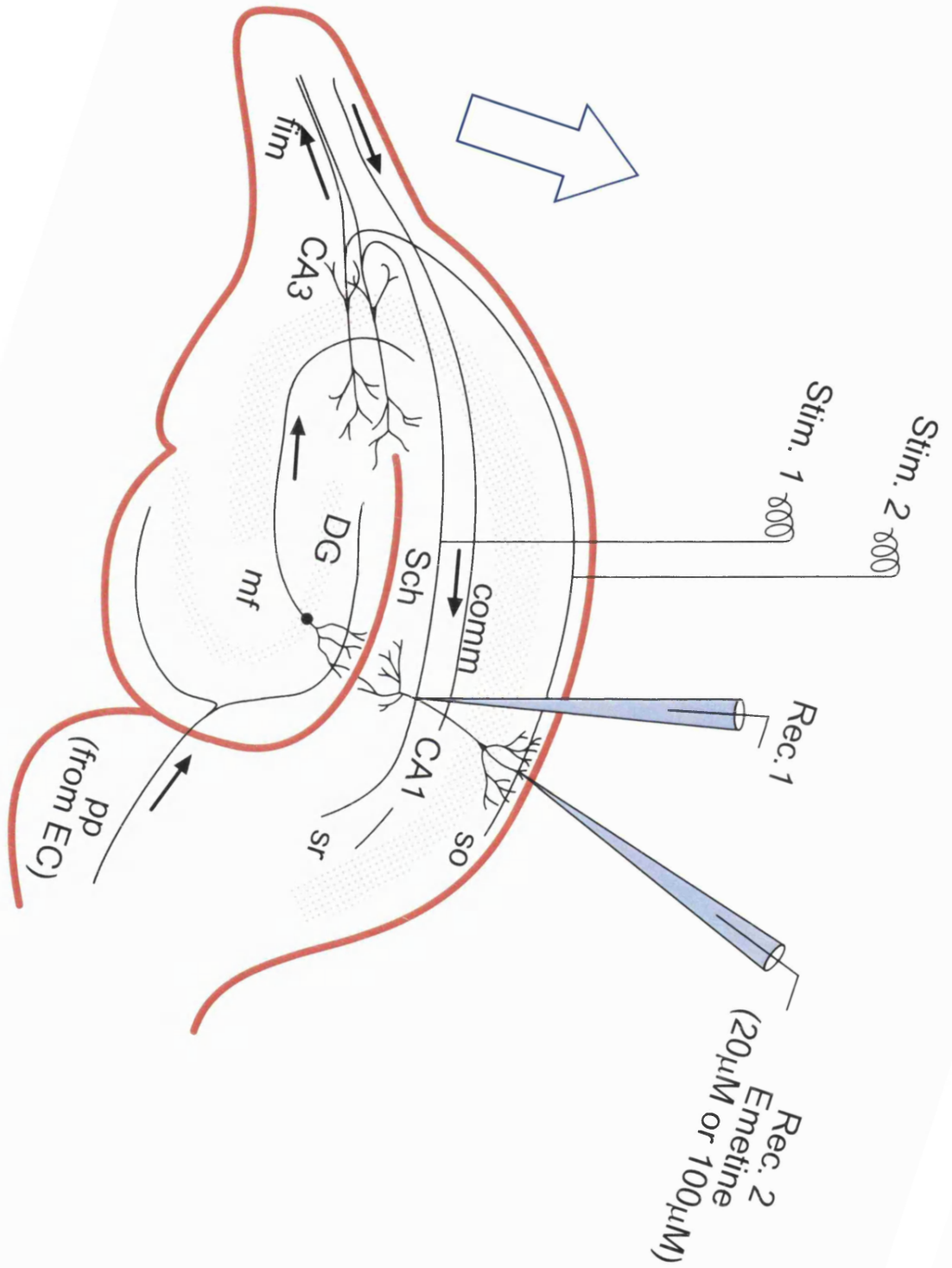

c
stratum radiatum + somatic emetine
stratum radiatum no drug
stratum oriens + somatic emetine
stratum oriens no drug
EPSP slope (% change)
200
150
100
50
0
-50
PTP
30 min
8 hrs

Figure 6.16 Schematic representation of electrode placement within the hippocampal slice during dendritic application of emetine to the stratum oriens region. In both the stratum radiatum (sr) and stratum oriens (so) regions, a single stimulating electrode (Stim.) is placed in the trajectory of the Schaffer collateral/commissural projections, whilst a single recording electrode (Rec.) is positioned in the dendritic region of the CA1 pyramidal cells. The recording electrodes are positioned opposite each other so as to maximise the chances of recording from two spatially separated dendritic regions (and thus independent synaptic populations) in the same population of CA1 pyramidal cells. In addition, the recording electrode in the stratum oriens (Rec.2) was filled with ACSF containing emetine (20μM or 100μM). Open arrow indicates the direction of ACSF flow across the slice. For key to abbreviations, see figure 2.1.


fim
CA3
DG
mf
Sch
comm
CA1
sr
so
pp
(from EC)
Stim. 1
Stim. 2
Rec. 1
Rec. 2
Emetine
(20μM or 100μM)

applied to the dendritic region of the stratum oriens via the glass recording microelectrode, at least 30 minutes prior to tetanisation (see section 2.1.3 and Fig. 6.16). The emetine filled recording electrode remained present for the duration of the experiment. Slices were oriented so that both the cell body and stratum radiatum region were upstream of the drug-containing electrode with respect to the flow of ACSF in the chamber, thereby minimising the spread of emetine to non-target regions (see Fig. 6.16, open arrow).

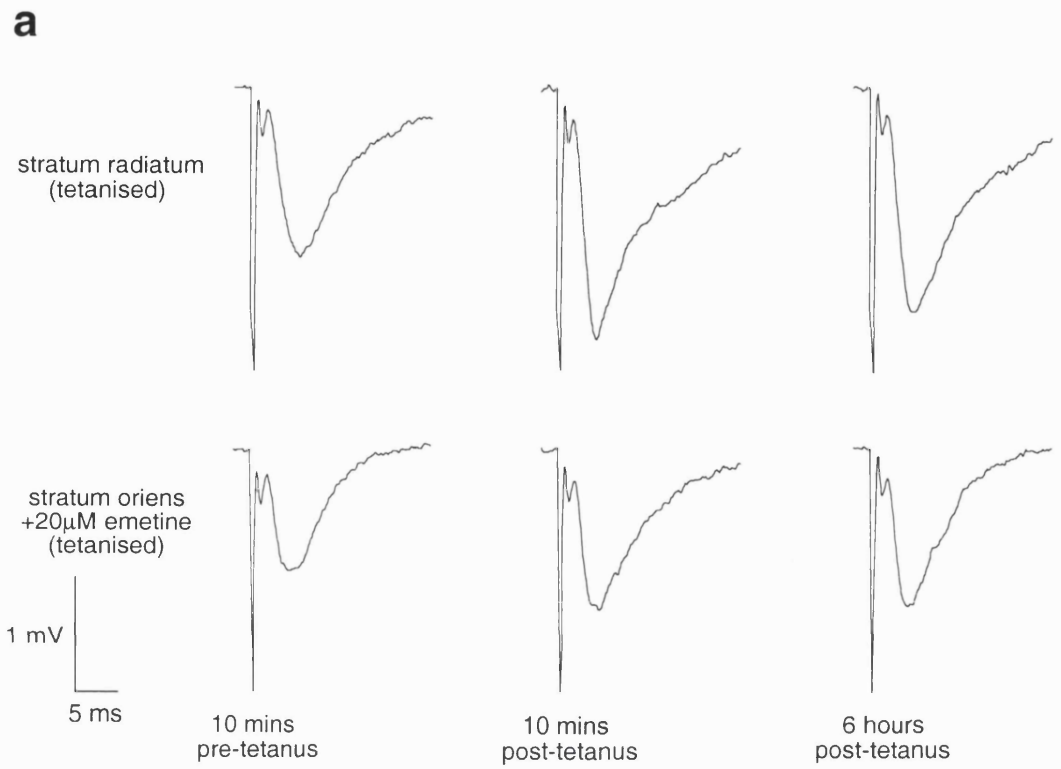
6.5.2 Results

Initial experiments, involving the focal application of 20μM emetine to the dendritic region of the stratum oriens, show no observable change in either pathway when compared to controls. The individual example in figure 6.17 demonstrates that both the stratum oriens (in the presence of 20μM emetine) and radiatum pathways show a similar profile throughout the experiment and exhibit a robust potentiation at the 6 hour time point (35% and 58% respectively; Fig. 6.17b). In addition, there is little change in afferent volley profile in either pathway (Fig. 6.17a).

In an attempt to test the efficacy of the drug, the concentration of emetine in the stratum oriens recording electrode was increased from 20μM to 100μM. A representative example is shown in figure 6.19. The stratum oriens pathway remains completely unaffected throughout, with EPSP slope values declining from an initial 154% to reach a value of 49% after 3 hours, that is maintained up to the 6 hour time point (49%). In contrast, the stratum radiatum pathway, which has not received focal emetine application, declines to 12% over 3 hours, returning to pre-tetanus levels by 6 hours (5%)(Fig. 6.19). Once again, EPSP profiles and afferent volley stability in both pathways, indicates good slice health throughout the experiment (Fig. 6.18).

A reduction in the magnitude of L-LTP in the stratum radiatum is also seen in the preliminary data (n=2), in response to focal application of 100μM emetine to the stratum oriens pathway (Fig. 6.20). EPSP slope values in both pathways are comparable for the first 2 hours following the tetanus, but after this point, potentiation in the stratum oriens remains stable whilst stratum radiatum LTP begins to decline. At

Figure 6.17 Induction of L-LTP in the stratum oriens is unaffected during focal application of emetine (20μM). a, Individual field EPSPs taken from a representative experiment, during focal application of emetine (20μM) to the stratum oriens pathway, at least 30 minutes prior to tetanisation. EPSPs from both the stratum radiatum and stratum oriens pathways are shown at various time points prior to, and following, a tetanus given to each pathway. Potentials are taken from the experiment shown in b. **b**, A representative example of L-LTP induction, during focal application of emetine. Electrodes were positioned as shown in figure 6.16. Field EPSP slope, expressed as percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to the first tetanus. Black arrow indicates tetanus applied to the stratum radiatum pathway (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs); grey arrow indicates tetanus applied to the stratum oriens pathway.


a
stratum radiatum
(tetanised)
stratum oriens
+20μM emetine
(tetanised)
1 mV
5 ms
10 mins
pre-tetanus
10 mins
post-tetanus
6 hours
post-tetanus

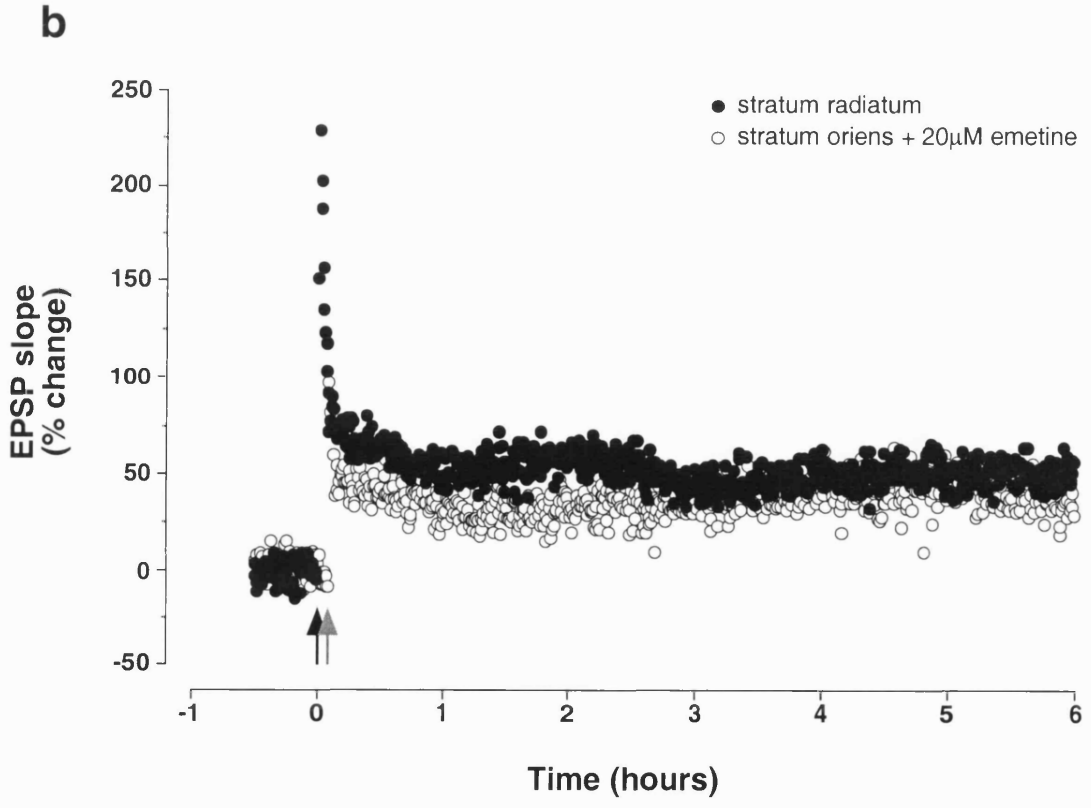

b
stratum radiatum
stratum oriens + 20μM emetine
EPSP slope
(% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
Time (hours)

Figure 6.18 Field EPSPs taken from an individual LTP induction experiment during focal application of emetine (100μM) to the stratum oriens pathway. Individual EPSPs from both the stratum radiatum and stratum oriens are shown at various time points prior to, and following, a tetanus given to each pathway. Emetine (100μM) was focally applied to the stratum oriens via the recording electrode, at least 30 minutes prior to tetanisation. The emetine filled recording electrode remained present for the duration of the experiment. Potentials are taken from the experiment shown in figure 6.19.

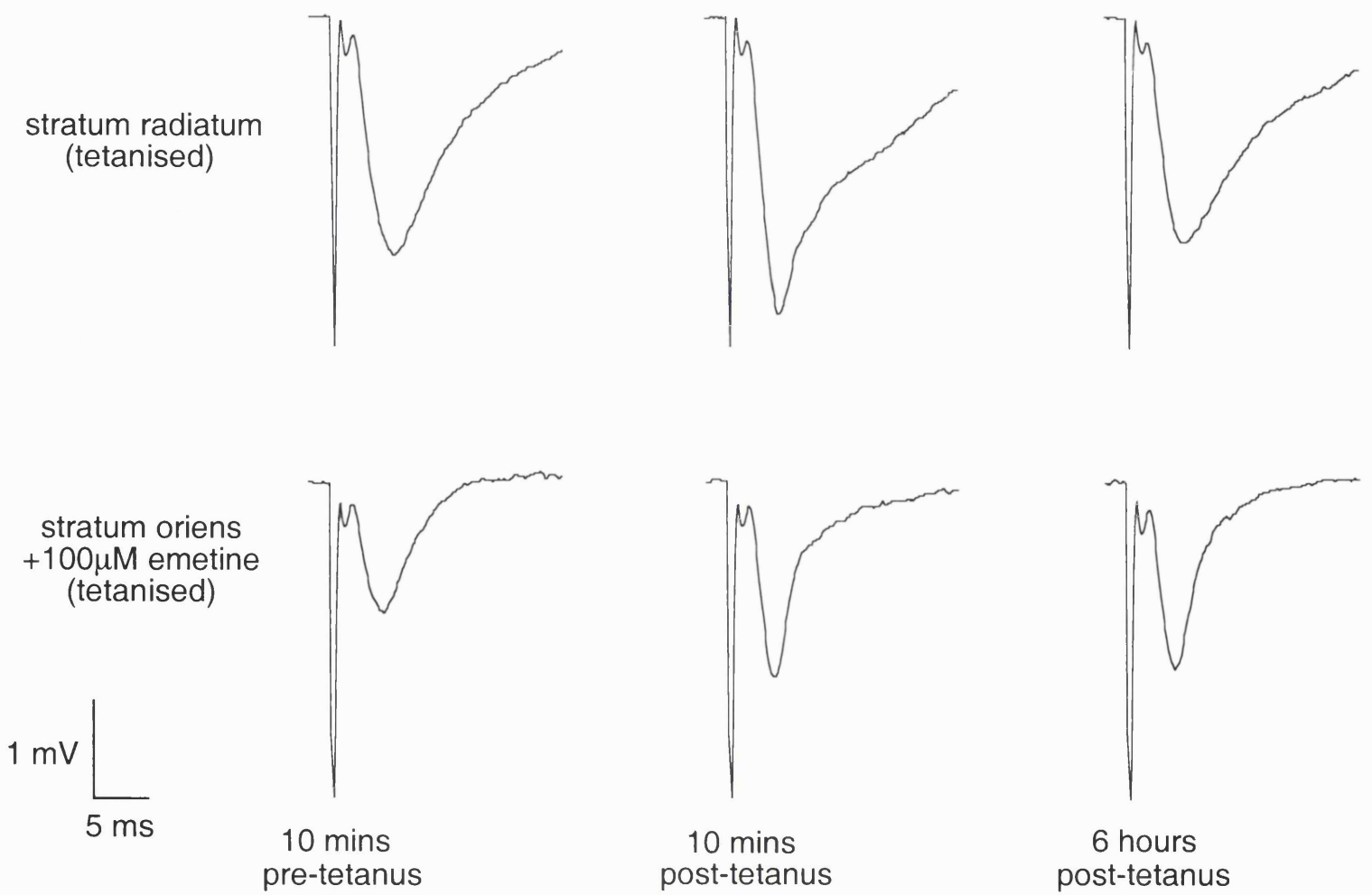

stratum radiatum
(tetanised)
stratum oriens
+100μM emetine
(tetanised)
1 mV
5 ms
10 mins
pre-tetanus
10 mins
post-tetanus
6 hours
post-tetanus

Figure 6.19 Individual example of L-LTP induction block in the stratum radiatum during focal application of emetine (100μM) to the stratum oriens. Electrodes were positioned as shown in figure 6.16. Field EPSP slope, expressed as percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to the first tetanus. Black arrow indicates tetanus applied to the stratum radiatum pathway (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs); grey arrow indicates tetanus applied to the stratum oriens pathway. Emetine (100μM) was focally applied to the stratum oriens via the recording electrode, at least 30 minutes prior to tetanisation. The emetine filled recording electrode remained present for the duration of the experiment.

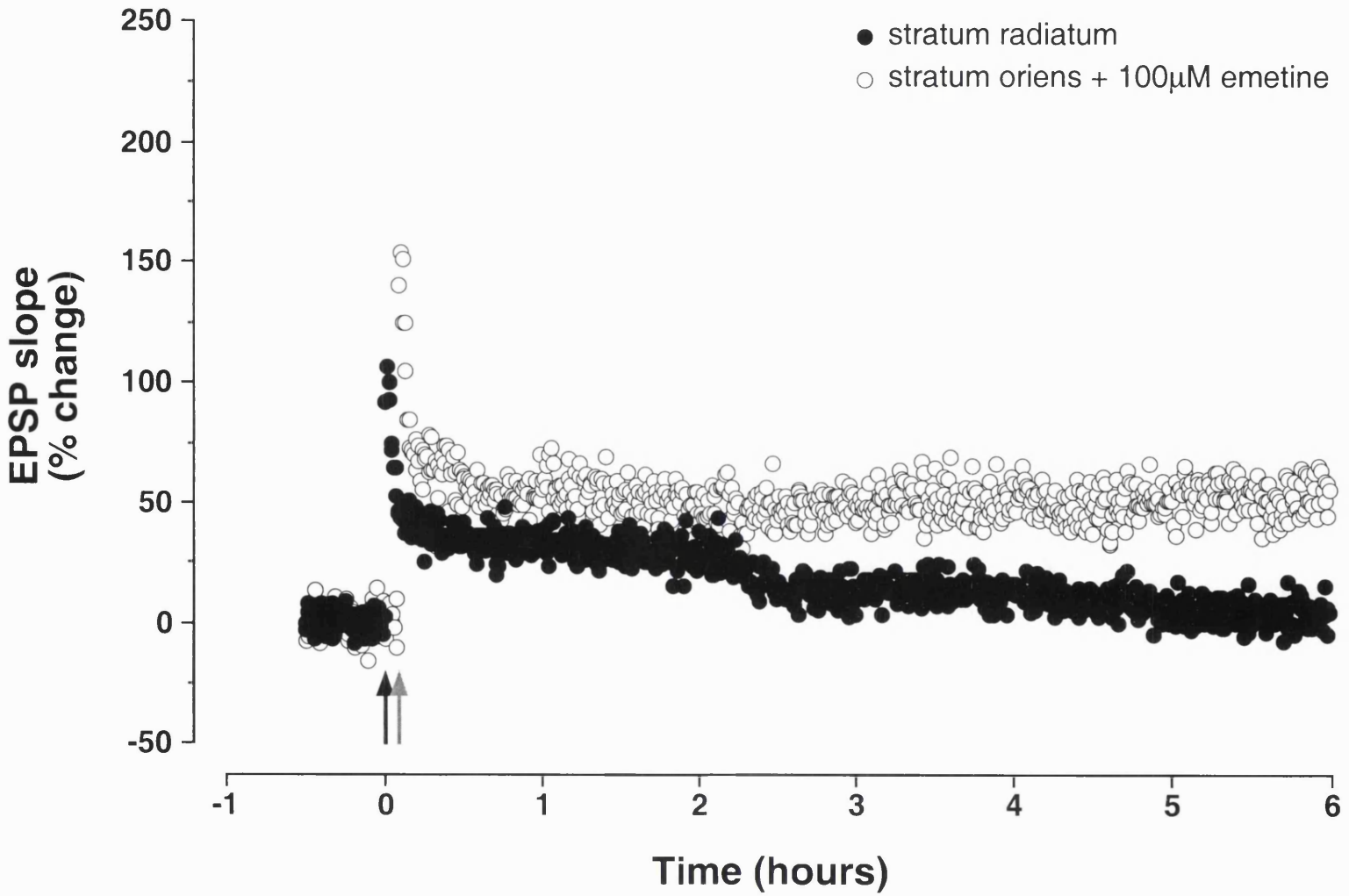
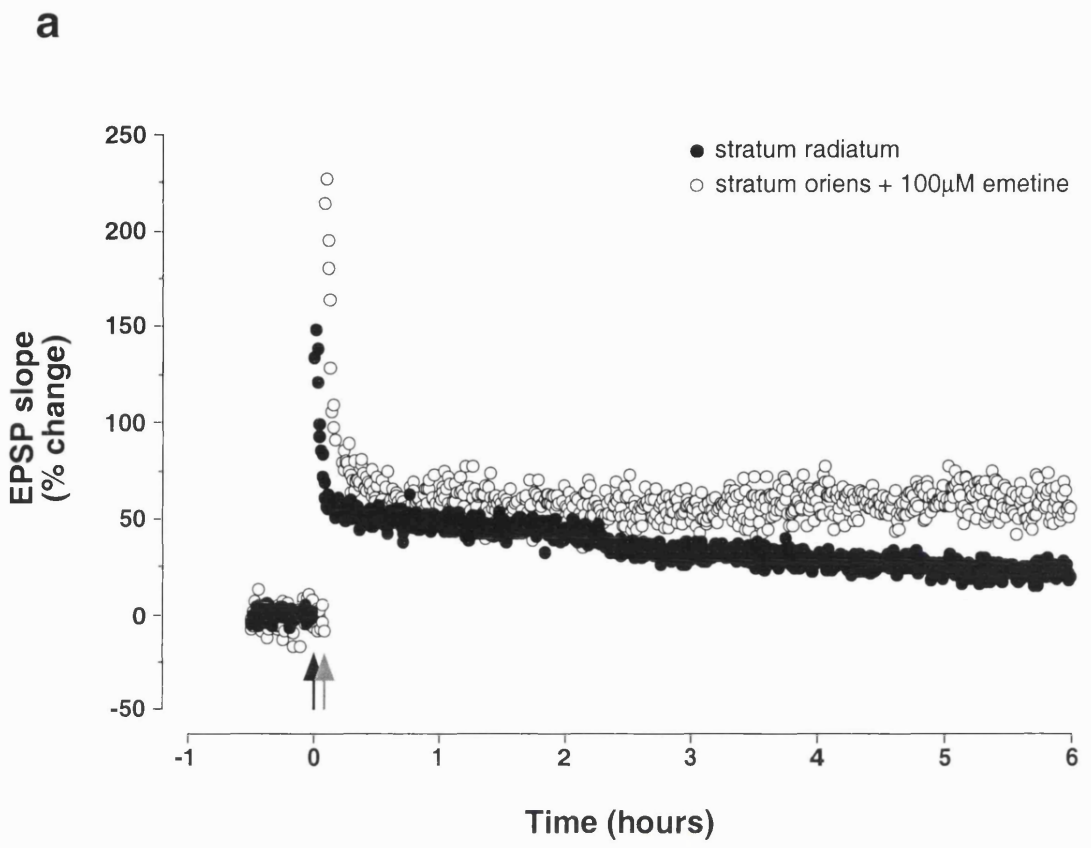
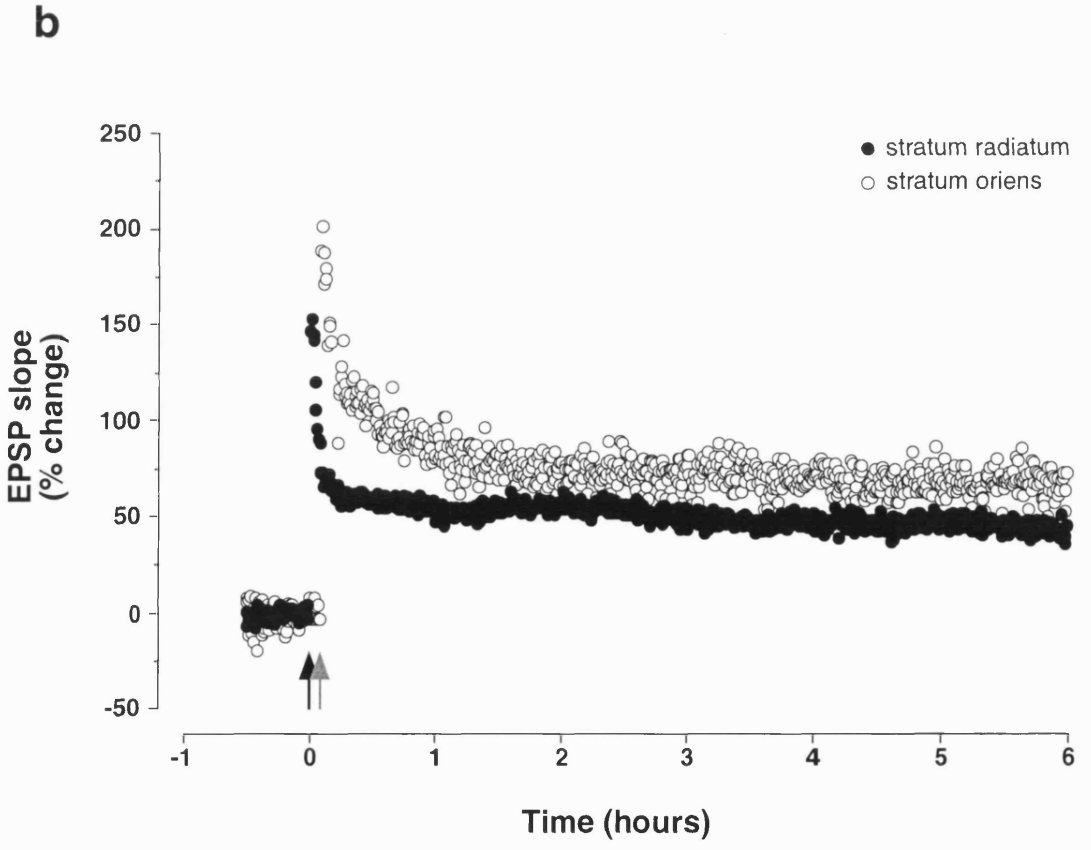

stratum radiatum
stratum oriens + 100μM emetine
EPSP slope
(% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
Time (hours)

Figure 6.20 The magnitude of L-LTP in the stratum radiatum is reduced during focal application of emetine (100μM) to the stratum oriens. Electrodes were positioned as shown in figure 6.16. Field EPSP slope, expressed as percentage change over time is shown. **a**, Reduction in L-LTP magnitude in the stratum radiatum pathway, in response to focal application of emetine (100μM) to the stratum oriens pathway, at least 30 minutes prior to tetanisation (n=2). **b**, Interleaved control experiments, performed in the absence of emetine (n=2). Zero percent was taken as the mean of the values recorded 10 minutes prior to the first tetanus. Black arrow indicates tetanus applied to the stratum radiatum pathway (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs); grey arrow indicates tetanus applied to the stratum oriens pathway.


a
stratum radiatum
stratum oriens + 100μM emetine
EPSP slope (% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
Time (hours)


b
stratum radiatum
stratum oriens
EPSP slope (% change)
250
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
Time (hours)

6 hours post-tetanus the level of potentiation in the stratum radiatum pathway (22%) is less than half that seen in the stratum oriens (57%Fig. 6.20a). Interleaved controls show stable, robust potentiation in both the stratum radiatum and stratum oriens pathways, up to the 6 hour time point (43% and 66% respectively)(Fig. 6.20b)

The preliminary data suggest that focal application of 20μM emetine (a concentration that has been shown to significantly block protein synthesis) has no effect on L-LTP in the stratum oriens pathway. Furthermore, increasing the emetine concentration to 100μM has the effect of dramatically reducing (presumably by drug diffusion) L-LTP in the second, nominally 'control' pathway. Even under these conditions there is still no observable effect on L-LTP in the stratum oriens pathway.

6.6 DISCUSSION

6.6.1 The Role of Local Protein Synthesis in Maintaining L-LTP in the Stratum Radiatum

In this chapter an experimental protocol was devised that would allow the locus of protein synthesis during L-LTP to be addressed. This experimental configuration (discussed in detail in section 6.2.1 and 6.3.1), allowed the sampling of two spatially separate dendritic populations (i.e. stratum radiatum and stratum oriens) within an almost identical population of CA1 pyramidal cells. However, for this arrangement to be useful as a method for addressing the role of local protein synthesis, it was necessary to be able to induce a stable 8 hour L-LTP in the stratum oriens pathway. Although L-LTP in the stratum radiatum has been demonstrated by a number of groups within the last 10 years (see section 1.5.1), it had not been demonstrated that the stratum oriens pathway could also support L-LTP. I have now shown that L-LTP can be produced in the stratum oriens region and that this L-LTP can remain stable and elevated for a least 8 hours. In addition, L-LTP in the stratum oriens was remarkably similar in time course and magnitude to that seen in the stratum radiatum, over the duration of the experiment.

Following optimisation of the experimental configuration, it was possible to ask specific questions concerning the locus of protein synthesis during L-LTP in these two pathways. Focal application of the irreversible translation inhibitor emetine (20μM), specifically to the stratum radiatum pathway, resulted in a ~50% reduction in the magnitude of L-LTP in that pathway at the 8 hour time point, when compared to interleaved controls. Furthermore, L-LTP in the stratum radiatum pathway appeared to still be declining at 8 hours, suggesting that it might have returned to pre-tetanus levels, had the recording period been extended. These data strongly suggest that dendritic protein synthesis is necessary for the induction of L-LTP in the stratum radiatum pathway.

The previous results suggest that local protein synthesis is necessary for L-LTP induction in the stratum radiatum, but is it also sufficient? To address this question, the effects on L-LTP induction, in response to a block of translation in the cell bodies, was investigated. Focal application of emetine (20μM) to the accompanying cell bodies of the sampled neuronal population, caused no significant block of L-LTP, in either the stratum radiatum, or the stratum oriens. This apparent lack of requirement for somatic protein synthesis suggests that in addition to being necessary, dendritic protein synthesis is also sufficient for the induction and maintenance of L-LTP in the stratum radiatum for at least 8 hours.

The data presented in this chapter is the first to provide evidence for a role of local protein synthesis in electrically induced homosynaptic L-LTP, and is consistent with a number of earlier studies that have attempted to address the role of local protein synthesis during heterosynaptic plasticity in hippocampal slices. Kang and Schuman (1996) showed that a protein synthesis dependent form of neurotrophin induced potentiation, in CA1 pyramidal cells, could still occur following excision of either the presynaptic or postsynaptic cell bodies (or both). In addition, long-term depression induced by the mGluR agonist DHPG (a translation dependent process), can also occur in CA1 dendrites that have been separated from their cell bodies (Huber *et al.*, 2000). These results, coupled with the rapidly growing body of data suggesting the occurrence of local translation from dendritically localised mRNA (see section 1.7),

provide compelling evidence for an involvement of local protein synthesis in L-LTP induction in the stratum radiatum.

6.6.2 L-LTP Induction in the Stratum Oriens is Independent of Protein Synthesis

Does the requirement for dendritic protein synthesis exhibited by L-LTP in the stratum radiatum, also occur in the stratum oriens? To investigate this, emetine (20μM) was focally applied to the dendritic region of the stratum oriens, in an identical manner to that used for the previous experiments in the stratum radiatum. However, initial studies showed that focal emetine application did not block L-LTP. Concerns about the efficacy of the drug were controlled for by increasing the concentration of emetine in the microelectrode, from 20μM to 100μM. Preliminary experiments using the increased concentration produced surprising results. Following focal application of emetine (100μM) to the stratum oriens, L-LTP in this pathway remained completely unaffected, whilst L-LTP in the stratum radiatum pathway was greatly reduced. This L-LTP reduction in the stratum radiatum can be explained by diffusion of emetine from the point of application (i.e. stratum oriens). Indeed the profile in the stratum radiatum is similar to that seen in earlier experiments involving focal application of emetine (20μM) to that pathway. Observations in the stratum oriens however, raise a much more interesting possibility. Taken together with the experiments employing somatic application of emetine, these results imply that the stratum oriens is capable of maintaining L-LTP over the course of 8 hours, that is independent of both somatic and dendritic protein synthesis. Although the data is preliminary, making it difficult to draw certain conclusions, the results provide the first evidence of a form of L-LTP that can be induced and maintained over 8 hours, without the need for protein synthesis.

It is not unreasonable to imagine that L-LTP in the stratum oriens could posses different properties of activity-dependent synaptic plasticity from that seen in the stratum radiatum. Indeed, previous reports suggest that there may be two mechanisms for LTP induction on a single CA1 pyramidal cell: a form exhibited at synapses on apical dendrites (stratum radiatum) that is dependent on nitric oxide synthase (NOS) and cGMP, and a form at synapses on basal dendrites that is NOS/cGMP independent

(Haley *et al.*, 1996; Son *et al.*, 1996; Son *et al.*, 1998). These two different types of LTP may reflect different mechanisms of information processing in these two dendritic regions.

LTP in the stratum oriens is NMDA-dependent (Haley *et al.*, 1996), but otherwise its mechanisms remain little studied. Furthermore, although results in this chapter suggest that the maintenance of L-LTP in the stratum oriens is unaffected over the course of 8 hours in the presence of protein synthesis inhibition, they provide no evidence as to the duration of this phenomenon. Can this potentiation be maintained in awake animals for timescales comparable to that seen for LTP in the stratum radiatum and dentate gyrus? Or does its lack of requirement for protein synthesis (at least over 8 hours) indicate that it is merely a temporary increase in efficacy and thus does not undergo the changes necessary for truly long-term maintenance (i.e. >8 hours)? Hopefully, further comparisons of LTP in the stratum radiatum and stratum oriens will enable these questions to be answered.

In conclusion, the data presented in this chapter provides evidence supporting a requirement for dendritic, but not somatic protein synthesis, in the induction of L-LTP in the stratum radiatum. In addition L-LTP induction in the stratum oriens has been demonstrated for the first time. Preliminary evidence suggests that unlike L-LTP in the stratum radiatum, induction and maintenance of stratum oriens L-LTP remains protein synthesis-independent for at least 8 hours.

THE ROLE OF PROTEIN KINASE C IN L-LTP

7.1 IS L-LTP DEPENDENT ON PKC?

Evidence has already been discussed regarding the involvement of a number of protein kinases in the induction of E-LTP (section 1.4.1). This chapter will concentrate on one of these kinases; protein kinase C (PKC) and investigate its involvement in protein synthesis-dependent L-LTP.

A large body of data exists to suggest that increased PKC activity is sufficient to cause synaptic potentiation. Application of phorbol esters, potent activators of PKC, has been shown to cause transient increases in synaptic transmission in the CA1 region of the hippocampus and in some cases to occlude LTP in this region (Malenka *et al.*, 1986; Malenka *et al.*, 1987; Hvalby *et al.*, 1988; Reymann *et al.*, 1988b). Furthermore, postsynaptic intracellular injection of PKC (but not inactivated PKC) causes an enhancement of synaptic transmission, with observed increases in EPSP amplitude and probability of firing (Hu *et al.*, 1987).

In addition to protein kinase activator studies, much of the data indicating a role for PKC in LTP comes from inhibitor studies that have attempted to block LTP. These studies have employed a range of different kinase inhibitors with varying specificity for PKC (i.e. mellitin, polymyxin B, H7, K-252a, K-252b and staurosporine). In the majority of cases, these experiments exhibited a critical time window <15-30 minutes post-tetanus, during which inhibitors must be applied to ensure the block of LTP and a decline in EPSP slope to pre-tetanus levels over 1-3 hours. Inhibitor application after this time window had no effect on LTP (Lovinger *et al.*, 1987; Malinow *et al.*, 1988; Reymann *et al.*, 1988a; Colley *et al.*, 1990; Denny *et al.*, 1990; Muller *et al.*, 1990; Reymann *et al.*, 1990).

However, the findings highlighted above are not consistent across the LTP field. A number of cases have been reported in which application of PKC inhibitors outside this time window have resulted in a block of LTP. Staurosporine has been shown to

cause a pre-established LTP to return to baseline levels following application at 90 minutes post-tetanus (Matthies *et al.*, 1991), whilst H7 inhibited the persistence of LTP, when delivered either 2 or 4 hours after the tetanus (Malinow *et al.*, 1988; Colley *et al.*, 1990). In addition, intracellular injection of a combination of a PKC inhibitor peptide ($PKC_{19\text{-}31}$) and polymyxin B, up to 3 hrs after the tetanus also caused LTP to return to baseline levels (Wang & Feng, 1992). These results, coupled with findings that NMDAR-dependent increases in PKC activation lasting 30-180 minutes have been observed following tetanic LTP induction, suggest that the continued activity of PKC may be required for LTP expression (Klann *et al.*, 1993; Sacktor *et al.*, 1993).

Although a role for PKC in the first 15 minutes of LTP is widely accepted, the conflicting pharmacological data described above makes it difficult to state whether PKC activity is also required during later stages for the continued maintenance of LTP. Indeed, many of the inconsistencies highlighted above may be due to the non-specific effects of the inhibitors used to block PKC (see section 7.4 for more detail).

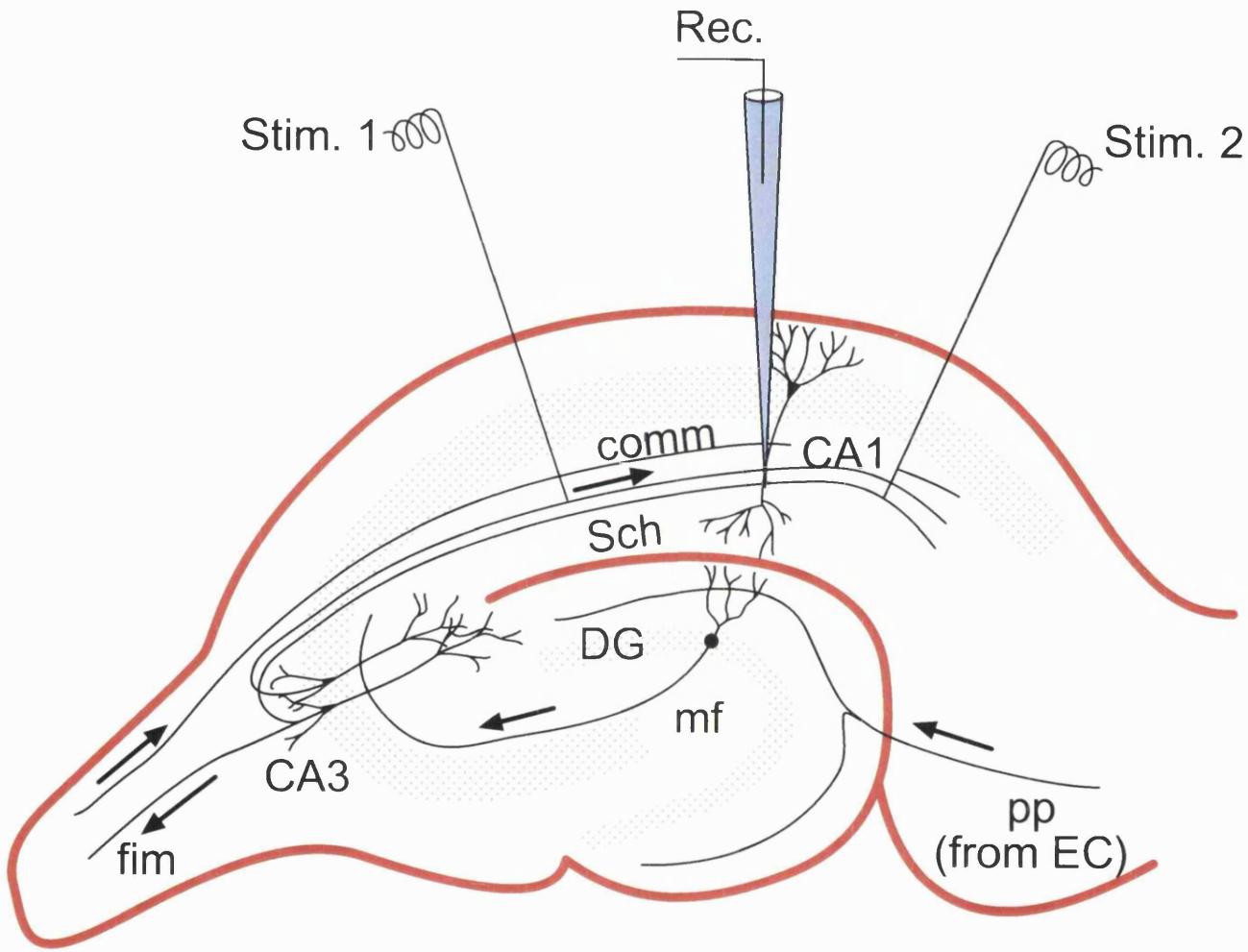
In this chapter, the role of PKC during LTP is further investigated, using the PKC inhibitor bisindolylmaleimide I (BDM I). BDM I is a staurosporine analogue that displays increased selectivity for PKC compared to inhibitors used previously (Toullec *et al.*, 1991; Martiny-Baron *et al.*, 1993). The use of BDM I, coupled with the ability to follow LTP for many hours, allows the role of PKC to be investigated both in the initial induction of LTP and during the induction and maintenance of protein synthesis-dependent L-LTP.

7.2 L-LTP INDUCTION IN THE PRESENCE OF THE PKC INHIBITOR BISINDOLYMALEIMIDE I APPLIED DURING TETANISATION

7.2.1 Experimental Design

Electrode placement within the hippocampal slice is illustrated in figure 7.1. Field EPSPs were recorded from two independent pathways in the stratum radiatum, in response to stimulation via the Schaffer collateral/commissural system. Test stimuli

Figure 7.1 Schematic representation of electrode placement within the CA1 region of the hippocampal slice. Two stimulating electrodes (Stim.) are positioned within the stratum radiatum of the CA1 region, on either side of the recording electrode (Rec.). This maximises the chances of stimulating two populations of convergent but non-overlapping Schaffer collateral/commissural fibres, thereby ensuring the stimulation of independent synaptic populations. For key to abbreviations, see figure 2.1.


Rec.
Stim. 1
Stim. 2
comm
CA1
Sch
DG
mf
CA3
fim
pp
(from EC)

were delivered alternately to each pathway at 15 second intervals and responses were monitored for in excess of 8 hours. Following a 1 hour baseline, a tetanus (20 pulses @ 100Hz repeated 6 times with an ITI of 3 seconds) was applied to one pathway whilst the other provided a non-tetanised control.

The PKC inhibitor, bisindolylmaleimide I (10μM) was bath applied to the hippocampal slices 1 hour prior to tetanisation and remained on the slices for the duration of the experiment.

7.2.2 Results

Bath application of bisindolylmaleimide I during the tetanus blocks the induction of L-LTP in the stratum radiatum of the CA1 region. Data from an individual experiment are shown in figures 7.2 and 7.3. Potentiation in the tetanised pathway gradually declines from a PTP value of 136%, back to baseline levels over the course of 2-3 hours (4% at 3 hours) and maintains this level for the remainder of the experiment (-1% at 8 hours; Fig. 7.3). Although some drift is seen in the non-tetanised pathway, the afferent volley profiles of the potentials in this pathway are relatively unchanged over the duration of the experiment. This suggests that the drift is likely to be due to biological fluctuations rather than electrode movement or poor slice health (Fig. 7.2).

The pooled data display a slightly extended time course for the block of LTP, compared to that seen in the individual experiment illustrated (Fig. 7.4a). Following the tetanus, LTP in the tetanised pathway declines from a PTP value of 90±22%, back to pre-tetanus levels over the course of 3-4 hours (-2±4% at 4 hours post-tetanus). At 8 hours, the level of potentiation in the tetanised pathway (-17±13%) and non-tetanised pathway (-15±4%) is not significantly different (Fig. 7.4b).

Application of the PKC inhibitor bisindolylmaleimide I during tetanisation causes EPSP slope values to return to baseline values over the course of 3-4 hours, resulting in the block of L-LTP induction. In light of these results, a role for persistent PKC

Figure 7.2 Field EPSPs taken from an individual LTP induction experiment in response to bath application of the PKC inhibitor bisindolylmaleimide I during the tetanus. Individual EPSPs from both the tetanised and non-tetanised pathways within the stratum radiatum are shown at various time points, prior to and following the tetanus. Bisindolylmaleimide I (10μM) was added 1 hour prior to tetanisation and remained present for the duration of the experiment. Potentials are taken from the experiment shown in figure 7.3.

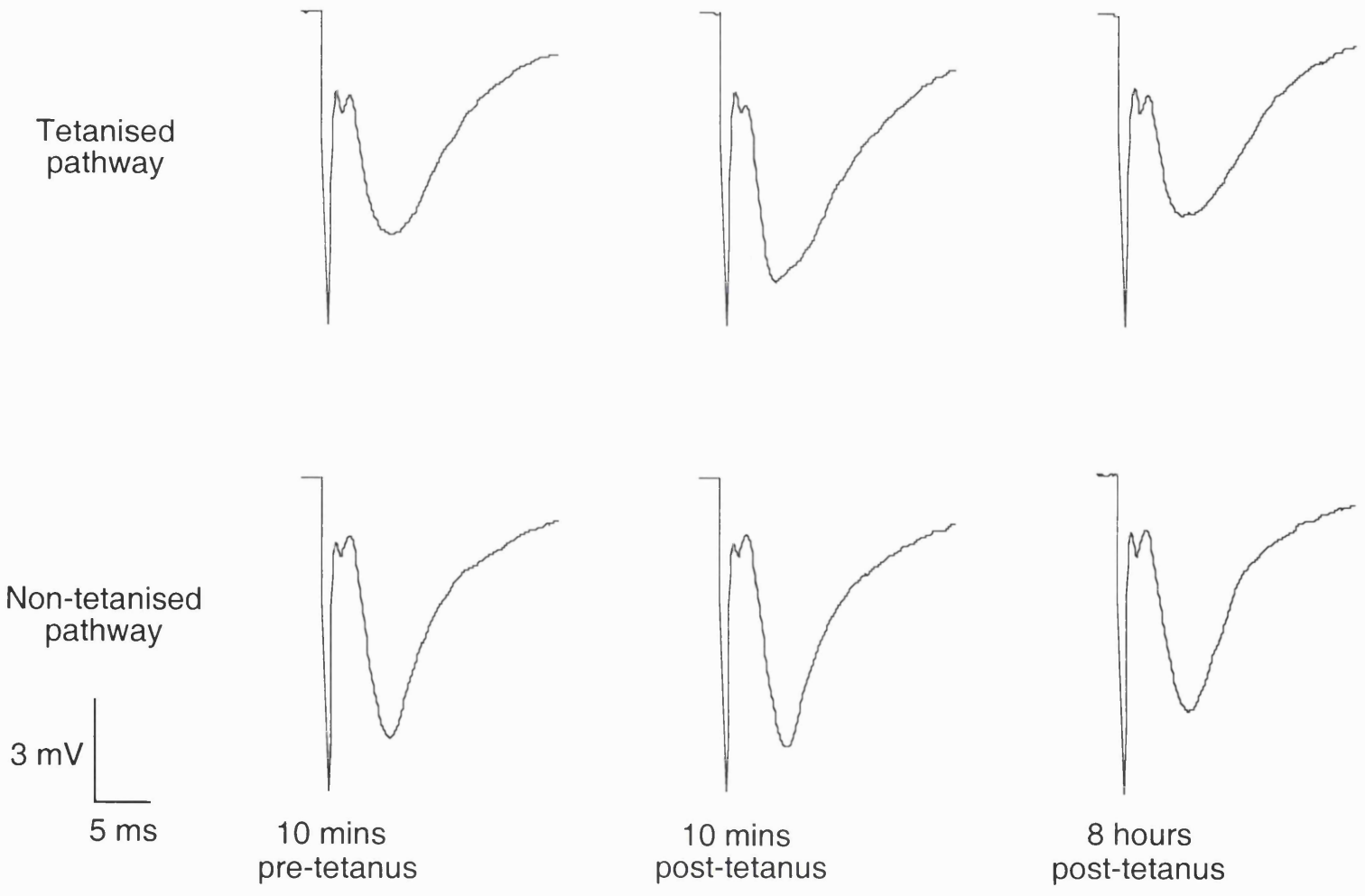

Tetanised pathway
Non-tetanised pathway
3 mV
5 ms
10 mins pre-tetanus
10 mins post-tetanus
8 hours post-tetanus

Figure 7.3 Individual example of L-LTP induction block in response to bath application of the PKC inhibitor bisindolylmaleimide I during the tetanus. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates duration of bisindolylmaleimide I application. Symbols: ● tetanised pathway; **O** non-tetanised pathway.

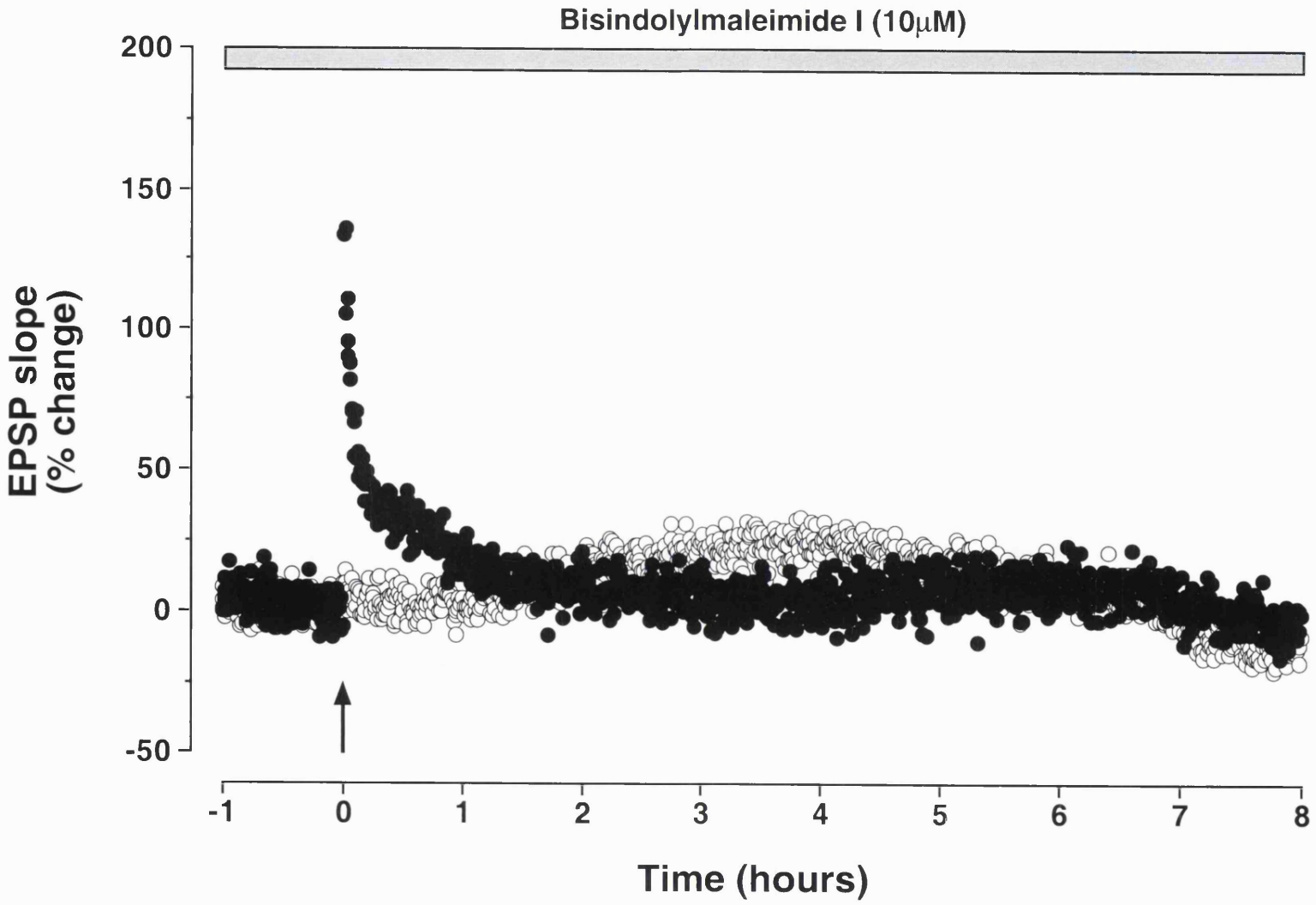
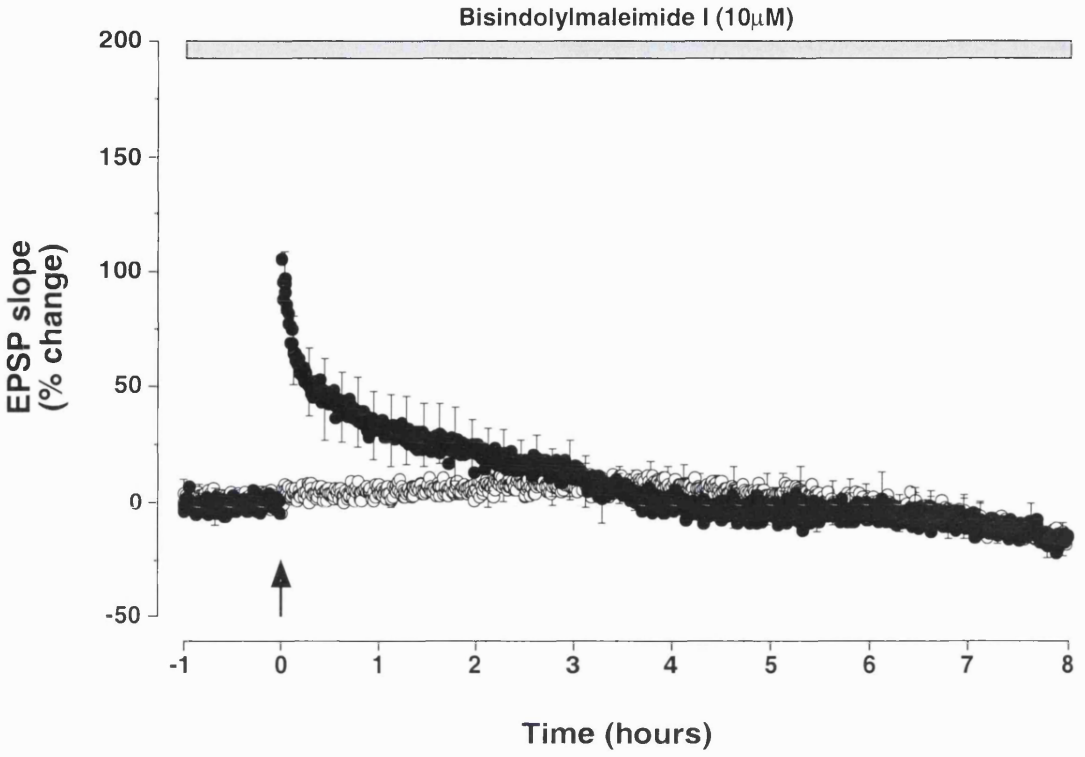
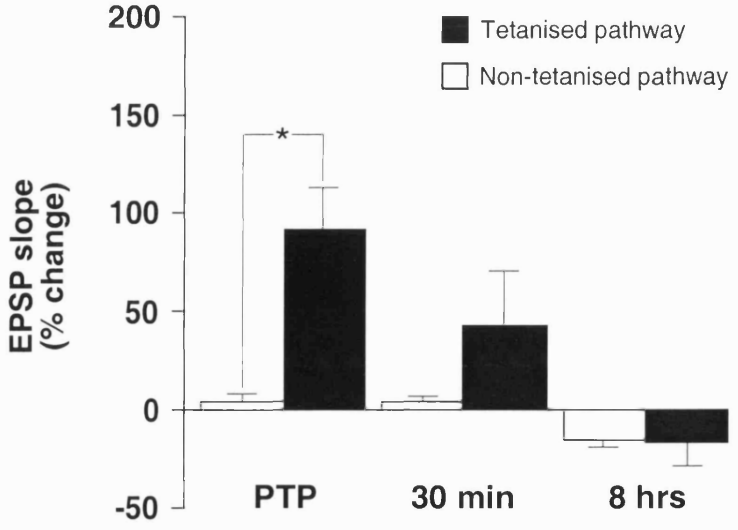

Bisindolylmaleimide I (10μM)
200
150
100
50
0
-50
EPSP slope
(% change)
-1
0
1
2
3
4
5
6
7
8
Time (hours)

Figure 7.4 Induction of L-LTP is blocked in response to bath application of the PKC inhibitor bisindolylmaleimide I during the tetanus. a, Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as percentage change over time is shown. Data shows L-LTP induction block in the presence of bisindolylmaleimide I (10μM), applied 1 hour pre-tetanus (n=3). Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Symbols: ● tetanised pathway; **O** non-tetanised pathway. Error bars indicate ±SEM. **b**, Statistical comparison of tetanised and non-tetanised pathways during L-LTP induction block in the presence of bisindolylmaleimide I (10μM), (n=3). Raw data sources are identical to those used for a. **PTP** (post-tetanic potentiation) corresponds to the 5 minute time period immediately following tetanisation; **30 min** corresponds to the 10 minute time period between 25-35 minutes post-tetanus; and **8 hrs** corresponds to the 10 minute time period between 7 hours 50 minutes - 8 hours post-tetanus. Values represent the mean EPSP slope measured during each time period. *$P<0.05$ (unpaired Student t test, two tailed). Error bars indicate ±SEM.

a


Bisindolylmaleimide I (10μM)
200
150
100
50
0
-50
EPSP slope
(% change)
-1
0
1
2
3
4
5
6
7
8
Time (hours)

b


200
150
100
50
0
-50
EPSP slope
(% change)
Tetanised pathway
Non-tetanised pathway
*
PTP
30 min
8 hrs

activity in LTP was investigated by applying bisindolylmaleimide I, 15 minutes after LTP onset.

7.3 L-LTP INDUCTION IN THE PRESENCE OF THE PKC INHIBITOR BISINDOLYMALEIMIDE I APPLIED 15 MINUTES AFTER TETANISATION

7.3.1 Experimental Design

Electrodes were placed to ensure the stimulation of two independent pathways within the stratum radiatum (Fig. 7.1). Test stimuli and tetanisation protocols were identical to those outlined in section 7.2.1. BDM I was bath applied to the hippocampal slices at a range of concentrations (0.25μM-10μM), 15 minutes after tetanisation and remained on the slices for the duration of the experiment.

7.3.2 Results

Bath application of bisindolylmaleimide I 15 minutes post-tetanus does not block L-LTP in the stratum radiatum of the CA1 region. In the representative experiment shown in figure 7.6, the tetanised pathway exhibits a initial potentiation of 160% that gradually declines to 50% at 4 hours, and maintains this level of potentiation up to the 8 hour time point (48%). Throughout the duration of the experiment the non-tetanised control pathway remains stable, reaching a value of 2% at 8 hours post-tetanus. Once again, afferent volley profiles for both the tetanised and non-tetanised pathways remain relatively unchanged throughout the duration of the experiment (Fig. 7.5).

Figure 7.7 shows pooled data from 10 experiments using concentrations of BDM I in the range of 0.25μM-10μM. No block of L-LTP was seen at any of the concentrations tested and data have been pooled (see figure legend for more detail). The data shown in figure 7.7 exhibit a similar profile to that seen in the individual experiment highlighted above. When applied 15 minutes after tetanisation, BDM I has no effect on the induction or maintenance of L-LTP (Fig. 7.7a). The tetanised pathway exhibits

Figure 7.5 Field EPSPs taken from an individual LTP induction experiment in response to bath application of the PKC inhibitor bisindolylmaleimide I 15 minutes after the tetanus. Individual EPSPs from both the tetanised and non-tetanised pathways within the stratum radiatum are shown at various time points, prior to and following the tetanus. Bisindolylmaleimide I (5μM) was added 15 minutes after tetanisation and remained present for the duration of the experiment. Potentials are taken from the experiment shown in figure 7.6.

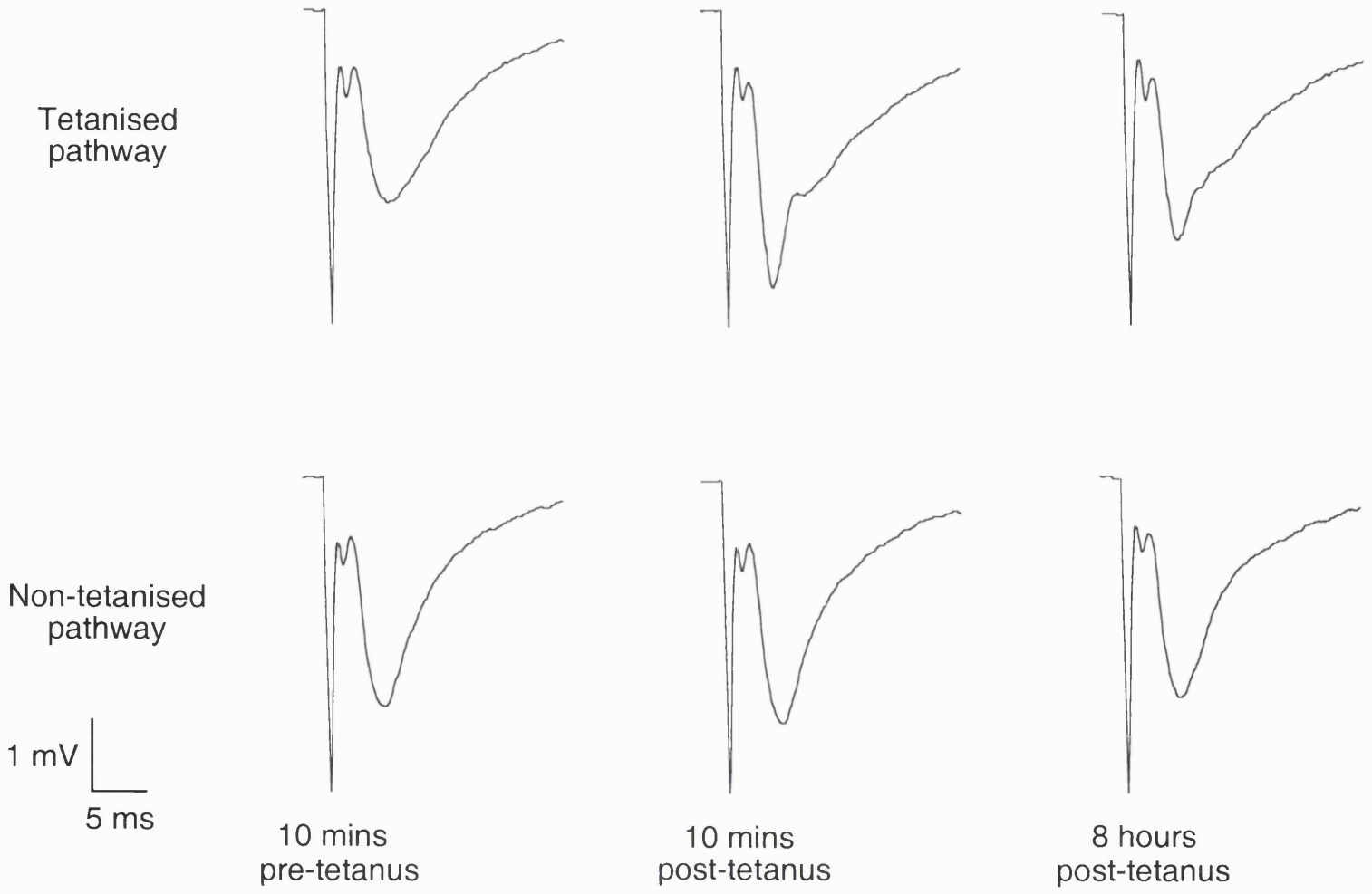

Tetanised pathway
Non-tetanised pathway
1 mV
5 ms
10 mins pre-tetanus
10 mins post-tetanus
8 hours post-tetanus

Figure 7.6 Individual example of L-LTP induction during bath application of the PKC inhibitor bisindolylmaleimide I 15 minutes after the tetanus. Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as percentage change over time is shown. Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Shaded bar indicates duration of bisindolylmaleimide I application. Symbols: ● tetanised pathway; **O** non-tetanised pathway.

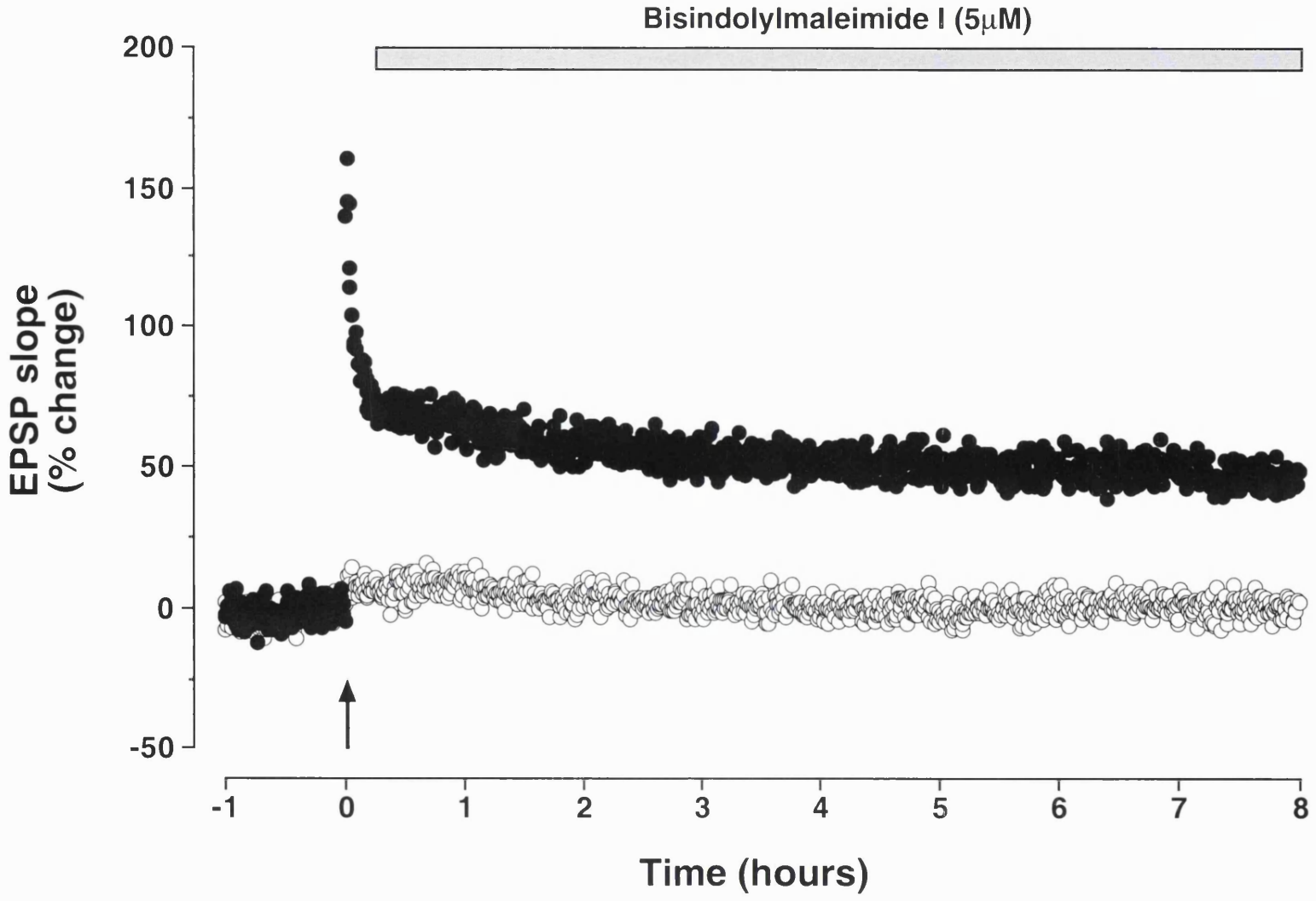
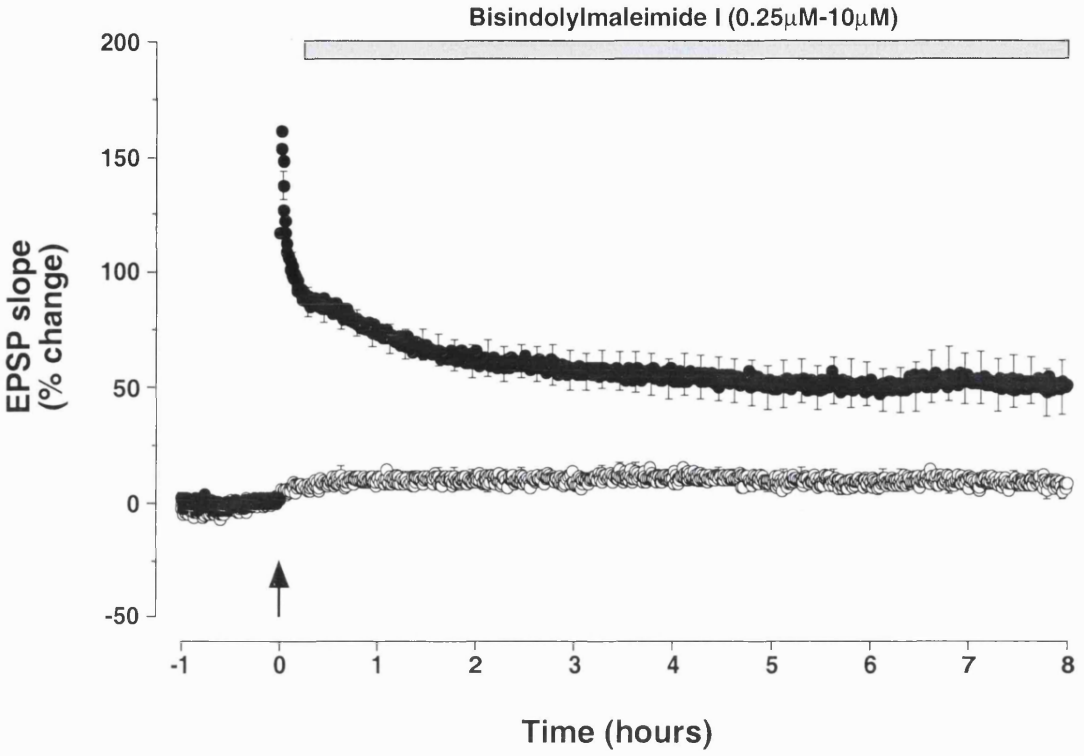
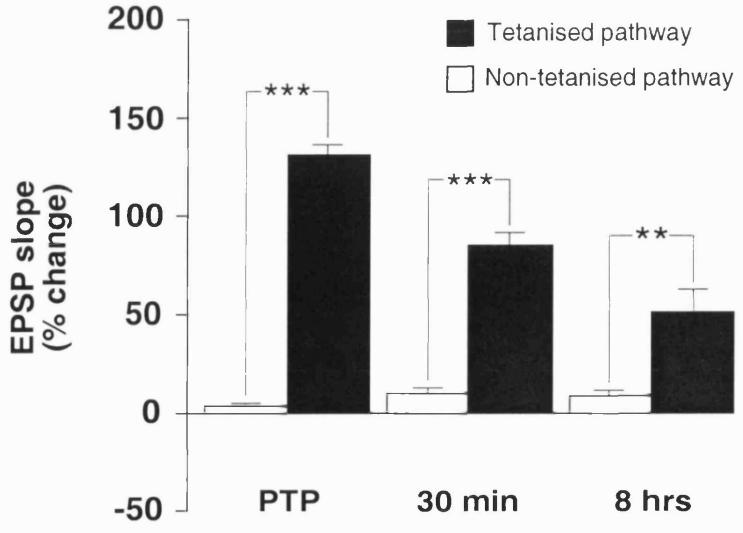

Bisindolylmaleimide I (5μM)
200
150
100
50
0
-50
EPSP slope
(% change)
-1
0
1
2
3
4
5
6
7
8
Time (hours)

Figure 7.7 Induction of L-LTP is unaffected by bath application of the PKC inhibitor bisindolylmaleimide I 15 minutes after the tetanus. **a**, Electrodes were positioned within the stratum radiatum of the CA1 region. Field EPSP slope, expressed as percentage change over time is shown. Data shows L-LTP induction in the presence of bisindolylmaleimide I (0.25μM-10μM), applied 15 minutes post-tetanus (n=10: n=4, 0.25μM; n=1, 0.5μM; n=1, 1μM; n=1, 2.5μM; n=2, 5μM; n=1, 10μM). Zero percent was taken as the mean of the values recorded 10 minutes prior to tetanisation. Arrow indicates tetanus (Tet: 6 trains 20 pulses @ 100 Hz ITI 3 secs). Symbols: ● tetanised pathway; O non-tetanised pathway. Error bars indicate ±SEM. **b**, Statistical comparison of tetanised and non-tetanised pathways during L-LTP induction in the presence of bisindolylmaleimide I (0.25μM-10μM), (n=10). Raw data sources are identical to those used for a. **PTP** (post-tetanic potentiation) corresponds to the 5 minute time period immediately following tetanisation; **30 min** corresponds to the 10 minute time period between 25-35 minutes post-tetanus; and **8 hrs** corresponds to the 10 minute time period between 7 hours 50 minutes - 8 hours post-tetanus. Values represent the mean EPSP slope measured during each time period. ***P<0.001, **P<0.01, (unpaired Student t test, two tailed). Error bars indicate ±SEM.

a


Bisindolylmaleimide I (0.25μM-10μM)
EPSP slope (% change)
200
150
100
50
0
-50
-1
0
1
2
3
4
5
6
7
8
Time (hours)

b


EPSP slope (% change)
200
150
100
50
0
-50
Tetanised pathway
Non-tetanised pathway
***
***
**
PTP
30 min
8 hrs

robust LTP that is maintained throughout the experiment; the difference at 8 hours between the tetanised (51±12%) and non-tetanised (8±4%) pathways, remains significant (t=3.4886, P≤0.0026)(Fig. 7.7b).

7.4 DISCUSSION

7.4.1 L-LTP is Dependent on a Critical Time Window of PKC Activity

In this chapter the protein kinase inhibitor bisindolylmaleimide I (BDM I), was employed to investigate the role of PKC in the induction and maintenance of L-LTP. BDM I was selected because it displays an increased selectivity for PKC compared with other kinase inhibitors used previously (Toullec *et al.*, 1991; Martiny-Baron *et al.*, 1993). Application of BDM I during the tetanus resulted in the block of both E-LTP and L-LTP induction, with EPSP slope values returning to pre-tetanus baseline levels over the course of 3-4 hours. This profile is indicative of a protein kinase independent short-term potentiation (see section 1.4.1 for more detail). These data suggest that although PKC activation during the tetanus does not appear to be necessary for the induction of short-term potentiation, it is required for the occurrence of the protein kinase-dependent E-LTP and the subsequent induction of protein synthesis-dependent L-LTP. Previous studies where LTP was followed for shorter timescales (typically 1-3 hours) show that application during the tetanus of a range of protein kinase inhibitors displaying varying degrees of selectivity for PKC, produce a decay of LTP back to pre-tetanus levels over the course of 1-3 hours (Lovinger *et al.*, 1987; Malinow *et al.*, 1988; Reymann *et al.*, 1988a; Malinow *et al.*, 1989; Colley *et al.*, 1990; Denny *et al.*, 1990; Muller *et al.*, 1990; Reymann *et al.*, 1990; Matthies *et al.*, 1991; Wang & Feng, 1992). In this study, BDM I application during the tetanus caused LTP to decline back to baseline levels over 3-4 hours, a timescale slightly longer than previously reported. This may be due to the fact that the slice health was optimised for the induction of L-LTP thereby rendering the tissue better able to tolerate a block in PKC activity for longer.

In addition to a requirement for PKC activity during the time of the tetanus, do E-LTP and L-LTP also display a requirement for persistent PKC activation after this initial

induction? (i.e. during LTP expression and maintenance). In this study, BDM I application to hippocampal slices 15 minutes after tetanisation, produced no discernible effect on E-LTP or L-LTP induction, resulting in the maintenance of a robust potentiation for at least 8 hours. This result is in agreement with the majority of previous studies, demonstrating that PKC activity required for LTP maintenance takes place only within a small time window, typically less than 15-30 minutes after tetanisation. Application of PKC inhibitors outside this time window does not affect LTP maintenance (Lovinger *et al.*, 1987; Malinow *et al.*, 1988; Reymann *et al.*, 1988a; Colley *et al.*, 1990; Denny *et al.*, 1990; Muller *et al.*, 1990; Reymann *et al.*, 1990). Because of the extended timescale used in these experiments, possible requirements for PKC activity in the later stages of LTP could also be investigated. During experiments in which BDM I application occurred 15 minutes post-tetanus, the slices were continually bathed in this drug for the remainder of the experiment. The fact that robust LTP was maintained for at least 8 hours under these conditions suggests that no further requirement for PKC activation occurs throughout the transition from E-LTP to L-LTP and during the subsequent maintenance of the late-phase over this time period.

The majority of the data within the field support the idea that no requirement for PKC activity occurs after 30 minutes post-tetanus. However, early experiments by Malinow *et al.* (1988) reported that the protein kinase inhibitor H7 could cause a reversible block on pre-established LTP when applied up to 2 hours after tetanisation, suggesting that LTP was maintained over this time period by persistently active PKC. Other studies have reported a similar effect in response to the application of a number of different PKC inhibitors (notably H7 and staurosporine), between 90 minutes and 4 hours after tetanisation (Malinow *et al.*, 1989; Colley *et al.*, 1990; Matthies *et al.*, 1991; Wang & Feng, 1992)(see section 7.1 for more detail).

The block of LTP in response to PKC inhibitor application outside the critical time window may be due to the non-specific effects of the inhibitors used in these studies. The most dramatic block on pre-established LTP was seen in response to application of either H7 or staurosporine. Both of these drugs block PKC activity via competitive inhibition for the ATP binding site on the PKC catalytic domain. However, the ATP

binding site of PKC exhibits a high level of homology with the ATP binding sites of other serine-threonine kinases, making it difficult to achieve a high degree of specificity using this inhibitory mechanism (Ruegg and Burgess, 1989; Hidaka and Kobayashi, 1992). Although the inhibitor H7 is often referred to as a protein kinase C inhibitor, it displays little, if any selectivity for PKC over a number of other protein kinases (IC_{50} values: PKA 3μM, PKG 5.8μM, PKC 6μM, MLC 97μM). The same is true for staurosporine (IC_{50} values: PKC 0.7nM, MLC 0.13nM, PKA 7nM, PKG 8.5nM). (Tamaoki *et al.*, 1986; Schachtele *et al.*, 1988; Ruegg & Burgess, 1989; Boulis and Davis, 1990; Takahashi *et al.*, 1990; Tischler *et al.*, 1990). Because the kinase inhibitor used in this study (BDM I) displays a greatly improved degree of selectivity for PKC when compared with H7 and staurosporine (BDM I, IC_{50} values: Mixed Rat Brain PKC 0.031μM, phosphorylase kinase 0.7μM, PKA 2μM)(Toullec *et al.*, 1991), unwanted side effects arising from non-specific actions of the drug were minimised. This may be the reason why a block of L-LTP outside the critical time window was not observed.

Results presented in this chapter, taken together with those published previously, suggest that PKC activity occurring within 30 minutes following LTP induction, is necessary for the induction of protein synthesis-dependent L-LTP. Is PKC therefore involved in the control of mRNA translation? Preliminary evidence suggests that this may be the case. Rapid increases in the size of synaptosomal polyribosomal aggregates have been seen in response to K^+ depolarisation or glutamate application (Weiler & Greenough, 1993). This apparent increase in synaptic translation appeared in part to be regulated via metabotropic glutamate receptor activation coupled to the phosphatidylinositol second messenger system (of which PKC is a component). Interestingly, experiments performed in rat hippocampal slices reported a rapid PKC-dependent translocation of the serine threonine kinase p90rsk to polyribosomes, following application of the mGluR agonist DHPG (Angenstein *et al.*, 1998). One of the substrates for p90rsk is glycogen synthase kinase 3β (gsk-3β), an enzyme involved in the regulation of translation efficiency (Angenstein *et al.*, 1998). Taken together, these results suggest a possible role for PKC in the regulation of synaptic activity-dependent translation.

Consistent with the above results, recent findings suggest that mGluR/PKC-mediated protein synthesis may also occur during LTP in the CA1 region of the hippocampus. Raymond *et al.* (2000) demonstrated that pharmacological or synaptic stimulation of group I mGluRs prior to a weak tetanic stimulation, could facilitate the production of an intermediate, protein synthesis-dependent, transcription-independent phase of LTP, via a mechanism dependent on PKC. LTP priming, via synaptic stimulation was input specific and, taken together with the rapidity of the priming effect (<20min), suggested that at least part of the protein synthesis responsible for this intermediate LTP occurred in the dendrites, close to the synaptically activated mGluRs (Raymond *et al.*, 2000). Recently, pharmacological mGluR activation has been shown to cause transfected mRNA for the ionotropic glutamate receptor GluR2, to become translated and incorporated into the membrane of isolated hippocampal neuronal dendrites (Kacharmina *et al.*, 2000). Finally, the mGluR antagonist AP3 blocks L-LTP induction in the CA1 region of hippocampal slices (Behnisch *et al.*, 1991). Taken together, these results provide strong evidence to suggest that an mGluR/PKC dependent mechanism could regulate dendritic protein synthesis from pre-existing mRNA in L-LTP.

In addition to a role in translation, PKC may also be involved in the regulation of transcription. Recently, Roberson *et al.* (1999) implicated PKC in the regulation of hippocampal CREB phosphorylation via the MAPK cascade. This result suggests that PKC may be involved in CREB-mediated alterations in gene expression. However, whether this PKC dependent process occurs in L-LTP induction is not yet known.

In conclusion, the data presented in this chapter provide evidence to suggest that L-LTP induction is dependent on PKC activity occurring exclusively within the first 15 minutes after tetanisation. To date, the role of PKC in L-LTP induction remains unclear. Preliminary data suggest that it may be involved in regulating dendritic translation via a mechanism dependent on mGluR activation. In addition, a role for PKC in CREB-mediated gene expression has also been proposed. These data, taken together with earlier literature suggest that protein kinase C may perform a dual role in LTP, both in the protein kinase dependent early-phase and in the macromolecular synthesis dependent late-phase.

GENERAL DISCUSSION

8.1 INDUCTION AND MAINTENANCE OF L-LTP

A large part of this study has involved the optimisation of an experimental configuration that was capable of maintaining physiologically stable, acute hippocampal slices for extended periods of time. Such a system is necessary for the investigation of the macromolecular synthesis-dependent phase of LTP. The technical difficulties encountered, and the methods developed to (partially) overcome them, have been highlighted in chapters 2 and 3. Although the induction and subsequent recording of L-LTP for periods of greater than 8 hours is not easily attainable, when it is achieved, the results are both reliable and reproducible. Due to the difficult nature of these experiments, the L-LTP field and associated literature is small and plagued with controversy. Many of the issues addressed in this project fall within this realm and the discrepancies in the field are highlighted where appropriate.

8.2 THE NATURE OF THE PROTEIN SYNTHESIS IN L-LTP

A requirement for protein synthesis in hippocampal L-LTP has been consistently shown both *in vivo* and *in vitro* (see section 1.5.1). An attempt to demonstrate a requirement for protein synthesis in L-LTP using anisomycin proved problematic in this study. Application of emetine however, was found to block L-LTP in the hippocampal CA1 region, thus confirming that protein synthesis is required for the successful induction and/or maintenance of L-LTP.

LTP in the CA1 region of hippocampal slices is not unique in its requirement for protein synthesis. L-LTP at both the mossy fibres and the medial perforant pathway is also blocked by application of translation inhibitors (Huang *et al.*, 1994; Nguyen & Kandel, 1996). Recently the dependence of LTP on protein synthesis in other brain structures has been investigated. L-LTP in the lateral amygdala (LA) of rat brain slices *in vitro* has been shown to be blocked by application of either anisomycin or

emetine during tetanisation (Huang *et al.*, 2000). Interestingly, intra-LA infusion of anisomycin also blocks long-term but not short-term memory for auditory fear conditioning (Schafe and LeDoux, 2000). Protein synthesis is also important during a number of other forms of synaptic plasticity. A late-phase of LTD has recently been described in the CA1 region of freely moving rats, that is dependent on translation but not transcription (Manahan-Vaughan *et al.*, 2000). In addition, the requirement for protein synthesis during long-term facilitation (lasting >24 hours) in the marine snail *Aplysia* is well documented (Montarolo *et al.*, 1986). It therefore appears that protein synthesis is necessary for the persistence of plasticity in a number of candidate memory models.

It is assumed that protein synthesis is responsible for the formation of long-term changes in synaptic function and morphology; however, little is known about the nature of the proteins involved. Are mechanisms such as L-LTP dependent on the synthesis of novel proteins, or are they reliant on the increased synthesis of pre-existing proteins. Furthermore, what is the locus of action of these proteins? The exact locus of LTP maintenance (i.e. presynaptic or postsynaptic) is a subject of intense debate with no definitive answer yet reached. Therefore, the locus of action of the proteins involved in L-LTP could be presynaptic, postsynaptic or both. Presynaptic mechanisms that result in LTP maintenance are thought to be due to factors that increase transmitter release. Therefore, the synthesis of new proteins involved in vesicle release could regulate quantal number, whilst quantal size could be affected by proteins controlling vesicle loading during endocytosis. Perhaps newly synthesised proteins modulate the presynaptic Ca^{2+} influx and may lead to an increase in the probability of release.

Postsynaptic modifications implicated in the maintenance of LTP are thought to be due to increases in the number, or functional characteristics of receptors or ion channels. Therefore proteins involved in the modification of receptor function (e.g. changes in affinity for glutamate, increases in probability of channel opening, or channel conductance) could lead to expression of L-LTP. Proteins involved in L-LTP could also form the receptors or ion channels themselves. Alternatively they may be involved in the insertion of pre-existing receptors into the postsynaptic membrane or

the rearrangement of these receptors to oppose presynaptic release sites. Each of these changes would enhance the postsynaptic currents generated by a fixed amount of neurotransmitter. Finally, changes in synaptic morphology could be involved in L-LTP maintenance. Either presynaptic boutons or postsynaptic spines (or both) could divide, enlarge or change shape so as to confer an increase in efficacy. This may involve the rearrangement of cytoskeletal proteins.

Although it is relatively easy to propose mechanisms that may be involved in the induction of L-LTP, the identity of the proteins involved and their precise role remains a mystery. A number of candidate proteins have been implicated in L-LTP. The best-characterised example is the activity regulated cytoskeletal-associated protein *Arc*. mRNA encoding for *Arc* is increased in response to LTP induction (Link *et al.*, 1995; Lyford *et al.*, 1995), whilst both *Arc* mRNA and protein have been shown to selectively localise to regions of recent synaptic activity in granule cell dendrites in the hippocampus (Steward *et al.*, 1998). Finally, inhibition of *Arc* protein expression, via hippocampal infusion of antisense oligodeoxynucleotides has been shown to block L-LTP and long-term but not short-term spatial memory in rats (Guzowski *et al.*, 2000). To date a precise role for *Arc* in L-LTP has not been determined, although its modest homology to α-spectrin (a cytoskeletal protein), suggests that it may be involved in anchoring or targeting processes and therefore might act in the modification or maintenance of postsynaptic structures (Lyford *et al.*, 1995).

8.3 THE ROLE OF TRANSCRIPTION IN L-LTP

Data implicating mRNA synthesis in L-LTP is far from conclusive. In the majority of studies, the broad range transcription inhibitor actinomycin-D was used. However, conflicting results obtained with this drug in both behavioural and L-LTP studies, have cast doubt on its reliability as an indicator for transcriptional dependence in either long-term memory or L-LTP (see section 1.5.1). Although actinomycin–D was found to block L-LTP in the CA1 region of the hippocampus in this study, concerns about the non–specific actions of this drug led to the use of the selective RNA polymerase II inhibitor, α-amanitin. α-amanitin was found to have no effect on

L-LTP, when applied at a concentration previously used to block mRNA synthesis, suggesting that mRNA synthesis is not required for the induction or maintenance of L-LTP over 8 hours. Unfortunately, an adequate verification of the block of mRNA synthesis by α-amanitin could not be provided, and consequently these data should be interpreted with caution.

The controversy surrounding the ability of actinomycin-D to block L-LTP could be a consequence of its broad substrate range. In addition to a block of mRNA synthesis actinomycin-D also blocks the synthesis of tRNA and rRNA. Therefore, actinomycin-D may block L-LTP, not via a direct block of mRNA transcription, but indirectly by limiting the abundance of the necessary components of translation (i.e. tRNA and rRNA; see section 5.7.1 for more detail). Therefore, during actinomycin-D application, the ability of the neurons to induce L-LTP would depend on the ability of the pre-existing rRNA and tRNA to meet the required demand for new protein synthesis. For example, one would predict that behavioural learning would produce a modest demand for new proteins that is easily met by the existing rRNA and tRNA, even in the presence of actinomycin-D. This is consistent with the observation that actinomycin-D failed to block long-term memory in the majority of the early behavioural studies (Barondes & Jarvik, 1964; Cohen & Barondes, 1966; Squire & Barondes, 1970). A similar situation may occur in the *in vivo* L-LTP studies where stimulation is kept to a minimum to avoid seizures. Consistent with this, actinomycin-D did not block L-LTP in the dentate gyrus of anaesthetised rats, causing the authors to hypothesise that proteins necessary for the maintenance of LTP are synthesised from pre-existing mRNA (Otani & Abraham, 1989; Otani *et al.*, 1989). A similar hypothesis has recently been proposed to explain the ineffectiveness of actinomycin-D at blocking L-LTD in the CA1 region of freely moving mice (Manahan-Vaughan *et al.*, 2000). It is notable that actinomycin-D seems most effective at blocking L-LTP occurring in acute hippocampal slices *in vitro*, a situation where strong stimulation paradigms are used and cells may be required to abruptly support LTP at many more synapses compared to distributed plasticity in the intact animal.

Although there is little doubt that a number of forms of synaptic plasticity are associated with an increase in gene expression, there is only a modest amount of evidence that this gene expression is necessary or sufficient for the electrophysiological changes occurring in these processes. Although a number of immediate early genes (IEGs) can be induced in response to seizure activity, the majority of these are poorly correlated with LTP induction or learning (section 1.5.3). In the few cases where IEGs are thought to be involved in L-LTP (e.g. *Arc*, *zif268*), it remains unclear whether their specific induction following potentiation is *required*, or whether protein synthesised from the translation of their pre-existing mRNA is sufficient for the maintenance of L-LTP over 8 hours.

Transgenic studies have attempted to demonstrate that gene expression, mediated by the transcription factor CREB, is necessary for L-LTP and long-term memory (see section 1.5.2). Hippocampal slices taken from mutant mice expressing a targeted disruption in the α and δ isoforms of CREB produced a decremental LTP that returned to pre-tetanus levels over 2 hours. These mice were also deficient in long-term but not short-term memory (Bourtchuladze *et al.*, 1994). Although this study is suggestive, the fact that CREB is absent from birth in these mice makes it impossible to determine whether CREB-induced gene expression, occurring specifically during the induction of L-LTP is required for its long-term maintenance, or whether these deficits are produced by a lack of constitutive CREB gene expression. In fact, contradictory reports have been published stating that $CREB^{\alpha\delta-}$ mice show no significant impairment in certain forms of memory, whilst LTP in both the CA1 and dentate gyrus of hippocampal slices taken from these animals was unaffected over 2 hours post tetanus (Gass *et al.*, 1998). To date, the most compelling evidence that a transcriptional response may be directly driving memory, is a study in *Drosophila* in which inducible expression of an activator isoform of CREB, prior to learning, caused the enhancement of long-term memory formation (Yin *et al.*, 1995).

Although a requirement for mRNA synthesis in L-LTP over 8 hours cannot be completely ruled out, it appears that transcription is not presynaptic in origin. Experiments performed in this study show that L-LTP occurring in the hippocampal pyramidal cells of the CA1 region is unaffected, following removal of the presynaptic

cell bodies. This finding is confirmed in a report by Nayak *et al.* (1998) that showed that L-LTP in rat CA1 mini-slices, could be maintained for at least 5 hours.

8.4 L-LTP IN THE STRATUM RADIATUM IS DEPENDENT ON LOCAL PROTEIN SYNTHESIS

In this study, the development of an experimental configuration that allowed the sampling of two spatially separate synaptic populations (i.e. stratum radiatum and stratum oriens) from a population of CA1 pyramidal cells, allowed the locus of protein synthesis during L-LTP to be addressed. In the stratum radiatum (i.e. apical dendrites), focal dendritic application of the translation inhibitor emetine resulted in a >50% reduction in the magnitude of L-LTP at 8 hours, strongly suggesting a role for dendritic protein synthesis in L-LTP. Furthermore, focal application of emetine to the cell bodies caused no significant block of L-LTP in either the stratum radiatum or the stratum oriens, suggesting that local protein synthesis is sufficient for L-LTP in the stratum radiatum for at least 8 hours.

The findings in this study are consistent with a rapidly growing literature suggesting that protein synthesis can occur in neuronal dendrites. A number of mRNA species, along with elements of the translational machinery, have been reported to be present in dendrites (see section 1.7.4). The observation that translation occurs in cultured dendrites, separated from their cell bodies suggests that this machinery is functional. Furthermore increases in dendritic protein synthesis have been observed in hippocampal slices in response to electrical and chemical stimulation suggesting that the translational machinery can be regulated by synaptic activity (see section 1.7.4).

Although there is much evidence supporting the occurrence of dendritic protein synthesis, the results presented in this study are the first to implicate a role for local protein synthesis in the induction of electrically induced homosynaptic L-LTP. These results are consistent with a number of recent findings suggesting that various forms of synaptic plasticity are dependent on local protein synthesis. Both neurotrophin-induced synaptic enhancement and mGluR-dependent LTD in the hippocampus have been shown to occur in dendrites that have been separated from their cell bodies

(Kang & Schuman, 1996; Huber *et al.*, 2000)(see section 1.7.6 for more detail). In addition studies in *Aplysia* demonstrate that long-term facilitation induced in a single sensory motor synapse can be blocked by the local application of the protein synthesis inhibitor emetine, specifically to the synapse. In addition, this local protein synthesis could occur in neurites that had been separated from their cell bodies (Martin *et al.*, 1997a) (see also section 1.7.6).

The results summarised above, taken together with the findings from this study, provide good evidence for local protein synthesis in synaptic plasticity. However, a number of wider issues have still not been adequately addressed. Is local protein synthesis from pre-existing dendritic mRNA sufficient to induce L-LTP, or does this phenomenon also require gene induction and the subsequent dendritic trafficking of newly synthesised mRNA? The results from Kang *et al.* (1996) and from Huber *et al.* (2000), suggest that new mRNA synthesis is not involved in at least these forms of synaptic plasticity. However, reports showing synaptic activity-regulated dendritic localisation of a number of mRNA species would appear to contradict this (section 1.7.2). For example, newly synthesised mRNA and protein for the IEG *Arc* show specific dendritic localisation to regions that have undergone recent synaptic activity (Steward *et al.*, 1998). Whether the induction of these genes is necessary for L-LTP however, is unknown.

Although little is known about the identity of the molecules involved in dendritic mRNA trafficking, some evidence does exist for the involvement of the RNA binding protein Staufen. Staufen has been implicated in the binding and localisation of *Drosophila* oocyte mRNA, whilst Staufen–GFP constructs transfected into hippocampal neurons, form RNA containing granules, some of which move in a bi-directional manner between the cell bodies and the dendrites (Kohrmann *et al.*, 1999). Despite ongoing research into the molecular mechanisms involved in mRNA trafficking, fundamental questions still remain about the nature of this trafficking. Are mRNA species transported specifically to activated synapses as proposed by the mail hypothesis (section 1.6.1), or is a synaptic tag required for the capture, or site specific translation of globally distributed mRNAs? (section 1.6.2). Furthermore, depending on the proximity of the protein synthetic machinery to the activated synapses, could

the capture of dendritically synthesised proteins by a synapse-specific tag be a further requirement?

8.5 LTP IN THE STRATUM ORIENS APPEARS INDEPENDENT OF PROTEIN SYNTHESIS FOR AT LEAST 8 HOURS

Surprisingly the requirement for dendritic protein synthesis exhibited by the apical dendrites of the stratum radiatum pathway is not seen in the basal dendrites of the stratum oriens. Preliminary data shows that focal application of emetine to the stratum oriens pathway does not effect L-LTP, even when concentrations are increased to a point at which L-LTP in the adjacent stratum radiatum pathway is affected. These results, taken together with the failure to block stratum oriens L-LTP in response to somatic emetine application, suggest that the stratum oriens is capable of maintaining L-LTP over the course of 8 hours, that is independent of both somatic and dendritic protein synthesis.

The results presented in this study are the first to suggest that L-LTP can be maintained in the stratum oriens pathway and that this maintenance over 8 hours occurs independently of protein synthesis. If confirmed, this protein synthesis independent L-LTP is unique with respect to L-LTP that has been described previously. To date, a protein synthesis-dependent phase has been characterised in all types of LTP discovered both in the hippocampus and the amygdala. In addition, this dependence on protein synthesis has also been extended to L-LTD, and to a number of chemically induced forms of synaptic enhancement and depression (see sections 1.5.1, 1.6.2, 1.7.6 and 6.2.2)

Although these findings were surprising, it is possible that a different mechanism for LTP induction and maintenance could occur in separate dendritic trees of the same cell. LTP induction in the stratum radiatum has been shown to be dependent on NOS and cGMP, while inhibition of each of these molecules has no effect on LTP in the stratum oriens (Haley *et al.*, 1996; Son *et al.*, 1996; Son *et al.*, 1998). The mechanistic differences between these two pathways may also extend into the later stages of LTP

expression and could play a role in differential storage of information in the hippocampus

Despite the preliminary nature of these results, it is intriguing to speculate on the mechanisms underlying protein synthesis independent L-LTP. The maintenance of this LTP over 8 hours may be dependent on changes in steady state protein phosphorylation, regulated via the interplay between kinases and phosphatases. During LTP induction in the stratum oriens, changes in the dynamic equilibrium between these groups of enzymes may favour an increase in long-term post-translational modifications that may be responsible for the protein synthesis independent maintenance of LTP in the stratum oriens, for in excess of 8 hours. Alternatively the autophosphorylation of a specific kinase (e.g. CaMKII) may play a role in the maintenance of stratum oriens L-LTP. Another explanation for the apparent protein synthesis-independence of L-LTP, seen in the presence of translation inhibitors, may arise from an indirect effect on the levels of protein phosphorylation. For example, the maintenance of LTP in the stratum oriens may be dependent on the breakdown of phosphatases, and a resulting increase in protein phosphorylation. Therefore, if protein synthesis is required to increase phosphatase levels, then during conditions of translational block low levels of these phosphatases could lead to an artifactual increase in the duration of this LTP.

Clearly further work needs to be carried out on the characterisation of this protein synthesis independent 'L-LTP'. Does this LTP display a requirement for protein synthesis during the later stages of maintenance (i.e. >8 hours), or does the protein synthesis independence reflect the fact that this LTP is 'short-lived', possibly returning to baseline levels shortly after 8 hours? If this LTP were indeed transient, then its maintenance by protein synthesis would be unnecessary, not to mention inefficient. Questions can also be asked about whether the absence of a requirement for protein synthesis in the basal dendrites, is reflected in their cellular architecture. Are polyribosomes or elements of the translational machinery present at the dendrites? Furthermore can dendritically localised mRNAs be identified in these dendrites?

8.6 CONCLUSION

The findings in this thesis, demonstrate that L-LTP in the stratum radiatum is dependent on dendritic protein synthesis and exhibits a requirement for postsynaptic RNA synthesis. In contrast, preliminary data suggest that a protein synthesis independent form of L-LTP may exist in the stratum oriens pathway.

The investigation of the later stages of candidate memory models such as LTP, provides important insights into the mechanisms involved in the persistence of memory. Consequently, these findings may enable the development of treatments to tackle diseases such as Alzheimer's and the more physiological consequences of ageing, such as benign senile forgetfulness, that are characterised by their impairments in memory.

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