PLASTICITY RELATED GENE EXPRESSION IN THE HIPPOCAMPUS

by Lindsay A. Roberts

A thesis submitted in part fulfillment for admission to the degree of Doctor of Philosophy

Faculty of Medicine
University of Glasgow
October 1996

ACKNOWLEDGEMENTS

I would like to thank Dr Brian Morris for his endless enthusiasm, encouragement and advice over the last three years. I would also like to thank Dr Heather Johnston and Dr Carol Simpson for helping me to find my way around the lab and showing me many of the techniques which I have learned. For the LTP work I would like to thank Dr Mike Higgins (*in vitro*) and Dr Charles Large (*in vivo*) for giving up their time to provide me with samples for my study.

I would also like to thank Glaxo Wellcome for their financial support, and everyone in the receptor systems unit for their help during my four month placement. In particular I express my gratitude to Dr Celestine O'Shaughnessy for her excellent supervision and guidance with my project.

Almost lastly I would like to give a big thank you to my friends and colleagues within and outwith the division who helped to keep me sane when I thought I was losing my grip. In particular Pasty (for sharing my love of food and Animal Hospital), Robert (for doing those manly things about the lab), Fiona (for guarding my snacks), Paul (for technical assistance), the lunchtime gang, Liz, Carol, my E-mail pals, my flatmate Katrine and especially David who made sure I never gave up.

Finally (!) and most importantly, I would like to thank my family for their emotional (and financial!) support over the last three years. I would like to dedicate this thesis to my mum, dad and brother, because if it were not for them there would be no thesis.

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SUMMARY

Learning and the retention of the information as a memory produces changes in synaptic efficiency, the formation of new synapses and remodelling of existing synapses in the central nervous system. These plasticity-related changes are brought about by the modification of existing proteins and the synthesis of new proteins. One structure in the brain which is particularly associated with learning and memory is the hippocampus. Manipulation of synaptic transmission in this structure (in particular the phenomenon of long-term potentiation or LTP) is widely believed to be the mechanism which underlies mammalian synaptic plasticity This work was undertaken to further investigate the proteins which are affected by plasticity-related changes within the hippocampus, to improve understanding of why these changes occur and uncover more details about the mechanisms involved.

The effects of intraperitoneal administration of pilocarpine, scopolamine, thioperamide, ondansetron and aniracetam on the expression of the two immediate early genes (IEGs), c-fos and zif/268, were studied in the hippocampus (**Chapter 3**). None of the compounds tested produced any significant changes in mRNA levels of either species except pilocarpine (8mg/kg) which significantly increased levels of c-fos mRNA in the CA1 region of the hippocampus 45 minutes after administration. These results suggest that the mechanisms by which these compounds affect cognitive function do not involve IEG expression. Pilocarpine-induced expression of c-fos has been previously demonstrated, and at higher doses induction of zif/268 expression is also seen.

Induction of LTP in the CA1 region of the hippocampus *in vitro* following high frequency stimulation of Schaffer collateral fibres (**Chapter 4**) produced

significant increases in zif/268 mRNA levels after 45 minutes, but no changes in c-fos or tubulin 26 (T26) mRNA levels. After 2 hours, levels of mRNA for the α subunit of Calcium/calmodulin-dependent protein kinase II (αCAMKII) were significantly increased. Levels of T26 remained unchanged. These results demonstrate a possible role for zif/268 and αCAMKII in the expression of LTP in this pathway.

The induction of LTP in perforant path/granule cell synapses *in vivo* (**Chapter 5**) produced significant increases in mRNA levels of αCAMKII, proenkephalin (pENK) and 25kD synaptosomal associated proteins A and B (SNAP A & SNAP B). These observations support the hypothesis that αCAMKII is important for the expression of LTP and that the induction of LTP in Schaffer collateral/CA1 synapses involves similar mechanisms to perforant path/granule cell synapse LTP. This study also revealed the previously unreported induction of pENK expression two hours after the induction of LTP in these synapses, and demonstrated the involvement of SNAP A and B, and the importance of pre-synaptic changes in the induction of LTP. In addition, this work revealed increases in dendritic mRNA levels of MAP2 and αCAMKII just 2 hours after the induction of LTP, which may reflect a mechanism whereby the induction of LTP can selectively stabilise mRNA levels in the affected synapses.

The expression of several genes was examined two and twenty four hours after microapplication of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), (+)-alpha-methyl-4-carboxyphenyl-glycine (MCPG), Sp-adenosine 3',5'-cyclic monophosphothioatetriethylamine (Sp-cAMPS) and 0.9% saline to hippocampal granule cell dendrites in vivo (**Chapter 6**). After two hours no changes in mRNA levels of any of the genes we were investigating was

observed. After twenty four hours levels of αCAMKII were found to be significantly increased, compared to saline, in the cell bodies of the granule cells following treatment with ACPD. This increased expression following metabotropic glutamate receptor activation by ACPD is most likely to be due to phospholipase C activation rather than a decrease in levels of cAMP, as activation of PKA by the cAMP analogue Sp-cAMPS also produced significant increases in αCAMKII mRNA levels after twenty four hours.

RT-PCR and related techniques were used to see how effectively these methods could be used to detect changes in mRNA levels (**Chapter 7**). Semi-quantitative information about developmental changes in αCAMKII, two isoforms of NCAM, Egr3 and T26 mRNA levels was obtained using these methods, but changes in mRNA levels following the induction of LTP in the hippocampus produced extremely variable results from animal to animal. It appears that for detection of small localised changes in mRNA levels,such as those associated with LTP, in-situ hybridisation is the method of choice.

The incorporation of variable alternatively spliced exon (VASE) into NCAM is associated with a loss of neurite outgrowth promoting ability of this molecule, and decreased plasticity in the CNS. We designed two oligonucleotide probes to the isoforms of NCAM which did and did not contain this exon (N-VASE and N-FLANK respectively) to see whether they were differentially distributed and developmentally regulated in the brain (**Chapter 8**). Levels of N-FLANK were higher in embryonic brain and N-VASE levels were higher in adult brain. However when the specificity of the two probes was tested, using displacement experiments, they were found to cross hybridise and it was not possible to determine exactly to which mRNA species each oligonucleotide was hybridising.

These results demonstrate that induction of αCAMKII expression is an important feature of LTP in 2 regions of the hippocampus and provide evidence that it may also be regulated by metabotropic receptor stimulation and activation of protein kinase A. In addition, we have demonstrated potential roles for SNAP A, SNAP B and pENK in the expression of LTP in the dentate gyrus. Increases in levels of dendritic mRNA species may be a reflection of a mechanism by which dendritic mRNA levels of MAP2 and αCAMKII are selectively enhanced in particular synapses following the induction of LTP. We have also demonstrated that in-situ hybridisation is the method of choice for detection of small localised changes in mRNA levels rather than RT-PCR. The induction of the IEG zif/268 appears to be a common feature of both granule cell LTP and CA1 LTP, but its expression is unaffected by compounds which affect cognition. These compounds probably produce their effects by enhancing existing processes rather than initiating new ones.

LTP has yet to be proved to be a definitive model for synaptic plasticity, but its properties and the changes in gene expression which it produces correlate well with other observations made about neuronal plasticity. In the mammalian hippocampus plasticity-related gene expression appears to involve the altered levels of mRNA derived from a variety of genes. However the significance of changes in gene expression which occur in response to electrically induced alterations in transmission in terms of physiological and biochemical changes associated with learning and memory remains to be determined.

ABBREVIATIONS

αCAMKII	the alpha subunit of calcium/calmodulin-dependent protein kinase II
ACPD	(1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	amyloid precursor protein
Arc	activity-regulated cytoskeleton-associated protein
BDNF	brain-derived neurotrophic factor
CAMKII	calcium/calmodulin-dependent protein kinase II
cAMP	adenosine 3': 5'-cyclic monophosphate
cDNA	complementary DNA
cGMP	guanosine 3': 5'-cyclic monophosphate
CNS	central nervous system
CPG	candidate plasticity gene
CRE	cAMP-response element
CREB	cAMP-response element binding protein
DAG	diacylglycerol
DEPC	diethylpyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E17	embryonic day 17
E19	embryonic day 19
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N, N, N', N'-tetra acetic acid
EPSP	excitatory postsynaptic potential
ERK	extracellular signal regulated kinase

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GluR	glutamate receptor
IEG	immediate early gene
IP_3	inositol (1,4,5)-triphosphate
LTD	long-term depression
LTP	long-term potentiation
MAP2	microtubule associated protein 2
MCPG	(+)-alpha-methyl-4-carboxyphenyl-glycine
mGluR	metabotropic glutamate receptor
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide
NT-3	neurotrophin-3
P1	postnatal day 1
P4	postnatal day 4
P8	postnatal day 8
P12	postnatal day 12
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDYN	prodynorphin
pENK	proenkephalin
PLC	phospholipase C
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PS	population spikes
RNA	ribonucleic acid
RT	reverse transcription

SDS	sodium dodecyl sulphate
SNAP A	25kD synaptosomal-associated protein a
SNAP B	25kD synaptosomal-associated protein b
Sp-cAMPS	Sp-adenosine 3',5'-cyclic monophosphothioatetriethylamine
SSC	standard saline citrate
STP	short-term potentiation
T26	tubulin 26
TAE	tris-acetate
TBE	tris-borate
TE	tris-EDTA
TIMP	tissue inhibitor of metalloproteinases
UV	ultra violet

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Synaptic plasticity

Synaptic plasticity is defined as adaptive changes in synaptic efficiency. These changes can be short term, involving localised modulation of transmitter release and alterations in the sensitivity of post-synaptic receptors, or longer term involving remodelling of existing synapses and the formation of new synapses. The ability of both invertebrates and vertebrates to adapt to their particular environments, learn by experience and to retain that information is due to plasticity-related changes in the brain.

1.2 Models used for studying learning and memory

Studies have shown that there are two distinct forms of learning. Explicit or declarative learning involves remembering people, places and things, and implicit or procedural forms of learning involve the acquisition of particular skills or conditioned responses (Polster *et al.*, 1991). Both these forms of learning are studied in a variety of species, both invertebrate and vertebrate using different experimental procedures.

1.2.1 Aplysia

Aplysia is a sea mollusc with a relatively simple central nervous system containing large easily identifiable neurones. Applying a noxious stimulus to the tail of this animal triggers the withdrawal of its gill, and this response can be used experimentally to study sensitisation and habituation, both of which are basic forms of learning. Habituation describes a situation whereby a living organism becomes accustomed to a particular stimulus following repeated use and no longer responds in the manner it would have done on first receiving the stimulus. When an animal is exposed to a particularly strong or noxious

stimulus which produces a marked reaction, it will, in some circumstances, over react to subsequent mild stimuli. This is known as sensitisation. It has been shown that for sensitisation to occur changes in synaptic efficacy between sensory and motor neurones has to take place, and 5-HT has been identified as mediating these changes (Frost *et al.*, 1985; Montarolo *et al.*, 1986; Emptage & Carew, 1993). Short term changes in synaptic efficacy in this model are due to increased presynaptic neurotransmitter release (Dale *et al.*, 1988) and depend on covalent modification of existing proteins whereas long-term changes require new protein and mRNA synthesis to allow the growth of new synaptic connections (Montarolo *et al.*, 1986; Castelucci *et al.*, 1989). In addition this long-term facilitation leads to activation of protein kinase A (PKA) (Bacskai *et al.*, 1993), and phosphorylation of cAMP responsive element binding protein-related (CREB-related) transcription factors which activate other transcription factors and hence cAMP inducible genes (Kaang *et al.*, 1993).

1.2.2 Drosophila melanogaster

Another species which has been used to study learning and memory is the small fruit fly *Drosophila melanogaster*. It is possible to look at classical and operant conditioning in these insects. Classical conditioning is the experimental induction of a reflex the most famous example of which are Pavlov's dogs which learned to associate the sound of a bell with being fed and eventually salivated to the sound of the bell in the absence of food. When an animal learns to do, or not to do, something in response to a cue, this is known as operant conditioning, as the animal has to consciously modify its behaviour. In *Drosophila* the most commonly used paradigm for studying learning and memory is olfactory avoidance (Tully & Quinn, 1985). Flies are presented with two fragrances and, during exposure to one of them, receive an electric shock.

The flies learn to avoid the odour associated with the electric shock. Several mutants of *Drosophila* occur naturally and have been used to study, amongst other things, the effect that mutations of particular genes have on learning and memory. It has been found that some of the mutants which perform badly in the olfactory avoidance learning model have mutations in components of the cAMP second messenger system. The *dunce* mutation results in a deficiency in cAMP phosphodiesterase (Byers *et al.*, 1981) and the *rutubaga* mutant has a defective adenylyl cyclase gene (Levin *et al.*, 1992). As with sensitisation in *Aplysia* this associative learning in *Drosophila* has a short term component which is independent of protein synthesis and a long term component which requires synthesis of new proteins (Tully *et al.*, 1994).

1.2.3 Chicks

One of the most widely used experimental procedures for studying learning in the chick is the one-trial passive avoidance test. When presented with a small bead chicks will peck at it randomly. They are then presented with another bead which has either been soaked in water or a bitter tasting substance such as methylanthranilate. The chicks again peck at the bead but, in the case of the methylanthranilate bead, show disgust. Presentation of subsequent beads with no coating should produce avoidance in the chicks which were exposed to the methylanthranilate coated bead, if the animal has learned. The effects of agents which improve or impair memory can be studied by intracerebral injection prior to the task, and changes in levels of protein or alterations in synaptic morphology can be examined. It has been shown that this learning paradigm produces several changes in the chick brain, including increased protein phosphorylation, gene expression and protein synthesis (Rose, 1991).

1.2.4 Mammals

There are large numbers of mazes and tasks available for studying learning and memory in rodents, and different types of learning can be investigated using different models. Inhibitory avoidance can be used as a model for examining basic learning. The principles are similar to those in olfactory avoidance which is used to look at cognition in *Drosophila*. Rats or mice are trained to reverse their instinctive behaviour, for example, use of mild electric shocks in a dark compartment of a cage containing dark and light compartments will hopefully lead to the animal learning to stay in the light compartment, although instinctively it would chose the dark compartment. Inhibitory avoidance is a straightforward learning paradigm whereas more complex models test more advanced cognitive function. The radial maze task requires some degree of spatial learning but is mainly used to assess working memory by examining the ability of an animal to remember where it has previously been. In humans working memory can be likened to the short term storage of information e.g. remembering a telephone number for a few seconds in between receiving it and using it. The variety of tasks available to test cognition illustrates the involvement of different pathways in different types of learning and memory. One of the most widely used models for studying spatial learning is the Morris' water maze. This consists of a pool of water made opaque by the addition of latex. A platform is submerged in the pool and rodents are tested for their ability to find the platform and subsequent retention of its position. The Morris' water maze tests spatial learning, retention and mapping of environmental information, all of which are thought to involve a structure in the brain known as the hippocampus (Morris *et al.*, 1982).

1.3 Neurotransmission in the hippocampus

The hippocampus is known to be associated with declarative memory (Scoville & Milner, 1957) and it is thought that, by virtue its connections with other structures in the medial temporal lobe system and neocortex, the hippocampus acquires information, stores it for a short period of time before memories are laid down in the neocortex (Squire & Zola Morgan, 1991). Figure 1.1 shows the location of the hippocampus in the rabbit brain (a) and (b) a cross section through it (taken from Andersen *et al.*, 1971). There are three main excitatory pathways in the hippocampus. The first originates in the entorhinal cortex and synapses with the granule cells of the dentate gyrus via the perforant path. The second arises from the granule cells of the dentate gyrus and synapses with the pyramidal cell dendrites of the CA3 region via the mossy fibres and the third pathway originates in the CA3 region and synapses with pyramidal cells of the CA1 region via Schaffer collateral fibres. An important feature of transmission in the hippocampus is that under certain conditions the efficiency of the synapses can be increased. This phenomenon is known as long-term potentiation (LTP) and is believed to be the mechanism which underlies synaptic plasticity in the hippocampus.

1.4 Long-term potentiation

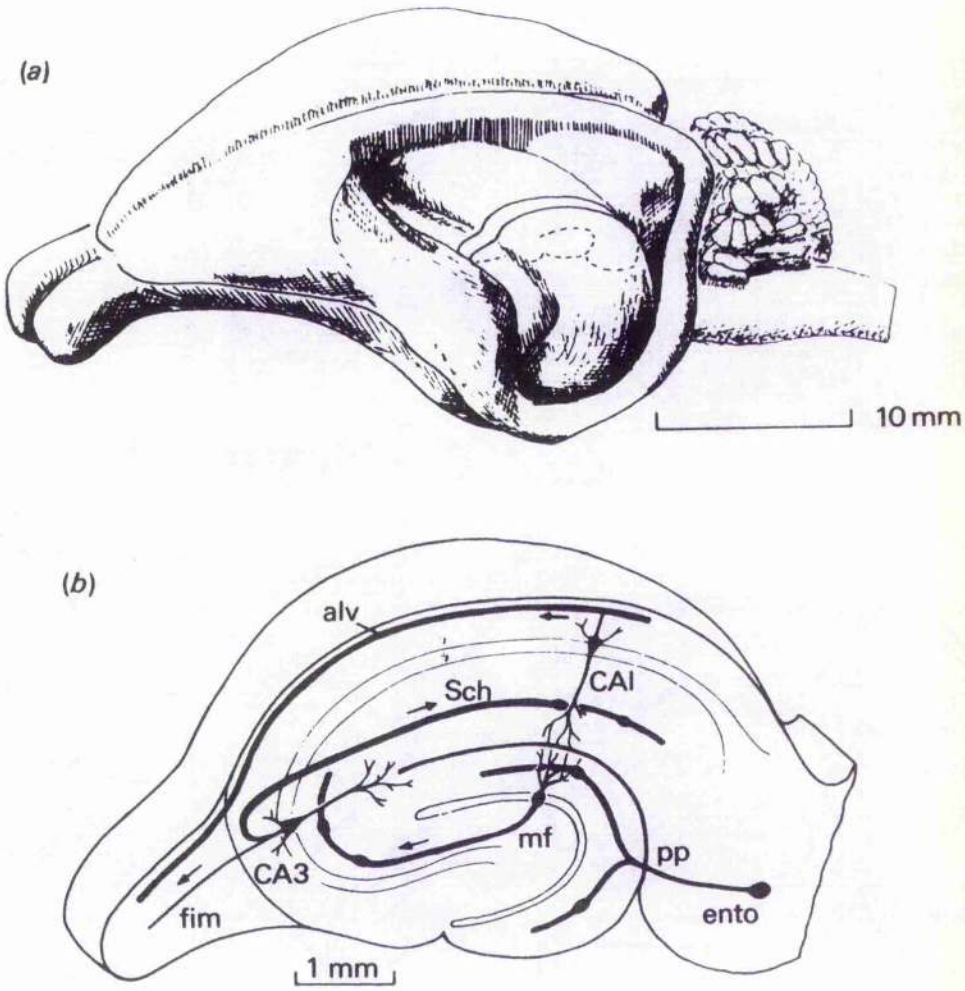
LTP is a lasting increase in synaptic efficacy following brief high frequency stimulation of monosynaptic excitatory pathways. It was first described by Bliss & Lømo (1973) who demonstrated a "long lasting potentiation of synaptic transmission" in the dentate gyrus of the rabbit. LTP has also been demonstrated in the CA3 region of the hippocampus following high frequency stimulation of the mossy fibres (Alger & Teyler, 1976) and in the CA1 region following high frequency stimulation of Schaffer collaterals (Schwartzkroin &

Figure 1.1 (from Andersen *et al.*, 1971)

The location of the hippocampus in the rabbit brain (a) and a transverse section through it (b).

Sch	Schaffer collateral	mf	mossy fibres
fim	fimbria	pp	perforant path
ento	entorhinal cortex	alv	alveus

Figure 1.1



Wester, 1975; Alger & Teyler, 1976; Andersen *et al.*, 1980). LTP can last for several hours *in vitro* and several days or weeks *in vivo*. There has been a great deal of research carried out to uncover the mechanisms involved in the induction and maintenance of LTP, mainly in Schaffer collateral/CA1 synapses and perforant path/granule cell synapses. Generation of mossy fibre/CA3 LTP appears to involve slightly different mechanisms. A diagram which summarises some of the mechanisms involved in Schaffer collateral/CA1 and perforant path/granule cell synapses is given in Figure 1.2 (taken from Bliss & Collingridge, 1993).

1.4.1 Induction of LTP

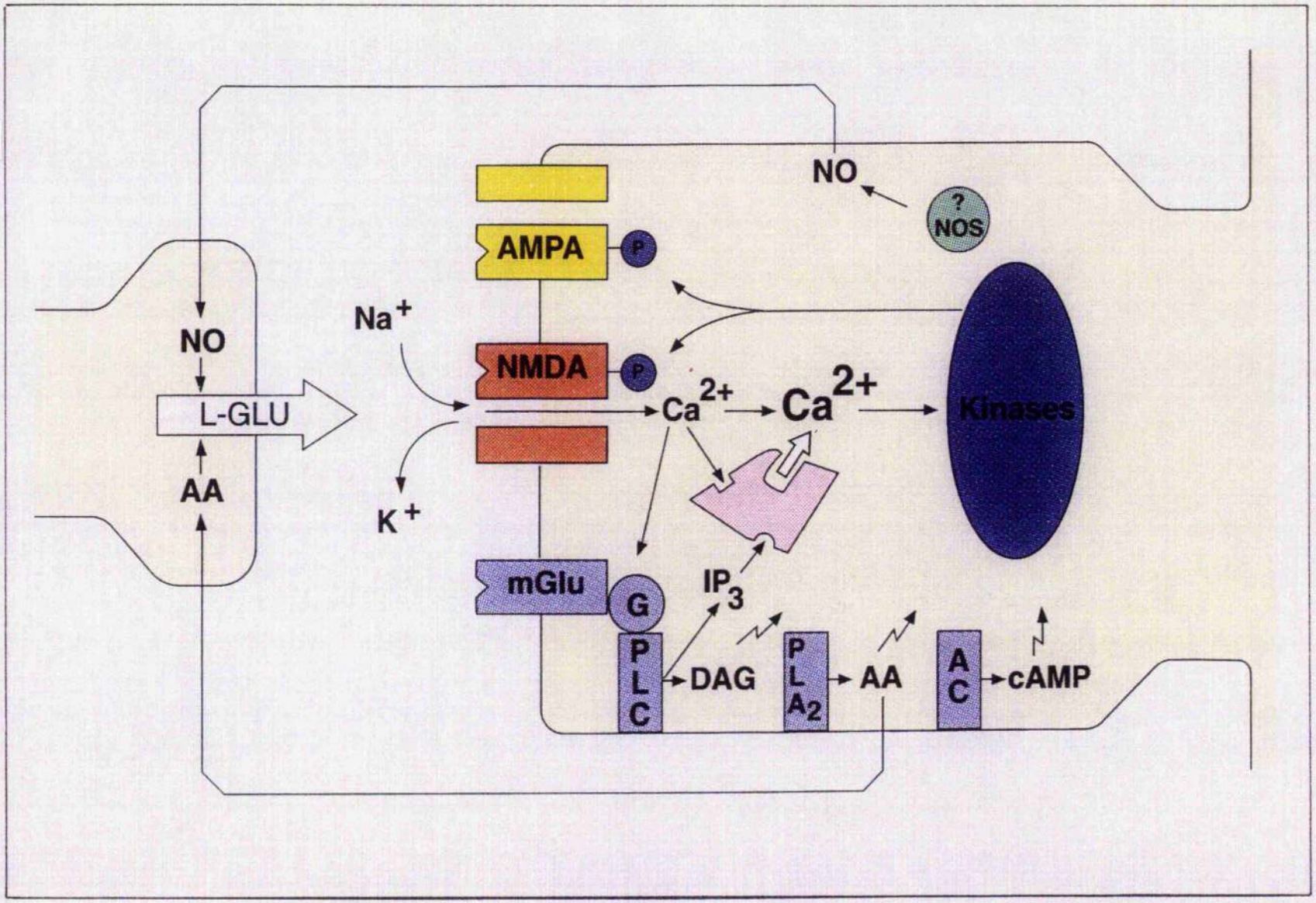
Nadler *et al.* (1976) carried out one of the first experiments which demonstrated that the excitatory amino acids glutamate and aspartate were involved in neurotransmission in the hippocampus. It was not until the discovery of selective antagonists for the three main subtypes of glutamate receptor (Watkins & Evans, 1981) that further progress was made in uncovering the roles these receptors might play in the induction of LTP. It is now thought that during high frequency (tetanic) stimulation of afferent fibres in the hippocampus, glutamate is released from the presynaptic nerve terminals and binds to, amongst other receptors, postsynaptic α-amino-3-hydroxy-5-methyl-4-isovazolepropionate (AMPA) receptors. AMPA receptors are responsible for mediating fast transmission in the hippocampus and their activation causes depolarisation of the postsynaptic membrane. The voltage-dependent Mg^{2+} blockade of N-methyl-D-aspartate (NMDA) receptor channels is reduced by membrane depolarisation, allowing Ca^{2+} entry into the cell. The size of the excitatory postsynaptic potential (EPSP) is increased above pre-stimulated levels and remains elevated for hours to weeks depending on the preparation used. Evidence which supports the involvement of both AMPA

Figure 1.2 (from Bliss & Collingridge, 1993)

A summary of some of the mechanisms involved in the induction of long-term potentiation in the hippocampus.

NO	nitric oxide	AA	arachidonic acid
P	phosphorylation site	NOS	nitric oxide synthase
AC	adenylate cyclase	PLC	phospholipase C
PLA2	phospholipase A2	IP3	inositol triphosphate
DAG	diacylglycerol		

Figure 1.2


NO
? NOS
AMPA
P
Na+
NO
NMDA
P
L-GLU
Ca2+
Ca2+
Kinases
AA
K+
mGlu
G
IP3
PLC
DAG
PLA2
AA
AC
cAMP

and NMDA receptors comes from work which has shown that the application of NMDA alone is not sufficient to induce LTP (Collingridge *et al.*, 1983), but only a short lived potentiation (STP) lasting up to one hour (Kauer *et al.*, 1988). Also NMDA receptor antagonists such as 2-amino-5-phosphonopentanoate (AP5 or APV) and MK-801 have been shown to prevent the induction of LTP (Collingridge *et al.*, 1983; Coan *et al.*, 1987). In addition the use of 6,7-dinitro-quinoxaline-2,3-dione (DNQX), an AMPA receptor antagonist, has been shown to prevent the induction of LTP following high frequency stimulation of Schaffer collateral fibres to CA1 (Muller *et al.*, 1988).

It has also been suggested that metabotropic glutamate receptors (mGluRs) may play a role in the induction of LTP. mGluRs differ from ionotropic (AMPA and NMDA) glutamate receptors in that they are coupled to a number of intracellular second messengers via G-proteins, and tend to be responsible for slower components of transmission in the hippocampus. Class I (mGluR1 and 5) stimulate phospholipase C (PLC) leading to formation of diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP_3). Class II (mGluR2 and 3) and class III (mGluR4, 6, 7 and 8) are negatively coupled to adenylate cyclase and reduce levels of cAMP. All sybtypes of mGluR are activated by aminocyclopentane decarboxylate (ACPD). Evidence to support a role for these receptors in LTP (Izumi *et al.*, 1991; McGuiness *et al.*, 1991; Otani & Ben-Ari, 1991; Bashir *et al.*, 1993; Borlotto & Collingridge, 1993; Musgrave *et al.*, 1993; Borlotto *et al.*, 1994; Richter-Levin *et al.*, 1994; Riedel *et al.*, 1994; Borlotto & Collingridge, 1995; O'Conner *et al.*, 1995; Riedel *et al.*, 1995a & b) appears to outweigh work which has disputed a role for these receptors in the induction of LTP (Chinestra *et al.*,1993; Bordi & Ugolini, 1995; Brown & Reymann, 1995). Work has shown that the selective metabotropic antagonist (RS)-α-methyl-4-carboxyphenylglycine (MCPG), known to competitively block ACPD mediated events (Eaton *et al.*, 1993), can

block the induction of LTP in the CA1 region (Bashir *et al.*, 1993). Although in the presence of MCPG there was evidence of some potentiation, it was limited to a duration of thirty minutes (similar to STP) which suggests that mGluR activation might be involved in the conversion of STP to LTP. This theory is strengthened by observations made by Borlotto & Collingridge (1993) who showed that activation of mGluRs by ACPD could induce slow onset LTP, without inducing STP, which took up to two hours to fully develop. A more detailed insight into the role of these receptors came from work by Borlotto *et al.* (1994) who used a number of different experiments to illustrate that NMDA receptors must be activated every time the induction of LTP takes place, whereas mGluR activation needed only to occur once for the induction of LTP.

Although the induction of LTP in Schaffer collateral/CA1 synapses and perforant path/granule cell synapses is dependent on NMDA receptor activation it has been shown that in mossy fibre/CA3 synapses the induction of LTP can still be achieved in the presence of NMDA receptor antagonists (Harris & Cotman, 1986). LTP in this region is less well characterised but it has been shown that transmission in this area involves opioid peptides derived from the proenkephalin and prodynorphin gene. Enkephalins and dynorphins are made in the granule cells of the dentate gyrus and transported to the terminals of the mossy fibres. Peptides derived from the proenkephalin gene preferentially act on μ and δ receptors and it has been shown that application of μ receptor agonists decreases the number of high frequency stimuli required for the induction of LTP (Derrick & Martinez, 1994). μ receptor antagonists also prevent the induction of LTP in this pathway (Derrick *et al.*, 1992) which suggests that enkephalins, rather than dynorphins, mediate the induction of LTP in the CA3 region. In addition, dynorphins increase the threshold for the

generation of LTP (Weisskopf *et al.*, 1993), suggesting that enkephalins and dynorphins have opposite modulatory effects on synaptic transmission.

It has been shown that the induction of LTP can be blocked by the Ca^{2+} chelator EGTA (Lynch *et al.*, 1983) and that high frequency stimulation raises Ca^{2+} levels in the dendrites of the CA3 region (Muller & Conner, 1991). It is widely assumed that raised intracellular Ca^{2+} levels associated with the induction of LTP occur as a direct result of Ca^{2+} influx through NMDA receptor channels. Evidence supporting this theory has come from work by Perkel *et al.* (1993). It is thought that the initial rise in Ca^{2+} levels triggers further release from intracellular stores and may also be necessary for the coupling mGluR activation to G-proteins, and subsequent alterations in second messenger levels (Irving *et al.*, 1992; see figure 1.2).

To summarise, the induction of a potentiation of transmission in Schaffer collateral/CA1 synapses and perforant path/granule cell synapses is dependent on the activation of NMDA receptors and entry of calcium ions into the post synaptic cell. The conversion of this potentiation from STP to LTP may involve the activation of metabotropic glutamate receptors. In mossy fibre/CA3 synapses an NMDA receptor-independent form of LTP exists which appears to involve opioid peptide transmission. Unless stated otherwise, most of the following will concern NMDA-receptor dependent LTP.

1.4.2 Maintenance of LTP

Once the initial induction of LTP has taken place it can last for hours *in vitro* and several weeks *in vivo*. The duration of LTP far outlasts the period of high frequency stimulation and brief increased intracellular calcium concentration

which are required for its induction, and attempts have been made to reveal the identity of factors which might contribute to this long maintenance.

It has been suggested that the maintenance of LTP may be due in part to presynaptic or post synaptic changes or both. The changes might involve an increase in presynaptic neurotransmitter release, alterations in neurotransmitter uptake, modifications in the number of receptors in the post synaptic membrane or the sensitivity of existing receptors to glutamate.

1.4.3 Retrograde messengers

Experiments have shown that there is a rise in glutamate concentration in the perfusates collected from animals in which LTP has been induced (Bliss *et al.*, 1986; Errington *et al.*, 1987), an observation which has been duplicated using a variety of other techniques (Dolphin *et al.*, 1982; Lynch *et al.*, 1985; Feasey *et al.*, 1986; Bliss *et al.*, 1987). It has been suggested that for this increase in transmitter release to occur generation of a messenger molecule from the post synaptic cell, which can effect changes in the presynaptic nerve terminal, is necessary. Several molecules have been proposed for this role including platelet activating factor, arachidonic acid, carbon monoxide and nitric oxide (Medina & Izquierdo, 1995), but at the moment all evidence is circumstantial and the existence of a retrograde messenger, however likely, remains unproven.

1.4.4 Role of protein kinases in LTP

Apart from STP, LTP can be subdivided into three components according to duration and requirements for maintenance (Bliss & Collingridge, 1993). LTP1 lasts 3-6 hours and is blocked by protein kinase inhibitors. Several protein

kinases have been found to be involved in the maintenance and/or induction of LTP.

Protein kinase C (PKC) is a Ca^{2+}/phospholipid-dependent protein kinase which controls the phosphorylation of serine and threonine residues of a variety of intracellular proteins and has been shown to play a role in the maintenance of LTP (Lovinger *et al.*, 1987; Reymann *et al.*, 1988a & b; Wang & Feng, 1992; Charlton-Sacktor *et al.*, 1993). PKC phosphorylates a protein kinase known as F1 (Akers & Routtenberg, 1985) which has been shown to be related to plasticity (Routtenberg *et al.*, 1985), and antibodies to another PKC substrate neurogranin prevent the induction of LTP in CA1 neurones (Federov *et al.*, 1995). It is thought that PKC may play a role in the conversion of STP to LTP. **Calcium/calmodulin-dependent protein kinase II** (CAMKII) has also been shown to be important for the induction and maintenance of LTP (Malenka *et al.*, 1989; Malinow *et al.*, 1989), and an increase in activity and phosphorylation of CAMKII and its substrates following the induction of LTP has also been reported (Fukunaga *et al.*, 1993, 1995). It is thought that the activation of CAMKII is both necessary and sufficient for the generation of LTP (Pettit *et al.*, 1994). Both PKC and CAMKII have been shown to phosphorylate AMPA receptors (McGlade-McCulloh *et al.*, 1993; Tan *et al.*, 1994) and increased activity of both is required for synaptic potentiation (Wang & Kelly, 1995). CAMKII has also been shown to phosphorylate NMDA receptor subunit 2B (Moon *et al.*, 1995). Most of the work implicating **protein kinase A** (PKA) as playing a role in the maintenance of LTP has come from observations made about cAMP, increased levels of which activate the catalytic subunit of PKA (Spatz, 1995). cAMP appears to partly mediate the phase of LTP lasting for periods in excess of 3 hours (Frey *et al.*, 1993; Matthies & Reymann, 1993; Nguyen *et al.*, 1994; Nguyen & Kandel, 1996), and its levels are increased following NMDA receptor activation (Chetkovich

et al., 1991). In addition the catalytic subunit of PKA can increase AMPA receptor function (Greengard *et al.*, 1991; Wang *et al.*, 1991).

These three protein kinases appear, therefore, to have important post synaptic roles in the maintenance of LTP for periods in excess of 3 hours. This may be due in part to their phosphorylation of NMDA and AMPA receptors which could result in increased sensitivity of these receptors to endogenously released glutamate.

Two protein kinases have been shown to be involved in the induction of LTP rather than its maintenance. **Tyrosine kinase** inhibitors prevent the induction of LTP when given prior to tetanic stimulation, but have no effect on established LTP (O'Dell *et al.*, 1991; Abe & Saito, 1993), and **protein kinase G** (PKG) is thought to be involved in the presynaptic mechanisms involved in the induction of LTP (Zhuo *et al.*, 1994). Some of the molecules suggested to act as retrograde messengers activate soluble guanylyl cyclase which catalyses the formation of cGMP, a PKG activator. cGMP has been shown to produce an enhancement of neurotransmitter release in cultured hippocampal neurones (Arancio *et al.*, 1995).

1.4.5 Protein synthesis and LTP

As mentioned previously LTP can be subdivided into 3 components. LTP1 is protein kinase dependent and LTP2 and LTP3 have been distinguished by the following observations. Agents which prevent the translation of mRNA into protein (such as anisomycin) reduce the duration of LTP to six hours or less (LTP2) (Krug *et al.*, 1984; Otani *et al.*, 1989). After this period the maintenance of LTP (LTP3) has been shown to require transcription of mRNA from DNA (revealed by the use of actinomycin) (Otani *et al.*, 1989). Duffy *et*

al. (1981) demonstrated that following the induction of LTP in hippocampal slices, newly synthesised proteins were secreted into the extracellular fluid and it has also been shown that the release of proteases accompanies the induction of LTP (Fazeli *et al.*, 1990). These observations suggested that the induction of LTP might stimulate the generation of new proteins and cleavage of existing proteins. Indeed more recent work (Fazeli *et al.*, 1993) revealed that the levels of several unidentified proteins were both increased and decreased three hours after the induction of LTP. Much effort has been channelled into identifying which genes are expressed following the induction of LTP and what role they might play in synaptic plasticity as a whole.

1.5 Gene expression following the induction of LTP

Protein and mRNA levels derived from a variety of genes have been examined after the induction of LTP. To some extent these genes can be grouped according to when their levels of expression are altered following the induction of LTP and in some cases genes encoding proteins with a particular function are regulated at the same time.

1.5.1 Immediate early genes

Immediate early genes (IEGs) represent a group of genes so called because they are rapidly activated in response to stimulation, but whose expression occurs independently of protein synthesis. Increased Ca^{2+} concentration and PKC activation have both been suggested to be involved in the induction of IEG expression (Morgan & Curran, 1986; Stumpo & Blackshaw, 1986) It is also thought that activation of calcium/cAMP response element binding protein (CREB) by phosphorylation may lead to IEG expression by its interaction with the calcium/cAMP response element (CRE) found within the promoter region

of some of these genes. Basal mRNA levels of many IEGs are low but they usually peak within an hour of stimulation and then decline to basal levels within the next two to three hours. Some of the proteins derived from IEGs, levels of which normally peak about two hours after stimulation, can act as transcription factors and up- or down-regulate the expression of other genes. There are several different types of immediate early gene. Most of the work carried out regarding LTP and IEG expression has concentrated on c-fos, members of the jun family and zif/268 (also known as krox-24, NGFIA and egr-1), and has involved the induction of LTP in perforant path/granule cell synapses *in vivo* in both conscious and anaesthetised rats using a variety of stimulation parameters. Detection of these changes in expression has been achieved by looking at mRNA or protein levels.

Increased levels of **c-fos** mRNA and/or fos-like immunoreactivity are only consistently seen in unanaesthetised animals when the parameters used produce LTP with a duration of several weeks (LTP3) (Douglas *et al.*, 1988; Jeffrey *et al.*, 1990; Abraham *et al.*, 1993; Demmer *et al.*, 1993; Worley *et al.*, 1993). In anaesthetised rats the induction of c-fos mRNA is less consistent or absent (Cole *et al.*, 1989; Wisden *et al.*, 1990; Demmer *et al.*, 1993). Some reports have shown that the induction of c-fos mRNA/protein is not specific to the generation of LTP as it can be achieved in both anaesthetised and unanaesthetised animal following certain patterns of high stimulation which do not lead to the induction of LTP (Douglas *et al.*, 1988; Dragunow *et al.*, 1989). A similar pattern is seen with **jun**-related proteins. Abraham *et al.* (1993) also showed that induction of LTP3 was accompanied by increased levels of c-jun, Jun-B and Jun-D protein which agreed with observations made by Demmer *et al.* (1993). Worley *et al.* (1993) only observed increased levels of jun-related mRNA with stimulation which produced a longer lasting LTP (LTP3).

Slightly different results have been obtained in these and other studies which have examined levels of **zif/268** mRNA and protein following the induction of LTP. The expression of this gene is not affected by the use of anaesthetics and increases in zif/268 mRNA levels have been reported in both anaesthetised (Cole *et al.*, 1989; Wisden *et al.*, 1990) and unanaesthetised animals (Richardson *et al.*, 1992; Worley *et al.*, 1993) following the induction of LTP in perforant path/granule cell synapses. However while Richardson *et al* (1992) suggested a correlation between the induction of zif/268 and the duration of LTP in unanaesthetised rats, Schrieber *et al.* (1991) claimed that the induction of LTP in the dentate gyrus of anaesthetised rats did not always lead to increased levels of zif/268 mRNA and in some cases levels were increased in the absence of the induction of LTP. *In vitro* studies have shown that zif/268 mRNA levels in CA1 pyramidal cells are also increased following the induction of LTP. Chinestra *et al.* (1994) used tetraethylammonium to elicit LTP in the CA1 region in hippocampal slices and found that levels of zif/268 mRNA were increased. Potentiation of EPSPs in single postsynaptic hippocampal CA1 neurones was also accompanied by increased levels of zif/268 mRNA (Mackler *et al.*, 1992).

Two more immediate early genes have recently been shown to be activated following the induction of LTP. Krox-20, a member of the same family of zinc finger containing transcription factors as zif/268, has been found to be expressed in the granule cells of the dentate gyrus following the induction of LTP (Williams *et al.*, 1995) and remains elevated for up to 24 hours post tetanus. Dragunow *et al.* (1996) showed that nur-77, a possible ligand activated transcription factor belonging to the steroid/thyroid hormone receptor family, was also expressed following the induction of LTP in perforant path/ granule cell synapses.

Fos and jun family proteins form heterodimers and jun family proteins form homodimers which bind to the AP-1 response element, and zif/268 has been shown to bind to the sequence -GCGTGGGGCG- on DNA (Christy & Nathans, 1989). Any genes containing either of these sequences can therefore be potentially regulated by these transcription factors. Repetitive induction of fos-related, jun-related and zif/268 protein does not prolong the maintenance of LTP (Abraham *et al.* 1995) and suggests that the mechanisms responsible for the maintenance of LTP, which are activated or repressed by IEG products, can potentially be saturated.

1.5.2 Protein kinases

The role of protein kinases in the induction and/or maintenance of LTP has been previously discussed (Section 1.4.4). Following the induction of LTP some of these kinases are not only activated, but levels of mRNA encoding them are altered. It has been shown that in the granule cells of the dentate gyrus in conscious rats, levels of the γ isoform of PKC are increased two hours after the induction of LTP and return to basal levels after 48 hours (Thomas *et al.*, 1994a). Levels of the α, βI, βII, δ and ε isoforms of PKC were unchanged in the same animals. Meberg *et al.* (1993) examined levels of mRNA for the β and γ isoforms of PKC and the PKC substrate F1 (GAP-43) three days after the induction of LTP in the CA3 region of the hippocampus. It was found that the greater the magnitude of synaptic enhancement the lower the levels of F1, βPKC, and γPKC and that 72 hours after the induction of LTP the expression of F1 and γPKC was lower than basal levels. It was suggested that three days after the induction of LTP synaptic stabilisation may take place and therefore decreased expression of F1 (leading to suppression of neurite outgrowth) might contribute to this. A more recent study (Meberg *et al.*, 1995) demonstrated that this negative correlation between LTP magnitude and F1 and γPKC

expression can be seen as early as one hour after the induction of LTP. However, the interpretation of these results is compounded by the use of anaesthetics in the second study, and by the unusual use of recordings made in the dentate gyrus as an indication of potentiation for the CA3 region.

Increased CAMKII activity has been shown to be both necessary and sufficient for the generation of LTP (Pettit *et al.*, 1994). Levels of mRNA for the α subunit of CAMKII (αCAMKII) are increased in the granule cells and proximal dendrites of the dentate gyrus between two and forty eight hours after the induction of LTP (Thomas *et al.*, 1994a), whereas levels of the β subunit are unchanged. *In vitro* studies (Mackler *et al.*, 1992) also showed that, following the induction of LTP in the Schaffer collateral/CA1 synapses, levels of αCAMKII mRNA were increased three hours post tetanus.

Thomas *et al.* (1994a) also found increases in extracellular signal-regulated kinase 2 (ERK2) and raf B 24 hours after the induction of LTP. These are two kinases which rely on phosphorylation rather than Ca^{2+} for their activation.

1.5.3 Neurotrophins

Neurotrophins are thought to induce neurite outgrowth and provide support for some neurons in the CNS (Hagg *et al.*, 1990; Alderson *et al.*, 1990).

The expression of brain-derived neurotrophic factor (BDNF) has been shown to be increased 2-3 hours after the induction of LTP in the dentate gyrus *in vivo* (Dragunow *et al.*, 1993; Castrén *et al.*, 1993). In addition Castrén *et al.* (1993) reported increases in nerve growth factor (NGF) mRNA, but decreases in neurotrophin-3 (NT-3) mRNA. Following induction of LTP in the CA1 region of the hippocampus *in vitro*, Patterson *et al.* (1992) found BDNF and

NT-3 mRNA levels were increased, but that NGF mRNA levels were unchanged.

1.5.4 Other genes

Expression of several other unrelated genes have been shown to be linked to the induction of LTP.

Consistent with the theory that increased presynaptic transmitter release occurs following the induction of LTP, Lynch *et al.* (1994) demonstrated that concentrations of three synaptic vesicle proteins (synapsin, synaptotagmin and synaptophysin) were increased three hours after the induction of LTP in the dentate gyrus. Also, Smirnova *et al.* (1993) demonstrated increased expression of GR33 mRNA, which encodes syntaxin, in the granule cells of the dentate gyrus. It was also found that GR33 protein levels were increased in the mossy fibre terminals demonstrating that the induction of LTP in one set of synapses may lead to alterations in downstream synapses. Although it is not known whether an increase in synaptic vesicle protein would necessarily lead to increased transmitter release, these changes may reflect the LTP induced increase in synapse number (Chang & Greenough, 1984).

Neural cell adhesion molecules (NCAM) are membrane spanning glycoproteins involved in cell-cell interactions and maintenance of neuronal structure. Fazeli *et al.* (1994) demonstrated that extracellular concentrations of NCAM and amyloid precursor protein (APP) were increased following the induction of LTP in the dentate gyrus of anaesthetised rats. In addition to this it has been shown that antibodies to NCAM reduce the magnitude of LTP in the CA1 region of the hippocampus (Lüthi *et al.*, 1994; Rønn *et al.*, 1995). These observations indicate a role for NCAM in LTP and suggest that alterations in

synaptic morphology which accompany LTP (Desmond & Levy, 1986a & b, 1990) may cause increases in extracellular concentrations of NCAM and APP as degradation of the extracellular matrix occurs.

Thomas *et al.* (1994b) showed that levels of NR1, a NMDA receptor subunit containing a site for phosphorylation of PKC, were increased following the induction of LTP in the dentate gyrus. It is possible that presynaptic glutamate receptors up-regulate transmitter release and that increased levels of PKC sensitive subunits of NMDA receptors reflect changes in the phosphorylation state of these receptors during LTP.

The release of proteases has been shown to accompany the induction of LTP *in vivo* (Fazeli *et al.*, 1990) and Qian *et al.* (1993) have demonstrated that mRNA levels of tissue plasminogen activator, an extracellular serine protease, were increased following the induction of LTP in the dentate gyrus *in vivo*.

Using subtractive hybridisation Nedivi *et al.* (1993) isolated and cloned several cDNAs of genes shown to be induced in the dentate gyrus following i.p administration of kainate. They then looked to see whether any of these genes were expressed following the induction of LTP in the dentate gyrus *in vivo*. Increased mRNA levels for candidate plasticity gene (cpg) and tissue inhibitor of metalloproteinases (TIMP) were observed following the induction of LTP in the dentate gyrus.

More recently differential cloning techniques identified a novel IEG termed activity regulated cytoskeletal protein (arc) shown to be induced following the induction of LTP in the dentate gyrus *in vivo* (Lyford *et al.*, 1995; Link *et al.*, 1995). Andreasson & Worley (1995) have reported increased expression of β-

A-activin, a transforming growth factor, following induction of LTP in the dentate gyrus.

To summarise, the induction of LTP appears to be accompanied by a wide variety of changes in gene expression in a wide range of genes. Initially the expression of IEGs and Ca^{2+} dependent protein kinases is increased, probably as a result of increased post synaptic calcium concentration. Alterations in levels of neurotrophins may reflect morphological changes occurring and increased expression of Ca^{2+}-independent protein kinases may reflect a role for such kinases once activation of Ca^{2+}-dependent processes has ceased. The reason for multiple induction of such a diverse range of genes is not known, and their functions remain to be fully resolved.

1.6 Long-term depression

Transmission in the hippocampus can also be manipulated to produce long lasting decreases in synaptic strength which have been collectively named as long-term depression or LTD. There are two main types of LTD, homosynaptic and heterosynaptic. Heterosynaptic LTD describes a long term decrease in the strength of inactive synapses and was first demonstrated in the hippocampus by Levy & Steward (1979). In the case of homosynaptic LTD only the strength of active synapses are depressed. This form of LTD has been most studied in recent years following the publication of a reliable method for its induction (Dudek & Bear, 1992). Homosynaptic LTD is generated by prolonged low frequency stimulation of afferent fibres and shares several features with LTP. Induction of both LTP and LTD is Ca^{2+}-dependent although it has been shown that in the case of LTD, Ca^{2+} entry through L-type voltage gated calcium channels paired with mGluR activation rather than NMDA receptor-mediated Ca^{2+} entry is required (Bolshakov & Siegelbaum,

1994). It is thought that the increase in postsynaptic Ca^{2+} required for the induction of LTD is less than the increase required for the induction of LTP and that this promotes the activation of phosphatases rather than kinases leading to a depression in transmission as dephosphorylation occurs (Mulkey *et al.*, 1993, 1994). Less is known about whether protein synthesis is involved in the maintenance of LTD, but one study has shown that heterosynaptic LTD in the dentate gyrus is associated with increased expression of zif/268, krox-20 and jun-B, but not c-fos or c-jun (Abraham *et al.*, 1994). It seems likely that LTD of synaptic transmission is also a form of synaptic plasticity which could potentially, given further investigation, prove to be as significant as LTP for cognitive function. The existence of phenomena which allow both up- and down-regulation of synaptic transmission suggests tight regulation of plasticity-related mechanisms in the hippocampus.

1.7 Relationship between LTP and cognition

In the same way that particular neurotransmitters are associated with certain functions according to their localisation in particular regions of the brain, the distribution of LTP appears to be concentrated in structures in the brain associated with learning and memory such as those in the limbic system and in particular the hippocampus which is known to be important for declarative memory, spatial learning and acquisition of information (Scoville & Milner, 1957; Morris *et al.*, 1982; Squire & Zola Morgan, 1991; Bunsey & Eichenbaum, 1996). Once the NMDA receptor-dependence of the induction of LTP had been suggested it was soon demonstrated that blockade of NMDA receptors could also impair spatial learning (Morris *et al.*, 1986) to the extent that comparable concentrations of AP5 were required for blockade of spatial learning *in vivo* and induction of LTP *in vivo* (Davis *et al.*, 1992). Concurrent blockade of NMDA receptors and mGluRs in the hippocampus has also been

shown to cause disruption of working memory (Ohno & Watanabe, 1996). Other behavioural experiments have shown that saturation of LTP (i.e. maximal generation of LTP in and entire population of synapses) can disrupt spatial learning (Barnes *et al.*, 1994) and Castro *et al.* (1989) also demonstrated that deficits in spatial learning were reversed as LTP decayed. These observations imply that processes underlying spatial learning and LTP overlap in some way. Biochemical studies have revealed a link between LTP and learning by demonstrating that the increase in glutamate release observed following the induction of LTP (Section 1.4.3) has also been shown to occur in the dentate gyrus following classical conditioning in the rat (Laroche *et al.*, 1987). Moser *et al.* (1993) also demonstrated that synaptic potentiation occurs in the dentate gyrus during exploratory learning. Several morphological changes are associated with the induction of LTP including increases in the number of synapses onto dendritic shafts (Lee *et al.*, 1980; Chang & Greenough, 1984) and changes in length and orientation of dendritic spines (Hosokawa *et al.*, 1995). It has also been shown that passive avoidance training in the rat leads to enhanced dendritic branching in the dentate gyrus (Vozeh & Myslivacek, 1996), and that an increase in dendritic spine density follows spatial learning (Moser *et al.*, 1994). This suggests that alterations in synapse morphology occur as a result of learning and following the induction of LTP.

It has recently been demonstrated that the AP5 induced deficit in spatial learning can be prevented by pretraining in a different Morris' water maze (Bannerman *et al.*, 1995). This observation led to the suggestion that NMDA receptors were necessary for acquisition of spatial information, but that retaining the information about the layout of an environment as a memory was less dependent on NMDA receptor activation. Saucier & Cain (1995) supported these observations by showing that although the NMDA receptor antagonist 2R,4R,5S-2-amino-4,5-(1,2-cyclohexyl)-7-phosphoheptano acid

(NPC17742) blocked the induction of LTP in the dentate gyrus it did not disrupt spatial learning in rats which had been pretrained. They concluded that NMDA receptor-dependent LTP in the dentate gyrus may not be necessary for spatial learning, but that it may make some contribution. These data illustrate that the relationship between LTP and cognition is far from straightforward and that NMDA receptor-dependent LTP may only be responsible for particular aspects of learning and memory.

All the evidence which points to LTP underlying the mechanisms by which we learn and remember is so far circumstantial and no definitive experiment has been carried out to confirm the link beyond all reasonable doubt. It appears that as with most scientific theories, in the absence of another explanation it is assumed, for the moment, that some relationship does exist.

1.8 Transgenic approaches

Recent advances in molecular biological techniques have led to the production of transgenic animals which lack a specific gene. Some of these genes code for proteins which have been shown to be involved in the induction and/or maintenance of LTP, reinforcing existing theories and introducing new theories about the mechanisms involved in the generation of LTP and gene expression associated with its induction.

1.8.1 Receptors

Mice lacking the metabotropic glutamate receptor mGluR1 display impaired cerebellar LTD and mossy fibre/CA3 LTP (Conquet *et al.*, 1994; Aiba *et al.*, 1994a). In addition they have severe deficits in motor co-ordination and spatial learning, although it was demonstrated that the deficit in spatial learning was

not due to their motor impairment. Aiba *et al.* (1994b) also demonstrated that LTP in the CA1 region of these animals was impaired (although STP was unaffected) and that context-specific associated learning was impaired (this model involves subjecting the animals to footshocks in a particular environment and then examining whether they freeze when replaced in that environment a day later as a result of them having remembered the previous experience). These observations support the theory that mGluRs may be involved in the development of slow onset potentiation and the conversion of STP to LTP (Bashir *et al.*, 1993; Borlotto & Collingridge, 1993). The NMDA receptor channel is formed by at least two members of the glutamate receptor channel subunit families (GluRε and GluRζ) The GluRε subunit can be further divided into four more subunits (1-4) and it has been shown that mice lacking the ε1 subunit of the NMDA receptor have spatial learning deficits and reduced hippocampal LTP in the CA1 region (Sakimura *et al.*, 1995). Ito *et al.* (1996) also demonstrated that NMDAε1 mutant mice were deficient in CA1 LTP but that the extent of the reduction increased with age. If the induction of LTP is NMDA receptor dependent (and the majority of reports support this theory) then it is reasonable to expect that alterations in its structure might affect the induction of LTP. It would be interesting to see how mossy fibre/CA3 LTP is affected in these mice as it is not though to be dependent on NMDA receptor activation.

1.8.2 Protein kinases

The involvement of protein kinases in the induction and maintenance of LTP has previously been addressed (Section 1.4.4, 1.5.2) and several transgenic mice exist with protein kinase deficits. It has now been demonstrated that mice lacking the γ isoform of PKC (PKCγ) display modified LTP in the CA1 region as the tetanus required for its induction has to be preceded with low frequency

stimulation (Abeliovich *et al.*, 1993a). In addition these mice had mild deficits in spatial learning (Abeliovich *et al.*, 1993b) which might be accounted for by the modified LTP which was seen in CA1. Thomas *et al.* (1994a) demonstrated that levels of PKC were increased following the induction of LTP in the dentate gyrus *in vivo* which supports observations made in the transgenic study. The expression of LTP has been shown to be dependent on CAMKII activity as well as PKC activity. The potential importance of the αCAMKII in LTP has been previously mentioned (Mackler *et al.*, 1992; Thomas *et al.*, 1994a) and it has now been shown that mice lacking this subunit have impaired spatial learning and CA1 region LTP (Silva *et al.*, 1992a & b). Chapman *et al.* (1995) also demonstrated that αCAMKII could, depending on the pattern of afferent stimulation, either enhance or depress synaptic transmission in the hippocampus. Two more studies examined site-directed mutagenesis of residue 268 of CAMKII. Substituting an aspartate residue for the threonine residue at this point results in expression of a Ca^{2+} independent form of CAMKII. Mayford *et al.* (1995) found that in the mutant mice high frequency stimulation still produced LTP, but that at lower frequencies the generation of LTD was favoured. The same animals also showed signs of deficits in spatial learning but not contextual learning, suggesting that these two forms of learning may be mediated by different synaptic mechanisms.

The involvement of tyrosine kinases in LTP has been previously demonstrated (O'Dell *et al.*, 1991; Abe & Saito, 1993). Mice with mutations in four tyrosine kinase genes *fyn*, *scr*, *yes* and *abl* were used to study the role of these kinases in LTP, learning and memory. Only *fyn* mutants were deficient in LTP and showed impaired spatial learning (Grant *et al.*, 1992). In addition, reduced phosphorylation of focal adhesion kinase was observed in *fyn* mutants (Grant *et al.*, 1995) suggesting that it may be involved in the expression of LTP. The involvement of CREB, CREB-related transcription factors, cAMP and cAMP

dependent protein kinase (PKA) in synaptic plasticity is well documented in invertebrate systems (Section 1.2.1, 1.2.2), but less is known about their role in LTP and plasticity in mammals. Initially it was demonstrated that long-term memory in mice lacking the α and δ isoforms of CREB was impaired although short-term memory was normal. Electrophysiological studies revealed that the duration and magnitude of LTP in these mice was reduced with potentiation returning to baseline levels after 1-2 hours (Bourtchuladze *et al.*, 1994). These results suggest that the processes activated by CREB are necessary for long-term memory storage and maintenance of LTP. LTD, but not LTP was found to be impaired in mice lacking the gene encoding the RIβ subunit of cAMP dependent protein kinase (Brandon *et al.*, 1995), but mice lacking the $C\beta_1$ subunit of PKA were found to be deficient in LTD and unable to sustain LTP for more than three hours (Qi *et al.*, 1996). These observations suggest that catalytic (C) and regulatory (R) subunits of PKA have different functions in synaptic plasticity. Disruption of type I adenylyl cyclase (which catalyses the formation of cAMP) resulted in mice deficient in LTP in the CA1 region and with spatial learning difficulties (Wu *et al.*, 1995). This may be due to a deficit in cAMP formation and therefore a lack of activation of CREB and CREB-related transcription factors.

1.8.3 Neurotrophins, glycoproteins and vesicle proteins

Increased expression of the neurotrophin BDNF has previously been demonstrated following the induction of LTP in the hippocampus (Patterson *et al.*, 1992; Dragunow *et al.*, 1993; Castrén *et al.*, 1993). It has now been shown that the induction of LTP is impaired in mice lacking BDNF, implicating this neurotrophin as having a functional role in plasticity (Korte *et al.*, 1995).

Involvement of glycoproteins in LTP has been reported by several groups (Fazeli *et al.*, 1994; Lüthi *et al.*, 1994; Rønn *et al.*, 1995). Cremer *et al.* (1994) have recently shown that NCAM deficient mice had impaired spatial learning ability, but did not report whether or not LTP was affected in these animals. Another glycoprotein Thy 1, whose expression results in an inhibition of neurite outgrowth, is thought to stabilise neuronal connections. (Tiveron *et al.*, 1992). Mice which lack the gene encoding Thy1 show impaired LTP in the dentate gyrus, but not the CA1 region of the hippocampus (Nostren-Bertrand *et al.*, 1996) and no deficits in spatial learning. These observations raise the possibility that LTP in the dentate gyrus is not required for the form of spatial learning tested in the Morris' water maze, but that Schaffer collateral/CA1 LTP may be.

Mice deficient in synapsin I, a synaptic vesicle protein, displayed no deficits in neurotransmitter release whereas mice lacking synaptotagmin I had such a severe impairment of Ca^{2+}-dependent neurotransmitter release that they died within 48 hours of birth (Rosahl *et al.*, 1993; Geppert *et al.*, 1994). LTP was not found to be impaired in synapsin I mutant mice (Rosahl *et al.*, 1993), but whether this means that the animal compensates for the lack of this protein or that its role in neurotransmitter release is less essential than the role played by synaptotagmin I is not known

Transgenic models have therefore reinforced data obtained from other experiments which have shown the induction of LTP to be dependent on expression of a particular gene. In addition they have revealed the importance of several other genes in plasticity. Some transgenic studies have revealed deficits in learning and LTP which adds further support to the theory that a relationship between the two exists. The importance of receptor activation, activation of transcription factors and protein kinases in the induction of LTP

and learning are documented in these studies. Additional information about proteins involved in neurite outgrowth, neurotransmitter release and structural proteins has also been obtained. Our lack of understanding of normal CNS function has made the development of animal models for the study of particular neurological diseases, such as Alzheimer's, rather problematic and has demonstrated how useful transgenics can be in this respect (Lathe & Morris, 1994). However, results obtained from transgenic studies, although very useful, have to be interpreted carefully as it is not clear whether compensatory mechanisms are involved in the development of these animals. In addition the background genotype may contribute to some of the observations made about these animals (Gerlai, 1996).

1.9 Aims of this research

This chapter has covered the study of cognitive function in a variety of animal models, the mechanisms involved in the induction of LTP and its relationship to cognition and plasticity. In addition evidence which reveals the identity of several genes thought to be involved in the expression of LTP has been presented.

The research carried out in this volume has been undertaken in an attempt to investigate further which genes are involved in plasticity-related changes within the hippocampus, confirm (or dispute!) the findings of other research groups and hopefully uncover the involvement of previously unreported genes in plasticity. Almost all the work has involved the use of in-situ hybridisation to look at mRNA levels, and two chapters have looked at changes in gene expression following the induction of LTP *in vivo* and *in vitro* in two regions of the hippocampus. We have also looked at whether drugs which affect cognition can produce changes in gene expression (Chapter 3), the effects of a

variety of glutamate receptor agonists and an analogue of cAMP on mRNA levels in the granule cells of the dentate gyrus *in vivo* (Chapter 6), the distribution of two isoforms of NCAM in the rat brain (Chapter 8) and have compared the use of in-situ hybridisation and PCR to study changes in mRNA levels (Chapter 7). Each chapter has a small introduction (more specific to the work in that particular chapter) and its own discussion. A general discussion which will attempt to link together some of the observations made can be found in chapter 9.

CHAPTER 2

MATERIALS AND METHODS

2.1 Treatment of animals/animal tissue

2.1.1 In vivo administration of compounds

Male Wistar rats (200-350g) were given intraperitoneal injections of the compounds under investigation or equivalent volumes of vehicle solutions. Animals were killed by a blow to the head followed by cervical dislocation, either 45min or 2hr after treatment. Their brains were rapidly removed and frozen on dry ice before being processed for in situ hybridisation.

Ondansetron (100ng/kg), thiopcramide maleate (10mg/kg), pilocarpine hydrochloride (8mg/kg) and scopolamine hydrobromide (1mg/kg) were all dissolved in 0.9% saline at a concentration such that 1ml/kg of solution was administered. Aniracetam (10mg/kg) was dissolved in 30% dimethylsulphoxide (DMSO)/0.9% saline solution at a concentration such that 2ml/kg of solution was injected into each animal. The pH of each solution was adjusted to between 6.5 and 7.5 before use.

2.1.2 Induction of LTP in vitro

Transverse hippocampal slices (450μm) were prepared from male Wistar rats (150-250g), overdosed with intraperitoneal urethane. After a minimum of one hour's incubation in artificial cerbrospinal fluid (aCSF) at room temperature in an atmosphere of 95%O_2/5% CO_2 saturated with water vapour, individual slices were removed to the recording chamber at 30°C and superfused with aCSF, composition (mM) KH_2PO_4 2.2, KCl 2, $NaHCO_3$ 25, NaCl 115, $CaCl_2$ 2.5, $MgSO_4$ 1.2, Glucose 10, gassed with 95%O_2/5% CO_2 at a flow rate of 2-4 ml/minute. Glass recording and concentric bipolar stimulating electrodes were positioned in the stratum pyramidale and the stratum radiatum respectively so that population spikes (PS) in area CA1 were recorded

following the stimulation of Schaffer collaterals. Only slices which exhibited stable PS which were of 6mV maximum amplitude and free of secondary potentials were used for the experiment.

All slices were stimulated continuously whilst in the chamber at 1 stimulation/minute. After 20 minutes LTP group slices were given a patterned stimulation based on theta rhythm at a stimulation strength which initially evoked 50% of the maximal PS (5 repetitions of a 20ms train delivered at 200Hz). Several more potentials were recorded and then the slices were transferred to an incubation chamber at 30°C. Each theta stimulated slice was alternated with a control to equalise factors such as time from dissection to stimulation. Control slices received 1 stimulation/minute continuously with no patterned stimulation.

After 45 minutes or 2 hours, depending on which mRNA species was to be studied, slices were removed from the incubation chamber and rapidly frozen on dry ice before being cut into 20µm sections and processed for in situ hybridisation.

2.1.3 Induction of LTP in vivo

Male Lister Hooded rats (240-300g) were anaesthetised with intraperitoneal urethane (1.8g/kg), before being placed in a stereotaxic frame with the incisor bar set at 3.3mm below the interaural line. Rectal temperature was maintained at 37°C with a heating lamp. The scalp was removed to expose the skull between bregma and lambda, and holes were drilled in the skull above the intended electrode positions. The dura was removed and the brain surface kept moist with phosphate buffered saline.

The tip of a glass microelectrode was positioned in the hilus of the right dentate gyrus (4mm posterior to bregma, 2.5mm lateral to the midline and approximately 3.2mm below the brain surface) to allow the recording of

evoked field potentials. Concentric bipolar electrodes (Clark Electromedical Instruments) were positioned bilaterally in the medial perforant path (4.4mm lateral to lambda, 2.5mm below the brain surface). Pulses of 200μA, 50μs were applied to each of the stimulating electrodes, and the depth of the recording and stimulating electrodes was adjusted to produce a maximal field excitatory postsynaptic potential. The amplitude of the test pulses was then adjusted to elicit a population spike of 1mV (typically 200-400μA).

Bilateral stimulation at the test amplitude was continued every 30 seconds for 30 minutes in order to establish baseline responses. Tetanic stimulation was then applied to the right perforant path only (three 200ms trains of pulses at 250Hz at twice the test pulse amplitude; inter-train interval was 60 seconds). A similar number of additional pulses were applied at low frequency to the control (left) perforant path. Test stimuli were then applied to both perforant paths every 30 seconds for two hours and both the slope of the excitatory post synaptic potential and population spike amplitude measured.

The animals were sacrificed 2hr after tetanisation, and their brains quickly frozen on dry ice. Once frozen the tissue was stored at -80°C until sectioned for in situ hybridisation.

2.1.4 Microapplication of compounds to hippocampal granule cell dendrites in vivo

Male Wistar rats (180-250g) were deeply anaesthetised with 4ml/kg equithesin, and placed in a Kopf stereotaxic frame. A small hole was drilled through the skull, and a 1μl Hamilton syringe needle then lowered slowly into the brain so that its position was approximately 1.8mm lateral, 4.5mm anterior and 6.0mm dorsal to the interaural point. Fine adjustments to the coordinates were made after consulting Paxinos & Watson's "The rat brain in stereotaxic coordinates" (2nd Edition, Academic Press). Two minutes after the needle had penetrated

the brain 75nl of either compound or saline was injected into the brain over a 3min period, and then after a further 2min the needle was removed and the wound closed using Ethibond polyester sutures. Marcain (0.5% bupivacaine hydrochloride) was infiltrated into the wound to provide post operative analgesia. Animals were sacrificed either 2hr or 24hr after the injection and their brains quickly frozen on dry ice. Cryostat sections (14 or 20µm) were collected and examined by dark field microscopy. Any brains found with the needle in an incorrect position were discarded. Sections were collected from the vicinity of the needle tip from all brains with correctly positioned injections and were processed for in-situ hybridisation.

Sp-Adenosine 3',5'-cyclic monophosphothioatetriethylamine (Sp-cAMPS) was prepared in 0.9% saline as a 10mM stock and 10µl aliquots were stored at -20°C until needed. Each aliquot was diluted with 90µl 0.9% saline before use to give a concentration of 1mM.

(1S,3R)-1-Aminocyclopentane-1,3-dicarboxylic acid (ACPD) was prepared in 0.9% saline as a 10mM stock and 50µl aliquots were stored at -20°C until needed. Each aliquot was diluted with 450µl 0.9% saline before use to give a concentration of 1mM.

(+)-α-Methyl-4-carboxyphenyl-glycine (MCPG) was prepared in 0.9% saline as a 20mM stock and 50ml aliquots stored at -20°C until needed. Each aliquot was diluted with 150µl 0.9% saline before use to give a concentration of 5mM.

(S)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was prepared in 0.9% saline as a 1mM stock and stored at -20°C until needed.

The pH of each solution was adjusted to between 6.5 and 7.5 before use.

As it is not possible to determine the active concentrations in this model it was assumed that all compounds were diluted 10-fold in the extracellular fluid.

2.2 In-situ hybridisation methodology

2.2.1 Preparation of tissue

14 or 20µm transverse sections of whole brain (at hippocampal level), hippocampal slices or, for distribution studies, horizontal whole brain were prepared at -24°C using a Reichert-Jung cryocut, and thaw mounted onto BDH twin frosted glass microscope slides which had been baked at 180°C for 3 hours, and coated with poly-l-lysine hydrobromide (0.1mg/ml in DEPC water). The sections were air dried and then immersed in ice-cold 4% (w/v) paraformaldehyde dissolved in 1x phosphate buffered saline (PBS) for 5 min, rinsed in 1x PBS for 2 min, and then dehydrated in rising concentrations of analytical grade ethanol (70, 90 and 100%). Slides were kept at 4°C in 100% ethanol until required for in situ hybridisation.

2.2.2 ^{35}S labelling of oligonucleotide probes

1.25µl 5x reaction buffer (Gibco), 1.3µl [^{35}S]dATP (NEN-DuPont), 1.2µl DEPC water and 1µl oligonucleotide (5ng/µl or 0.3pmol) were added to a 1.5ml eppendorf and the 0.5µl terminal transferase (Pharmacia) mixed in by pipetting. Terminal transferase was kept on ice after removal from the freezer to prevent its loss of activity.

The eppendorf was incubated at 35°C for at least 2hr, then removed and 100µl DEPC water added to stop the reaction.

The labelled oligonucleotide was separated from the unincorporated nucleotides in the 100µl sample using Nick spin columns (Pharmacia) prepared according to the instructions provided with the columns using DEPC water as a buffer.

2µl of the column eluate was analysed by liquid scintillation counting. The oligonucleotide was considered "labelled" for use if the 2µl analysed produced counts per minute of between 100 000 and 300 000. 2µl of dithiothreitol (1M) was added to the labelled oligonucleotide and it was stored at -20°C for up to a fortnight until required.

2.2.3 In-situ hybridisation reaction

In a 1.5ml eppendorf tube ^{35}S-dATP-labelled oligonucleotide probes (specific activity $1x10^7$-$2x10^7$Bq/µg) were diluted 50-fold in "minimal hybridisation buffer" (50% formamide, 20% 20xSSC) and 4µl/100µl 1M dithiothreitol (DTT) added. For displacement experiments a 25-fold excess of an unlabelled oligonucleotide was added to the mixture. The mixture was vortexed and 120µl pipetted in small drops onto each slide. Coverslips made from Parafilm (pre-cut to match the size of the slide) were then gently lowered on top of the slides using blunt forceps so that all the air was excluded. Slides were placed horizontally in 15cm closed petri dishes in an atmosphere humidified by 4x standard saline citrate (SSC) at 42°C and left overnight.

2.2.4 Post hybridisation washing

Slides were removed from the petri dishes, transferred to a rack and immersed in 1xSSC at 55°C and agitated for 30min, their Parafilm coverslips removed, then immersed in fresh 1xSSC at 55°C and agitated for 30min before being transferred to 0.1xSSC for 30min at room temperature. Slides were dehydrated in increasing concentrations of analytical grade ethanol (70, 90, 100%) and allowed to air dry before exposure to X-AR film. The length of exposure time was determined by the specific activity of the oligonucleotide probe and the abundance of that particular mRNA in the tissue.

2.2.5 *Photographic emulsion coating of slides*

Under Ilford safelight, Ilford K5 photographic emulsion was dissolved in the ratio 1:1 with 0.5% glycerol solution at 45°C . Once dissolved the solution was filtered through a gauze (to remove air bubbles) into a dipping chamber (an open ended glass chamber just large enough to contain a glass slide) at 45°C. Individual slides were then dipped once into the emulsion and allowed to air dry for at least 3hr on damp tissue paper. When dry they were placed in a light tight box with a small amount of silica gel and kept at 4°C until due for developing (usually 5x the length of exposure to film).

2.2.6 *Developing films and slides*

Film: Under safelight film was removed from cassette and placed in D-19 (2min), water (1min), UNIFIX (5min), water (15min), and then allowed to dry.
Slides: Under safelight slides were transferred to a rack and immersed in D-19 (10min), water (1min), 30% sodium thiosulphate (5min) and water (15-30min).

2.2.7 *Staining developed slides*

Slides were taken from the final wash after developing and immersed in 0.2% neutral red solution for up to 5min, then placed in water for 1min and increasing concentrations of ethanol (70, 90, 100, 100, 100%) until a suitable degree of staining was achieved. Slides were then placed in Histoclear for 5min and then fresh Histoclear for 5min before they were coverslipped using Histomount and BDH 22x64mm N° 1 coverslips, and allowed to dry overnight.

2.3 Quantification and Statistics

Developed slides or film were quantified using a computerised image analysis system NIH Image 1.52 linked to an Olympus BH-2 microscope.

2.3.1 Intraperitoneal injections

mRNA levels were quantified in area CA1 and the dentate gyrus of the hippocampus under dark field illumination (x4 magnification) except when slides were not available (scopolamine/pilocarpine) and the film was quantified under bright field illumination (x2 magnification). In both cases the background level, obtained from measurement of the white matter of the corpus callosum, was subtracted from each reading.

Results for drug treated animals were expressed as a percentage of mean values for corresponding saline treated animals. Any changes were analysed for significance using a Mann Whitney U-test or, in the case of c-fos/pilocarpine, a Wilcoxon test.

2.3.2 LTP in vitro

mRNA levels were quantified in area CA1 for 3 sections of each hippocampal slice under dark field illumination (x4 magnification) except when slides were not available and the film was quantified under bright field illumination (x2 magnification). The background level, obtained from measurement of an area of the slide/film with no tissue present was subtracted from each reading. In the case of MAP2 and αCAMKII mRNA levels were also measured in the proximal dendrites (stratum oriens and stratum radiatum).

Results were expressed as a percentage of corresponding control slices and analysed for significance using a Wilcoxon test.

2.3.3 LTP in vivo

Levels of mRNA were quantified over the cell bodies of both dentate gyri. Values were obtained from measuring the corpus callosum optical density and subtracted appropriately. In the case of αCAMKII and MAP2 mRNA levels were also measured in the proximal dendrites. The number of cells expressing proenkephalin (pENK) mRNA were counted in control and potentiated dentate gyri and used to give an indication of changes in mRNA levels.

Results were expressed as a percentage of control and any changes analysed for significance using a one (MAP2, αCAMKII) or two sided Wilcoxon test.

2.3.4 Microapplication of compounds to hippocampal cell dendrites in vivo

mRNA levels were measured in the granule cells immediately below the tip of the needle under bright field illumination (x40 magnification). For comparison mRNA levels of an adjacent part of the dentate gyrus away from the needle tip were measured and levels in a corresponding area on the contralateral side of the hippocampus. A background measurement was taken from the corpus callosum and subtracted from each set of readings.

Results for the area under the site of injection were expressed as a percentage of the part of the dentate gyrus adjacent to the site of injection, and the significance of any changes assessed using analysis of variance (ANOVA) with a post-hoc Fisher's test for multiple pairwise comparison.

2.4 cDNA preparation

2.4.1a RNA extraction (whole brain)

For developmental studies whole brain tissue was obtained from embryonic day 19 (E19), postnatal day 1 (P1), P4, P8, P12 and adult Wistar rats and frozen on dry ice. The tissue was homogenised in RNAzol B (Biogenesis) using 2ml per 100mg tissue and a volume of chloroform added (0.2ml/100mg tissue). The resulting suspension was vigorously shaken and kept at 4°C for 30min, then centrifuged at 4°C for 15min at a speed of 12000g. The upper (colourless) aqueous phase was then removed in 0.5ml aliquots to clean 1.5ml eppendorfs and an equal volume of isopropanol added. The mixture was left for at least 30min at 4°C then centrifuged for 15min at the same temperature at a speed of 12000g. The supernatant was then removed and discarded, and the pellet washed with 1ml 75% ethanol in DEPC-water. The tubes were then centrifuged for 8min at 4°C at a speed of 7500g and the supernatant removed and discarded. The final pellet of RNA was allowed to air dry for 5-10min before being dissolved in 50µl DEPC water by heating at 60°C if necessary. 10µl of the RNA solution was removed to a clean tube and used for measuring the RNA concentration and verifying the integrity of the RNA on an agarose gel.

2.4.1b RNA extraction (hippocampi)

Both hippocampi were removed from Lister hooded rats two hours after the induction of LTP (see section 2.1.3 for experimental details) and frozen on dry ice. Using Micro RNA isolation kit (Stratagene) total RNA was extracted from the tissue. Briefly, the tissue was weighed (approx. 20mg) and then homogenised in a mixture of 200µl denaturing solution (4M guanidium

isothiocyanate, 0.02M sodium citrate, and 0.5% sarcosyl) and 1.44µl β-mercaptoethanol. The homogenate was then transferred to a 1.5ml microfuge tube and 20µl 2M sodium acetate added. 200µl water saturated phenol was added and 40µl chloroform:isoamyl alcohol before vortexing the mixture vigorously. The mixture was the microcentrifuged at 13000g and the upper phase removed into a clean microcentrifuge tube. 200µl isopropanol was added, the mixture inverted, and left on ice for 30 minutes. The mixture was then centrifuged at 13000g for 5 minutes, the supernatant removed and the pellet washed with 75% ethanol in DEPC-treated water (400µl). The pellet was allowed to dry and then resuspended in 20µl DEPC treated water. The concentration and purity of the RNA was then analysed using a Gene Quant RNA/DNA calculator (Pharmacia). The optical density of each sample was measured at 260 and 280nm. From this the concentration of RNA in µg/µl was calculated and the purity of the sample (ratio) obtained.

2.4.2 Agarose gel analysis of RNA samples

Using 1x DEPC-treated TBE, a 1.2% agarose gel was prepared with 1µl/100mls ethidium bromide (10mg/ml). 1µg of RNA from each sample was mixed with 5µl RNA sample loading buffer (62.5% deionised formamide, 1.14M formaldehyde, 200µg/ml bromphenol blue, 1.25x MOPS-EDTA-sodium acetate, 50µg/ml ethidium bromide, Sigma), and the volume made up to 20µl with DEPC-water. Each sample was heated at 70°C for 10min prior to loading onto the gel to denature the RNA. A volume of 1x DEPC-treated TBE was added to the hardened gel so that it was just submerged under buffer, and each lane loaded with the whole of the 20µl sample. A voltage sufficient to produce a current of 70mA was applied to the gel for 20min and the presence of 18S and 28S bands confirmed by examination of the gel under UV light.

2.4.3 Reverse Transcription

Rat cDNA was prepared from total RNA using Superscript preamplification system (Gibco). 1µg RNA and 1µl random hexamers (50ng/µl) were mixed and the volume made up to 12µl using nuclease free water (Sigma). Each sample was then incubated at 70°C for 10 minutes and at 4°C for at least 1 minute. A reaction mixture for the reverse transcription was prepared (per µg RNA) as follows: 2µl 10xPCR buffer (200mM Tris-HCl, pH 8.4, 500mM KCl), 2µl $MgCl_2$ (25mM), 1µl 10mM dNTP mix (10mM each of dATP, dCTP, dGTP, dTTP) and 2µl DTT (0.1M), and was added to each RNA/hexamer mixture. The solution was then incubated at 25°C for 5 minutes and 1µl superscript II RT added to each tube (200units/µl). Using a Perkin Elmer Gene Amp PCR system, each sample was incubated at 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes and then chilled on ice. 1µl of E.coli RNase H (2units/µl) was added to each tube and incubated at 37°C for 20 minutes to degrade any untranscribed RNA before freezing each sample at -20°C.

2.5 Amplification of cDNA and quantification of DNA

2.5.1 Polymerase Chain Reaction (PCR)

For each cDNA sample a PCR mix was prepared as follows:

		Final Conc.
10x PCR buffer (Gibco)	5µl	1x
25mM $MgCl_2$ (Gibco)	3µl	1.5mM
nuclease free water (Sigma)	29.5µl	
GAPDH primer mix (5µM)	1µl	0.1µM
sense primer (15µM)	1µl	0.3µM

antisense primer (15μM)	1μl	0.3μM
1.25 mM dNTPs (Pharmacia)	8μl	0.2mM
cDNA (obtained from RT of 1μg RNA)	1μl	
Taq polymerase (Promega)	0.5μl	(3 units)

In practice for *n* cDNA samples the ingredients in the "mix" (all components except Taq and cDNA) were made up to (*n*+1) x volume for one reaction and 48.5μl mix added to the 1μl cDNA sample before adding 0.5μl Taq. This helped to reduce errors introduced by pipetting inaccuracies.

The samples were placed in a Perkin Elmer Gene Amp system and run for between 20 and 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 2 minutes preceded by 94°C for 2 minutes and followed by 72°C for 15 minutes, then stored at -20°C.

2.5.2 *TBE/agarose gel analysis of PCR products*

To analyse PCR fragments the products were run on a 1xTBE gel (2% agarose). 1g agarose (Gibco) was dissolved in 50mls 1xTBE by microwaving to boil, and 0.5μl ethidium bromide added. Enough 1xTBE was poured onto the gel to immerse its surface, and approx. 5μl PCR product/gel loading solution (0.005% bromphenol blue, 40% sucrose, 0.1M EDTA pH 8.0, 0.5% SDS, Sigma) in a 5:1 ratio was loaded into each lane along with a PCR marker or 100bp DNA ladder. A voltage sufficient to produce a 40mA current through the gel was applied to the gel for 20 minutes. The gel was viewed under UV light and photographed to identify the bands produced. In some cases the DNA was extracted from the gel by Southern Blotting.

To obtain good separation of the 2 NCAM fragments gels of 4% NuSieve (FMC Biochemicals) dissolved in 1xTBE were prepared.

2.5.3 *Southern Blotting*

The 2% TBE/agarose gel was denatured in 0.4M NaOH for 15 minutes then inverted and placed on top of 2 pieces of Whatman filter paper which acted as wicks immersed in 0.4M NaOH. A piece of Hybond (Amersham) nylon membrane was placed on top of the gel below the level of the slots and then covered with 2 pieces of Whatman filter paper soaked in 0.4M NaOH which completely covered the gel. About 5cm depth of disposable hand towels, cut to match the size of the whole gel, were placed on top of the Whatman paper and a weight (approx. 1kg) then placed on top of the hand towels. The arrangement was left for 3 hours to allow the DNA from the gel to be transferred to the nylon membrane. After this time the membrane was removed and rinsed in 2xSSC for a few minutes. Although it was unnecessary to do so (as NaOH is a fixative) the fragments were fixed to the membrane by cross linking using a UV Stratalinker.

2.5.4 *^{32}P labelling of DNA fragments*

20ng of each DNA fragment (obtained from the digestion reaction in section 2.5.8) was placed in 45µl 1xTE and heated at 95°C for 5 minutes. This was then added to a Rediprime tube containing a buffered solution of dATP, dGTP and dTTP, exonuclease free Klenow enzyme and 9mer random primers (Amersham), and gently mixed. 5µl ^{32}P dCTP was added and mixed by tapping before incubating at 37°C for 10 minutes. 100µl of 10mg/ml salmon sperm was added (Sigma) and the mixture incubated at 95°C for 5 minutes.

2.5.5 *Hybridisation of Southern blots*

Each blot was rinsed in 2xSSC then rolled around the inside of a hybridisation tube (DNA side inwards) and 10 or 20ml (depending on the size of the tube) prewarmed hybridisation solution added. Tubes were placed in a rolling hybridisation oven at 60°C and left for one hour.

1ml of fresh hybridisation solution was added to each ^{32}P-labelled DNA probe and 200/400µl of the mixture added to 20/10ml hybridisation solution in each tube so that each tube contained GAPDH and one other DNA probe The blots were left to hybridise overnight at 60°C.

Control blots were hybridised to establish whether there was any cross reactivity between the DNA probes.

2.5.6 *Post hybridisation washing (Southerns)*

Blots were washed with fresh solutions of 2xSSC and 0.1%SDS in a large container (30 min/wash) at 60°C until all the non-specific labelling had been removed (estimated by monitoring the blots using a hand held series-900 minimonitor). Blots were wrapped in cling film and exposed to a phosphor screen overnight.

2.5.7 *Analysis of results*

The phosphor screens were scanned to produce images of each Southern blot and the strength of signal quantified using Image Quant. In most cases the strength of signal of each band of interest was expressed as a percentage of GAPDH signal.

2.6 DNA manipulation for sequencing and ^{32}P labelling

2.6.1 TAE/agarose gel analysis of PCR products

To obtain PCR fragments for ligation, the products were run on a 1xTAE gel (2% agarose). 1g agarose (Gibco) was dissolved in 50mls 1xTAE by microwaving, and 0.5µl ethidium bromide added. Enough 1xTAE was poured onto the gel to immerse its surface, and approx. 50µl PCR product/gel loading solution (0.005% bromophenol blue, 40% sucrose, 0.1M EDTA pH 8.0, 0.5% SDS, Sigma) in a 5:1 ratio was loaded into each lane along with a PCR marker or 100bp DNA ladder (Promega). The PCR products were separated according to size by applying 40-60mV across the gel for 20-30minutes. Products of the appropriate size were cut out of the gel using a new clean scalpel blade for each band, and placed in a 1.5ml microcentrifuge tube, and stored at 4°C until purified for the ligation reaction.

2.6.2 Recovery of PCR fragments from the TAE gel

PCR fragments were recovered from the gel using the Wizard DNA Clean Up kit (Promega). Briefly, the gel containing the fragment was melted in 400µl sodium iodide (6M) at 80°C and 1ml Clean-Up resin added. The mixture was then inverted and filtered on a vacuum manifold using a 5ml syringe and Wizard minicolumn. 2ml 80% isopropanol was added to the syringe barrel and drawn through the column followed by air for 1-2minutes. The minicolumn was then placed in a microcentrifuge tube and centrifuged for 2 minutes at 12000g to remove any residual isopropanol. 15µl prewarmed nuclease free water (70°C) was applied to the minicolumn and left for 1minute. The column was then centrifuged again at 12000g for 2minutes to elute the DNA.

2.6.3 *Ligation into the pTAg vector*

The purified fragments were inserted into the pTAg vector using The LigATor (R & D systems). For each ligation a reaction mixture was prepared as follows: 1µl 10x ligase buffer (200mM Tris-HCl pH 7.6, 50mM $MgCl_2$), 0.5µl 100mM DTT, 0.5µl 10mM ATP and 1µl pTAg vector (50ng/µl), and added to a 2µl of recovered fragment and 4.5 µl nuclease free water. The mixture was microfuged, vortexed and microfuged, and 0.5µl T4 DNA ligase added by careful mixing. The tubes were left at 16°C for 72hr.

2.6.4 *Transformation Reaction*

For each transformation a 1.5ml microcentrifuge tube was cooled on ice and 20µl of competent cells (provided with The LigATor) carefully pipetted into the tube. 1µl of each ligation reaction was added to each 20µl aliquot of competent cells and mixed by gentle tapping. The mixture was incubated on ice for 30 minutes and then heat shocked at 42°C for 50 seconds, before incubating on ice again for 2 minutes. 80µl SOC medium was then added to each tube and the mixture shaken (200-250rpm) at 37°C for 1 hr.

2.6.5 *Plating the transformants*

Each LB agar (+ampicillin 50mg/ml) plate was pre-treated with 40µl X-Gal (50mg/ml) and 40µl IPTG (100mM). 50µl of each of the transformation reactions was spread onto each plate, and the plates incubated overnight at 37°C.

2.6.6 *Screening of colonies*

pTAg contains a LacZα peptide sequence, which, when functionally produced, complements the N-terminal truncated LacZ peptide synthesised in the competent cells provided. The resulting enzyme, β-galactisidase, cleaves X-Gal to give blue colonies. When an insert is cloned into the cut pTAg vector the LacZα peptide sequence is interrupted and the resultant interference with the function of the peptide produces white colonies.

Several white colonies from each plate were removed and placed into 10µl of nuclease free water. 5µl of each aliquot was removed and heated at 96°C for 10minutes to denature the DNA. A PCR reaction mixture was prepared as follows: 2.5µl 10x PCR buffer (200mM Tris-HCl, pH 8.4, 500mM KCl; Gibco), 2µl MgCl2 (25mM; Gibco), 2µl dNTP mix (1.25mM each of dATP, dCTP, dGTP, dTTP; Pharmacia), 1µl each of appropriate primers (15pmol/µl), 13.5µl nuclease free water, and added to 2.5µl of the denatured DNA. 0.5µl (3units) Taq polymerase (Promega) was added and the tubes placed in a Perkin Elmer Gene Amp system for 25 cycles of 95°C for 45 seconds, 55°C for 45 seconds and 72°C for 45 seconds, preceded by 95°C for 3 minutes. 5µl of each PCR product was mixed with 1µl gel loading solution (Sigma) and loaded onto a 2% agarose gel (dissolved in 1xTBE; 1µl/100mls ethidium bromide added). The separated products were examined under UV light and compared to a 100bp ladder (Promega) to see whether the correct sized fragments had been successfully inserted into the pTAg vector.

For colonies that were found to contain the correct insert, the remaining 5µl of colony and nuclease free water was placed into 5mls L-Broth with ampicillin (50mg/ml) and shaken overnight at 37°C (200-250 rpm).

2.6.7 *Purification of vector containing the DNA insert*

DNA was purified using Wizard minipreps purification system (Promega). 3ml of each L-Broth/colony mixture was microcentrifuged for 1-2 minutes at 12000g to obtain a pellet which was then resuspended in 200µl cell resuspension solution (50mM Tris-HCl pH 7.5, 10mM EDTA, 100µg/ml RNase A). 200µl of cell lysis solution was added (0.2M NaOH, 1% SDS) and the tube inverted several times until the suspension cleared. 200µl neutralisation solution was added (1.32M potassium acetate pH 4.8) and mixed by inversion. The tube was microcentrifuged at 12000g for 5 minutes and the clear supernatant removed into a clean microcentrifuge tube. 1ml of Wizard minipreps DNA purification resin was added to the supernatant and mixed by inverting the tube. The mixture was then filtered using a vacuum manifold and a 5ml syringe with a Wizard mini column. 2ml Column Wash solution (80mM NaCl, 8mM Tris-HCl pH 7.5, 2mM EDTA in 55% Ethanol) was added to the syringe barrel and drawn through the column using the vacuum. The resin was dried by drawing air through for a further 30 seconds, placing the minicolumn in a microcentrifuge tube and centrifuging at 12000g for a further 2 minutes. After transferring the column to a fresh microcentrifuge tube 50µl hot water was added and left for 1 minute. The minicolumn was then finally centrifuged at 12000g and the eluent containing the DNA stored at -20°C.

Production of larger quantities of DNA was achieved using Wizard midipreps (Promega). The same method was used, but larger volumes of reagents. Colonies containing the insert were seeded into 50ml L-broth and 3mls resuspension, lysis and neutralisation solution used, then 10mls purification resin and 30mls column wash solution. The DNA was eluted from the column using 300µl hot water, and the concentration measured using a Gene Quant RNA/DNA calculator (Pharmacia). The DNA obtained was used for automated sequencing.

2.6.8 *Extraction of the DNA insert from the pTAg vector (for probing Southern Blots)*

Restriction maps of each inserted fragment of DNA and pTAg were compared so that suitable enzymes could be chosen for "cutting out" each DNA insert from the vector whilst leaving the insert intact.

A digestion reaction was prepared by mixing 10µg of whole DNA obtained from midiprep purification, 5µl SuRE/Cut 10x buffer B for restriction enzymes (100mM TrisHCl, 50mM $MgCl_2$, 1M NaCl, pH 8.0; Boehringer Mannheim) and 2.5µl each of EcoRI and HindIII. The volume was made up to 50ml using nuclease free water (Sigma) and the reaction incubated at 37°C for 2hours.

The whole of the 50µl mixture was added to 10µl gel loading solution (Sigma) and separated on a 2% agarose/1xTBE gel (section 2.5.2). Bands of the appropriate size were cut out of the gel under UV light and the DNA extracted from them using Wizard DNA clean up kit (Promega) (section 2.6.2) eluting the DNA in 50µl hot nuclease free water. The concentration of DNA in the sample was measure using a Gene Quant RNA/DNA calculator (Pharmacia).

2.6.9 *Preparation of DNA sample for automated sequencing*

Using the purified DNA obtained using Wizard midipreps (section 2.6.7), 0.5µg each sample was mixed with 8µl Premix (ABI), 1µl T7 promoter primer (Promega) and the volume made up to 20µl using nuclease free water. The Perkin Elmer Gene Amp was used for amplification of the sequence. Tubes were added to the machine once it had reached 94°C then 25 cycles of 95°C for 15 seconds, 50°C for 1 second and 60°C for 4 minutes.

90µl of water was added to the reaction products and 100µl of ABI phenol:water:chloroform (68:18:14) added. The mixture was vortexed and centrifuged at 14000g for 2 minutes. 105µl of the top aqueous layer was removed and placed into a clean eppendorf. 100µl of phenol:water:chloroform was added to the aqueous layer and the mixture vortexed and centrifuged at 14000g for 2 minutes. 93µl of the top aqueous layer was removed into a clean eppendorf tube and 10µl 3M sodium acetate and 300µl of 100% cold ethanol added. The solution was mixed and placed on ice for 15 minutes. The tubes were centrifuged at 14000g for 15-20minutes and the supernatant removed. The pellet was washed in 70% ethanol, and then dried in a thermal cycler for 1 minute at 90°C. The pellet was then stored at -20°C until required for automated sequencing (ABI).

2.7 Materials

2.7.1 Drugs

Pilocarpine hydrochloride and scopolamine hydrobromide were purchased from Sigma. Ondansetron and thioperamide maleate were a gift from Glaxo. Aniracetam was purchased from Research Biochemicals International.

Sp-cAMPS was purchased from Research Biochemicals International. ACPD, MCPG, and AMPA were purchased from Tocris.

Equithesin: 16.2ml sodium pentobarbitone (60mg/ml), 4.25g chloral hydrate, 2.1g MgSO4, 39.6ml propylene glycol, 10ml ethanol made up to 100ml with water.

2.7.2 *PCR primers*

These were designed by entering the full sequence of T26, αCAMKII and Egr3 mRNA into Gene Works and specifying ranges for the length of the amplified sequence (100-300bp), the length of the primers (18-22bp), and the percentage GC content (45-55%).

Optimum primers were all screened for homology with sequences in the GenBank database using FastA criteria and only specific primers were used.

αCAMKII (Accession code J02942):

Antisense primer complementary to bases 309-289 of rat calcium/calmodulin-dependent protein kinase II α-subunit mRNA, 21mer.

- CGAAGATAAGGTAGTGGTGCC-

Sense primer corresponding to bases 175-195 of calcium/calmodulin-dependent protein kinase II α-subunit mRNA 20mer.

-ACACCAAGAAGCTCTCAGCC-

Egr3 (Accession code U12428):

Antisense primer complementary to bases 331-312 of Egr 3/pilot mRNA, 20mer.

-ATACATGGCCTCCACGTCTC-

Sense primer corresponding to bases 194-214 of Egr3/pilot mRNA, 21mer.

-ATCCGGAACTCTCTTATTCGG-

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Accession code X02231):

Antisense primer complementary to bases 775-756 of rat mRNA for GAPDH, 20mer.

-GTAGGAACACCGAAGGCCAT-

Sense primer corresponding to bases 283-302 of rat mRNA for GAPDH, 20mer.

-CCATCACCATCTTCCAGGAG-

NCAM (Accession code X06564):

Primers for NCAM (which produced PCR products with and without the VASE exon) were obtained from Small & Ackeson (1990).

Antisense primer complementary to bases 1346-1317 of rat mRNA for 140kD NCAM polypeptide, 30mer.

-AGGACACACGAGCATGGCTGCGTACCACCA-

Sense primer corresponding to bases 1229-1256 of rat mRNA for 140kD NCAM polypeptide, 28mer.

-ACCTGGAGAACGTCCACCCGAAACATCA-

Tubulin 26 (Accession code V01226):

Antisense primer complementary to bases 507-488 of the non-coding region of rat tubulin 26, 20mer.

-CATCGATGCCTGCAGCTAGT-

Sense primer corresponding to bases 380-398 of α-tubulin, 19mer.

-AGGAGGTTGGCGTTGATTC-

2.7.3 Oligonucleotides for in- situ hybridisation

All 45-mer unless stated otherwise

activity-regulated cytoskeletal protein (arc) (42mer):complementary to nucleotides spanning amino acid residues 26-39 (Lyford *et al.*, 1995)

c-fos and zif/268: complementary to nucleotides spanning amino acid residues 1-15 and 2-16 respectively (Wisden *et al.*, 1989).

α-calcium/calmodulin-dependent protein kinase II (αCAMKII): complementary to nucleotides spanning amino acid residues 408-422 (Lin *et al.*, 1989).

candidate plasticity gene (cpg): complementary to nucleotides 219-264 (Nedivi *et al.*, 1993)

microtubule associated protein 2 (MAP2): complementary to the coding region for the N-terminal 15 amino acid region common to all MAP2 isoforms (Kindler *et al.*, 1990).

N-FLANK (42-mer): complementary to nucleotides spanning amino acid residues 329-335 and 346-351 on NCAM (Small & Akeson, 1990).

N-VASE (42-mer): complementary to nucleotides spanning amino acid residues 334-347 on NCAM (Small & Akeson,1990).

prodynorphin (pDYN): complementary to bases 862-906 (Civelli *et al.*, 1985).

proenkephalin (pENK) (36-mer): complementary to bases 400-435 (Yoshikawa *et al.*, 1984).

tissue inhibitor of metalloproteinases (TIMP) (54mer): complementary to nucleotides 76-130 in the mouse (Edwards *et al.*, 1986)

Tubulin T_{26}: complementary to part of the 3' non-coding region

-GGGAAACAGCATAGAAGCATCGATGCCTGCAGCTAGTGCTGGAGC-

(Miller et al., 1987).

25kD synaptosomal-associated proteins a & b (Boschert *et al.*, 1996):

(SNAPA) (50-mer): bases 60-11, exon 5a,

5'-CGGCCTCCTTCATGTCTTGGTTGATCTGGTTCATGCCTTCTTCGACACGG-3'

(SNAPB) (50-mer): idem exon 5b,

5'-CTGCTTCTTTCATATCCTTATTGATTTGGTCCATCCCTTCCTCAATGCGT-3'

2.7.4 Solutions

Diethylpyrocarbonate (DEPC) (Sigma) treated water:

- 1ml DEPC (1.12g/l) per litre deionised water. The solution is then autoclaved to inactivate the DEPC.

10xPBS:

- 75.97g NaCl, 9.94g Na_2HPO_4, and 4.68g NaH_2PO_4 dissolved in 1 litre deionised water then treated with DEPC (1ml/l) and autoclaved.

20xSSC:

- 175.3g NaCl and 88.1g Na citrate dissolved in 800ml deionised water. pH adjusted to 7.0 using 1M NaOH and the volume made up to 1 litre using deionised water. The solution is then treated with DEPC (1ml/l) and autoclaved.

1M Dithiothreitol (DTT) (Sigma):

- 3.09g DTT dissolved in 20ml 0.01M Na acetate buffer (pH5.2), and sterilised by filtration.

Minimal hybridisation buffer for in situ hybridisation:

- 25ml formamide (Fluka), deionised with 2.5g Amberlite monobed MB-1 resin (BDH).
- 10ml 20xSSC
- 2.5ml Na phosphate buffer (0.5M, pH7.0). Buffer is prepared by mixing 0.5M NaH_2PO_4 and 0.5M Na_2HPO_4, until the pH reaches 7.0 then DEPC treated (1ml/l) and autoclaved (approx. ml NaH_2PO_4 and ml Na_2HPO_4).
- 0.5ml Na pyrophosphate (0.1M).
- 1ml polyadenylic acid [5'], potassium salt (5mg/ml) (Sigma).
- 5g dextran sulphate (Pharmacia)

The solution is then made up to 50mls with DEPC treated water.

Poly-l-lysine hydrobromide (Sigma):

- 25mg dissolved in 5ml DEPC treated water then aliquoted into 1ml microcentrifuge tubes. Each aliquot was added to 50ml DEPC treated water before use and used to coat microscope slides.

0.2% Neutral Red (BDH):

- 1g Neutral Red dissolved in 500ml distilled water and 1ml glacial acetic acid added.

Hybridisation solution (Southern blots)

- 200mls 5M NaCl, 100ml 10% SDS, 2ml EDTA (0.5M), 100g dextran sulphate and 700ml deionised water.

L-Broth

- 10g bacto-tryptone, 5g bacto-yeast extract and 10g NaCl dissolved in deionised water and the pH adjusted to 7.0 using NaOH. The volume is then made up to 1 litre and the solution autoclaved.

L-Broth Agar

- 15g agar added to 1 litre L-Broth and autoclaved. The solution is then cooled to 40-50°C and 1ml of a 50mg/ml ampicillin solution added. 30-35ml of the mixture is then poured in to each 10cm petri dish and allowed to harden. The plates can then be stored for up to a month at 4°C.

Tris-EDTA (TE)

- 10mls of 1M TrisCl (pH 8.0) and 2mls 0.5M EDTA (pH 8.0) mixed and the volume made up to 1 litre using deionised water.

10x Tris-borate (TBE)

- 108g Tris base, 55g boric acid and 40ml 0.5M EDTA (pH8.0) in 1 litre of deionised water. Dilutions of the stock are made as required.

10x Tris-acetate (TAE)

- 48.4g Tris base, 11.42ml glacial acetic acid and 20mls 0.5M EDTA (pH 8.0) in 1 litre deionised water. Dilutions of the stock are made as required.

SOC medium (per litre)

- 20g bactotryptone, 5g bacto-yeast extract and 0.5g NaCl dissolved in 950mls deionised water and 10mls KCl (250mM) added. pH adjusted to 7.0 with NaOH (5M) then volume made up to 1 litre with deionised water

and autoclaved. Before use add 5ml sterile 2M $MgCl_2$ and 20ml of sterile 1M glucose.

2.7.5 Chemicals

X-AR film, D-19 and UNIFIX (Kodak)

DMSO, paraformaldehyde, glycerol (Sigma)

^{35}S-dATP (NEN)

K5 photographic emulsion (Ilford)

Histoclear and Histomount (National Diagnostics)

Analytical Grade Ethanol (AnalaR)

X-Gal (Promega)

IPTG (Promega)

CHAPTER 3

THE EFFECT OF DRUGS WHICH AFFECT COGNITION ON IMMEDIATE EARLY GENE EXPRESSION

3.1 INTRODUCTION

3.1.1 The hippocampus and cognition

The hippocampus has been known to be important for the initial storage of declarative memory in humans since 1957 (Scoville & Milner). Since then it has been suggested, following studies in primates, that the hippocampus is used initially for the storage of information before it is consolidated in the neocortex (Squire & Zola-Morgan, 1991), and that it is also involved in spatial learning (Morris *et al.*, 1982). Although its exact role has not yet been fully defined it is widely believed to be involved in cognitive function.

3.1.2 Pharmacological manipulation of cognitive function

Several classes of compound have been shown to affect the acquisition and/or storage of information. It is known that central cholinergic systems are involved in the modulation of learning and memory, and it is thought that the loss of memory associated with Alzheimer's disease is due to decreased cortical concentrations of choline acetyl transferase and degeneration of cholinergic cells (Whitehouse *et al.*, 1982; Coyle *et al.*, 1983). Muscarinic receptor antagonists such as scopolamine are used experimentally to produce amnesia and impair learning (Bartus & Johnson, 1976; Beninger et al., 1986), and muscarinic agonists have been shown to improve memory (Haroutunian *et al.*, 1985; Perio *et al.*, 1989), and improve spatial learning in in rats with lesions of the forebrain cholinergic system (Hodges *et al.*, 1996).

Other compounds which have been shown to improve memory are piracetam-like nootropics. Although these compounds were discovered almost 30 years ago their mechanism(s) of action have yet to be fully determined. Aniracetam has been shown to improve social (short-term) memory in rats (Perio *et al.*,

1989), and improves cognitive function following impairment by a wide variety of experimental procedures (Cumin *et al.*, 1982). Although it is also known that aniracetam is capable of potentiating responses induced by the activation of AMPA receptors and EPSPs in mossy fibre-CA3 and Schaffer collateral-CA1 synapses (Ito *et al.*, 1990) it is not known whether these effects contribute to its ability to improve cognitive performance.

Two compounds which have been shown to affect cognitive processes indirectly are ondansetron (a 5-HT_3 receptor antagonist) and thioperamide (a H_3 receptor antagonist). Using in vivo microdialysis they have both been shown to increase basal acetylcholine levels in the rat hippocampus (O'Shaughnessy, unpublished observations). Ondansetron has also been shown to protect against cognitive deficits induced by scopolamine in the marmoset (Carey *et al.*, 1992) and to improve spatial learning in rats with lesions of the forebrain cholinergic system (Hodges *et al.*, 1996). It also increases the magnitude and duration of LTP in the CA1 region of the hippocampus (Staübli & Xu, 1995). Thioperamide ameliorates scopolamine induced learning deficits and increases levels of choline in mouse brain (Miyazaki *et al.*, 1995a & b).

3.1.3 Aims of the study

A strong relationship appears to exist between LTP and cognition (Morris *et al.*, 1986; Davis *et al.*, 1992) although the underlying mechanisms are still unclear, and it has recently been reported that the induction of LTP and activation of NMDA receptors may not necessarily underlie all forms of learning (Bannerman *et al.*, 1995; Saucier & Cain, 1995). As it is known that the maintenance of LTP is accompanied by the expression of several immediate early genes (Cole *et al.*, 1989; Wisden *et al.*, 1990; Richardson *et al.*, 1992; Chapter 4, this volume) and c-fos deficient mice show impairment of certain

forms of learning (Paylor *et al.*, 1994), it is conceivable that some of the compounds which affect cognitive function might do so by modulating this process. We have tested this hypothesis by looking for evidence of changes in mRNA levels of zif/268 and c-fos in the hippocampus following intraperitoneal administration of several compounds from the different classes previously mentioned.

3.2 RESULTS

3.2.1 zif/268 mRNA levels

Figure 3.2.1a shows zif/268 mRNA levels in the dentate gyrus 45 minutes after i.p. administration of thioperamide, ondansetron and aniracetam. Results are expressed as mean ± sem compared to saline treated animals, and mRNA levels were not significantly different from those seen in saline treated animals.

No data were available for zif/268 expression in the dentate gyrus following scopolamine and pilocarpine treatment as levels of mRNA were not high enough to be distinguished from background levels.

Figure 3.2.1b shows zif/268 mRNA levels in the CA1 region of the hippocampus 45 minutes after i.p. administration of scopolamine, pilocarpine, thioperamide, ondansetron and aniracetam. Results are expressed as mean ± sem compared to saline treated animals, and mRNA levels were not significantly different from those seen in saline treated animals.

3.2.2 c-fos mRNA levels

Basal levels of c-fos mRNA are almost zero. No c-fos mRNA was detected in either the CA1 or dentate gyrus areas of the hippocampus 45 minutes after i.p. administration of saline, scopolamine, thioperamide, ondansetron and aniracetam. Figure 3.2.2.1 shows levels of c-fos mRNA in the CA1 region of the hippocampus 45 minutes after i.p. administration of 0.9% saline (a) and 8mg/kg pilocarpine (b). Levels of c-fos mRNA were shown to be significantly increased 45 minutes after administration of pilocarpine (Figure 3.2.2.2).

Figure 3.2.1a

Percentage change in zif/268 mRNA levels in the dentate gyrus 45 minutes after i.p administration of thioperamide (10mg/kg), ondansetron (100ng/kg) and aniracetam (10mg/kg). Results shown are mean $\pm$ sem compared to results obtained from saline treated animals. Changes were not significant (Mann-Whitney U test).

(n = 2-11)

Figure 3.2.1a

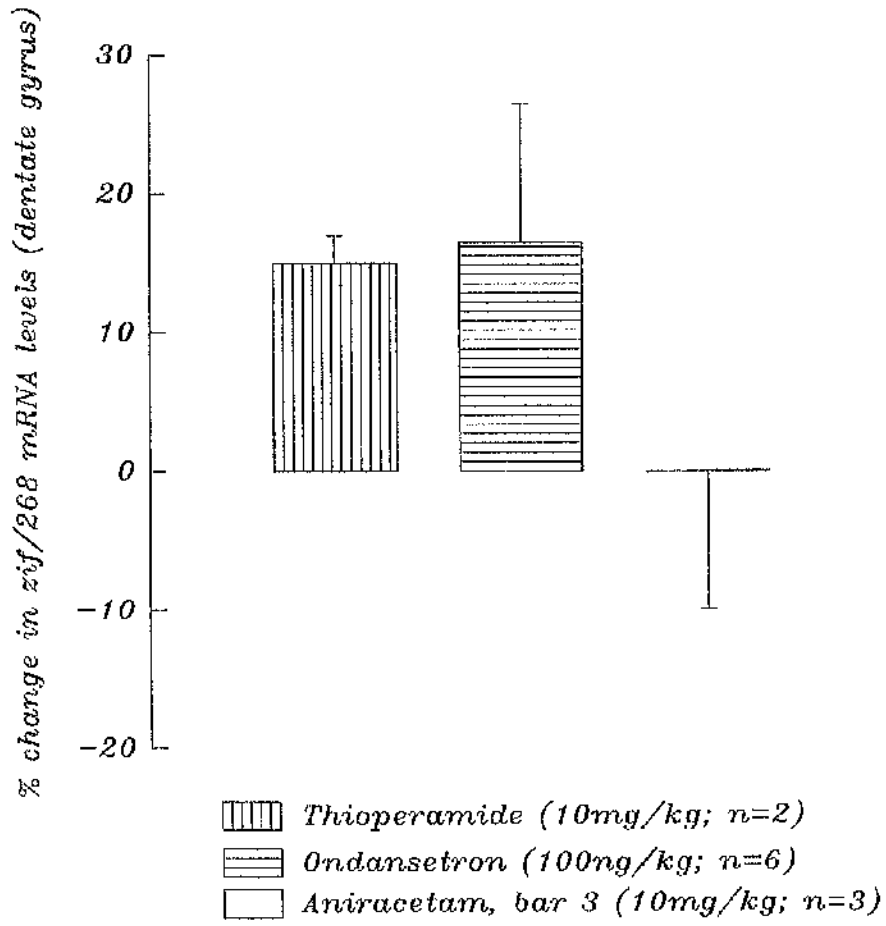

% change in zif/268 mRNA levels (dentate gyrus)
30
20
10
0
−10
−20
Thioperamide (10mg/kg; n=2)
Ondansetron (100ng/kg; n=6)
Aniracetam, bar 3 (10mg/kg; n=3)

Figure 3.2.1b.

Percentage change in zif/268 mRNA levels in the CA1 region of the hippocampus after i.p. administration of scopolamine (1mg/kg), pilocarpine (8mg/kg), thioperamide (10mg/kg), ondansetron (100ng/kg) and aniracetam (10mg/kg). Results shown are mean ± sem compared to results obtained from saline treated animals. Changes were not significant (Mann-Witney U test).

(n = 2-16)

Figure 3.2.1b

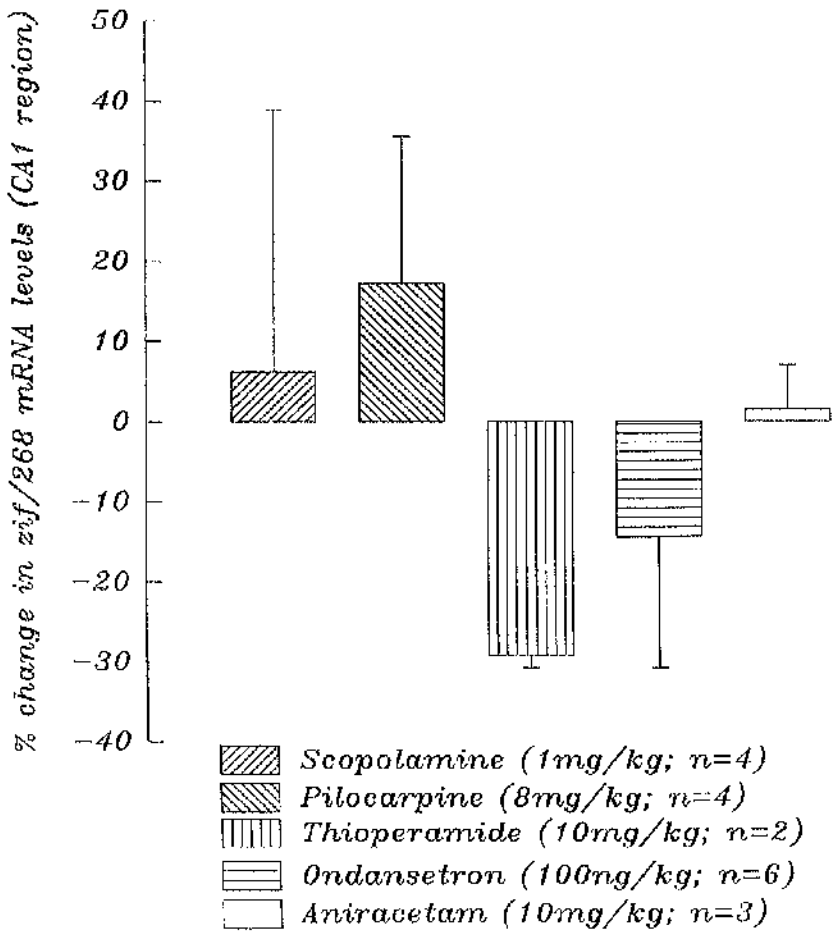

% change in zif/268 mRNA levels (CA1 region)
50
40
30
20
10
0
−10
−20
−30
−40
Scopolamine (1mg/kg; n=4)
Pilocarpine (8mg/kg; n=4)
Thioperamide (10mg/kg; n=2)
Ondansetron (100ng/kg; n=6)
Aniracetam (10mg/kg; n=3)

Figure 3.2.2.1

Photomicrograph showing c-fos mRNA levels in the CA1 region of the hippocampus 45 minutes after administration of 0.9% saline (1ml/kg) (a) and 8mg/kg pilocarpine (b). Scale bar = 50μm

Figure 3.2.2.1

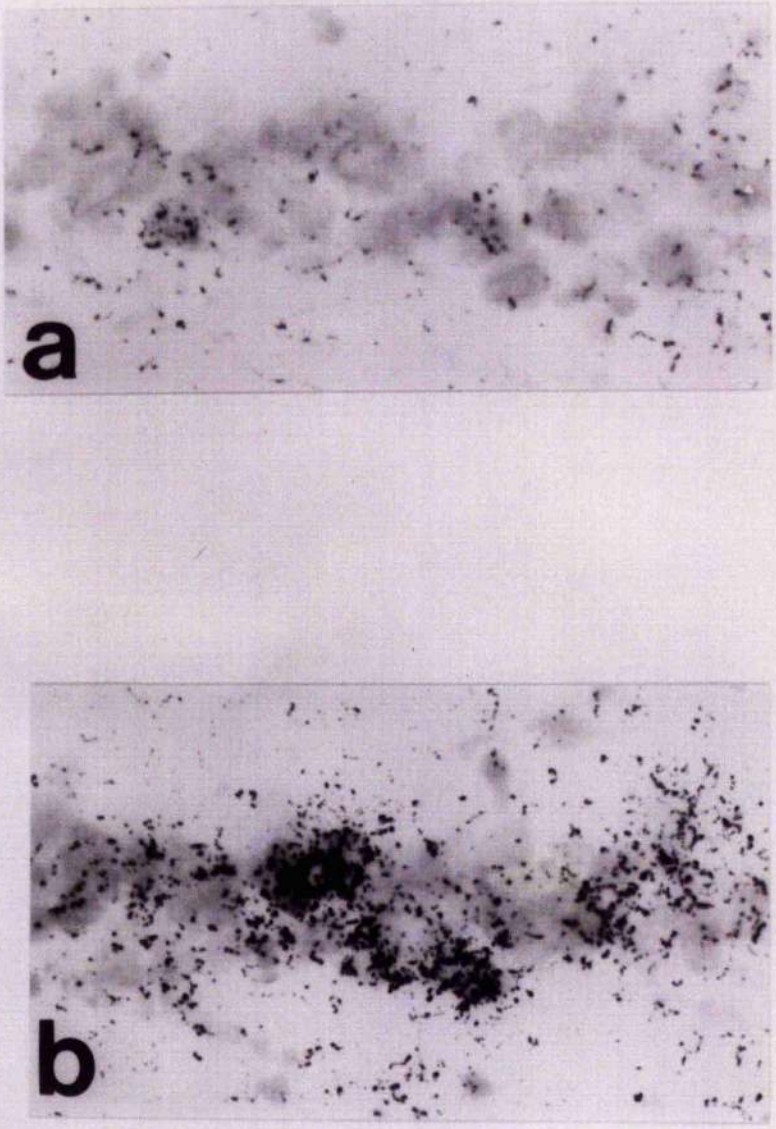
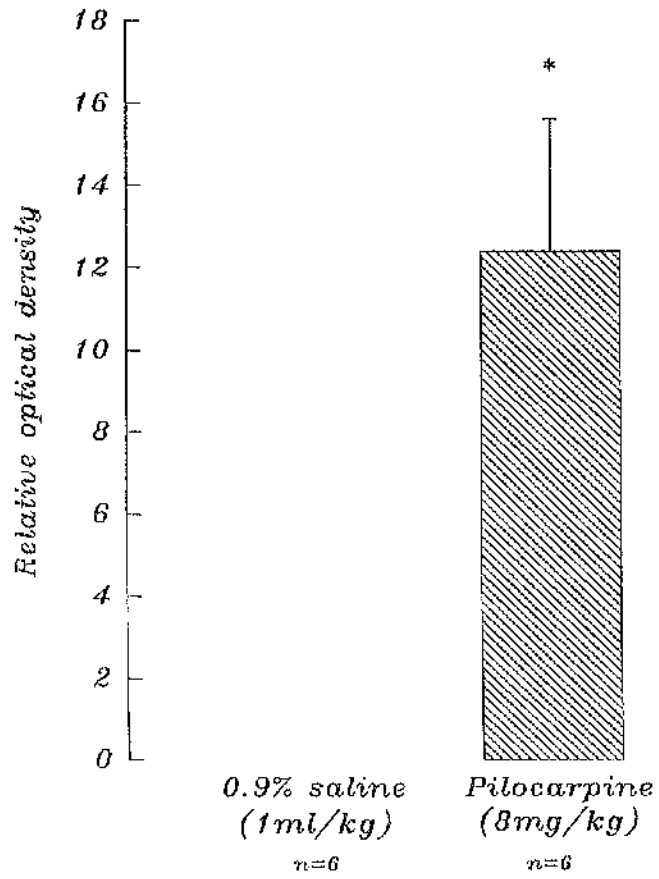


Figure 3.2.2.2

Levels of c-fos mRNA in the CA1 region of the hippocampus 45 minutes after i.p. administration of 8mg/kg pilocarpine and 0.9% saline (1ml/kg). Results are expressed as relative optical density $\pm$ s.e.m from 6 animals.

(*$p<0.05$, Wilcoxon test)

Figure 3.2.2.2


18
16
14
12
10
8
6
4
2
0
Relative optical density
*
0.9% saline
(1ml/kg)
n=6
Pilocarpine
(8mg/kg)
n=6

3.3 DISCUSSION

The aim of this work was to test the hypothesis that the mechanism of action of some or all of the compounds we administered might involve modulation of zif/268 and c-fos expression.

3.3.1 Effects of pilocarpine on c-fos and zif/268 expression

We have shown that 45 minutes after ip administration of 8mg/kg pilocarpine c-fos mRNA levels in the CA1 region of the hippocampus are significantly increased. Previous studies (Hughes & Dragunow, 1993) have shown that levels of c-fos protein are increased in area CA1 following ip administration of 25mg/kg pilocarpine. This higher dose induces seizures in rats and is also sufficient to increase zif/268 mRNA levels in the hippocampus (Hughes & Dragunow, 1994). We did not observe any changes in zif/268 mRNA levels in CA1 at our lower dose, although it was still a pharmacologically active dose as the animals displayed signs of increased gastric motility and lacrimation. This might suggest that the threshold for induction of zif/268 expression following muscarinic receptor activation is higher than for c-fos, and that increased expression of zif/268 mRNA is brought about by the seizures rather than by muscarinic receptor activation (Dragunow *et al.*, 1994). Muscarinic agonists have been shown to enhance LTP in area CA1 (Blitzer *et al.*, 1990) and it is therefore possible that the induction of c-fos following muscarinic receptor stimulation is involved in this enhancement. It has also been shown that c-fos mRNA expression is altered by certain behavioural situations (Nikolaev *et al.*, 1992; Hess *et al.*, 1995a & b), but is unclear how important these results are given the difficulty in designing experiments in which behavioural and biochemical observations correspond. Although it is generally agreed that cholinergic transmission and therefore muscarinic (and nicotinic) receptors are

fundamental for correct functioning of cognitive processes, their exact roles are unclear and this makes the drawing of firm conclusions regarding these observations difficult.

3.3.2 zif/268 mRNA levels

Levels of zif/268 mRNA have been shown to be increased following the induction of LTP in both the dentate gyrus and CA1 in vivo and in vitro (Cole *et al.*, 1989; Wisden *et al.*, 1990; Mackler *et al.*, 1992; Richardson *et al.*, 1992; Chapter 4, this volume). Our observations demonstrate the failure of administration of a single ip dose of each compound to affect zif/268 expression in the hippocampus. Taken together these data might suggest that LTP is not linked to cognition, but it seems more likely that the relationship between induction of LTP, expression of IEGs, learning and memory is not straightforward and unlikely to be revealed by one simple experiment such as this. There is also the strong possibility that other genes, characterised or not may be involved in the mechanism of action of some or all of these compounds. Although Wisden *et al.* (1990) also demonstrated that zif/268 mRNA levels were no different in animals which had been trained in the Morris' water maze, Wallace *et al.* (1995) showed that animals which were group housed in a complex environment had significantly higher levels of zif/268 mRNA than those housed individually. It is difficult to estimate the importance of these observations when designing an experiment in which the time courses for peak expression of a particular gene and demonstration of an improvement/deficit in cognitive function overlap is so problematic. Once the mechanisms of action of some of these compounds are known it may make interpretation of these results easier. If the mechanisms of action of these compounds are due purely to localised enhancements of neurotransmission, as has been reported for aniracetam (Ito *et al.*, 1990), and are not sufficient to bring about changes in

gene expression then this might account for our not observing any increases or decreases in IEG expression. With so much evidence in favour of a role for zif/268 in the maintenance of LTP the absence of any regulation of its expression by the compounds which we have studied is not sufficient to dismiss its importance.

3.3.3 Conclusions

Increased expression of c-fos mRNA following muscarinic receptor stimulation may explain the ability of muscarinic receptor agonists to enhance LTP in vitro. The lack of effect of the majority of compounds tested in producing changes in zif/268 and c-fos mRNA could be interpreted in several different ways, but the most likely explanation is that while these compounds enhance learning processes in progress they do not themselves bring about learning in the absence of a behavioural stimulus. These results are not sufficient evidence to abolish or question the theory that LTP and cognition are linked, and merely seem to further illustrate of how complex the relationship between LTP, cognition and gene expression is.

CHAPTER 4

CHANGES IN HIPPOCAMPAL GENE EXPRESSION ASSOCIATED WITH THE INDUCTION OF LTP IN VITRO

4.1 INTRODUCTION

4.1.1 Long-term potentiation

Long-term potentiation (LTP) is a lasting increase in synaptic efficiency which occurs following brief, high frequency stimulation of monosynaptic excitatory pathways (Bliss & Lømo, 1973). Hippocampal LTP has been extensively studied, and it has been clearly demonstrated that in Schaffer collateral/CA1 pyramidal cell synapses and perforant path/dentate gyrus granule cell synapses, the induction of LTP is dependent on the activation of NMDA receptors, by glutamate, and the influx of calcium ions into the post-synaptic cell (Collingridge *et al.*, 1983; Zalutsky & Nicoll, 1990). The importance of the hippocampus in the initial storage of declarative memory (Scoville & Milner, 1957), and the way in which distribution of LTP within the brain can be correlated to areas known to be important for storage of information, strongly supports the hypothesis that LTP may be linked in some way to learning and memory and be the underlying mechanism for synaptic plasticity. In addition, it has been demonstrated that the blockade of NMDA receptors by D-AP5 *in vivo* impairs spatial learning at concentrations comparable to those required for blockade of LTP *in vivo* and *in vitro* (Morris *et al.*, 1986; Davis *et al.*, 1992).

4.1.2 Gene expression following the induction of LTP

There are distinct temporal phases involved in the expression of LTP. The activation of several protein kinases and enzymes follows the initial rise in postsynaptic calcium concentration (Linden & Routtenberg, 1989; Malinow *et al.*, 1989), and leads to protein phosphorylation (Pasinelli *et al.*, 1995). Although these changes are capable of initially sustaining LTP, its maintenance for periods in excess of 3 hours is dependent on de-novo protein synthesis

(Fazeli *et al.*, 1993; Krug *et al.*, 1984; Otani *et al.*, 1989; Stanton & Sarvey 1984). Efforts have been focused on identifying which particular proteins are the most crucial for the maintenance of LTP. Most of the work on LTP has involved its induction in the perforant path/dentate gyrus granule cell synapses *in vivo*, where it has been shown that within 1 hour of the induction of LTP, mRNA and protein levels of the immediate early gene (IEG) zif/268 are increased (Cole *et al.*, 1989; Richardson *et al.*, 1992; Wisden *et al.*, 1990). It has also been shown that mRNA levels of another IEG, c-fos, are increased following induction of LTP in the same region, although the increase does appear to be dependent on the strength and type of stimulation used to induce LTP (Worley *et al.*, 1993). It is known that zif/268 and c-fos are able to act as transcription factors and regulate the expression of other genes, although the role which other downstream genes might play in the maintenance of LTP is unclear.

Several kinases have been shown to play roles in the induction of LTP. One of these is αCalcium/Calmodulin-dependent protein kinase II (αCAMKII) (Malinow *et al.*, 1989), whose activation is necessary and sufficient for the generation of LTP (Pettit *et al.*, 1994). In addition to this it has been demonstrated that transmission in the hippocampi of mice deficient in the αCAMKII gene cannot be potentiated (Silva *et al.*, 1992a). Increases in mRNA levels of αCAMKII are observed in the cell bodies of the dentate gyrus granule cells 2 hours after the induction of LTP in the perforant path/dentate gyrus granule cell synapses *in vivo*. This increase spreads to the proximal and distal dendrites where it persists for up to 48 hours (Thomas *et al.*, 1994a). Similarly levels of αCAMKII mRNA are increased in the hippocampal dentate gyrus following the stimulation of NMDA receptors (Johnston & Morris, 1995).

It has been shown that the induction of LTP triggers synaptic remodelling (Chang & Greenough, 1984; Desmond & Levy, 1986a & b; Geinisman *et al.*, 1991; Hosokawa *et al.*, 1995) and it therefore seems reasonable to assume that altered levels of certain proteins might underlie these structural changes. Although the identity of the genes which might be involved in these changes has not yet been resolved it is quite possible that cytoskeletal proteins play a role. Of particular interest is microtubule associated protein 2 (MAP2) as it has been shown that its expression promotes neurite outgrowth (Caceres *et al.*, 1988; Ferriera *et al.*, 1990), and is regulated by NMDA receptor stimulation and nitric oxide (Johnston & Morris, 1994b & e).

4.1.3 Aims of this study

Most of the changes in gene expression observed following the induction of LTP have thus been observed in the dentate gyrus granule cells following stimulation of the perforant path, and it is unclear whether similar changes occur during stimulation of Schaffer collateral-commissural pathway/CA1 pyramidal cell synapses, or if other genes might contribute to LTP in this area. It has been shown that induction of LTP in area CA1 of the hippocampus following stimulation of the commisural input did not cause an increase in zif/268 mRNA levels although stimulation of the same input to the granule cells, prevented the induction of LTP in response to perforant path stimulation, but failed to supress increases in zif/268 mRNA levels (Wisden *et al.*, 1990). If similar changes to those in the dentate gyrus were to be found following the induction of LTP in area CA1 then this would be evidence that such changes might be a fundamental part of the processes involved in the expression of LTP rather than regional-specific responses of particular cell types. We have used hippocampal slices maintained *in vitro* to study the mRNA levels of 5 genes

(zif/268, c-fos, αCAMKII, MAP2 and tubulin 26) following the induction of LTP in Schaffer collateral/CA1 pyramidal cell synapses.

4.2 RESULTS

All the slices exposed to high frequency theta rhythm stimulation exhibited a marked potentiation of population spike amplitude (83.6±5.1%; n=17), which remained elevated for at least 90 minutes after stimulation.

4.2.1 Changes in mRNA levels 45 minutes after the induction of LTP

Photomicrographs showing zif/268 hybridisation signal over the CA1 region of the hippocampus in control (a) and potentiated slices (b) 45 minutes after the induction of LTP can be seen in Figure 4.2.1.1. It is clear from these that zif/268 mRNA levels are increased 45 minutes post-tetanus. Figure 4.2.1.2 shows c-fos hybridisation signal in the CA1 region in (a) control and (b) potentiated slices. Levels were unchanged 45 minutes after the induction of LTP. The distribution of zif/268 and T26 mRNA appears to be widespread throughout area CA1 whereas c-fos mRNA is found to be localised in scattered neurones within the same region. The hybridisation signals for zif/268, c-fos and T26 were quantified and the % change in mRNA levels compared to control for each species 45 minutes after the induction of LTP are shown in Figure 4.2.1.3. While levels of T26 and c-fos were not significantly altered 45 minutes post-tetanus, zif/268 mRNA levels were significantly increased (22.2±7.6%; *$p<0.05$, n=6).

4.2.2 Changes in mRNA levels 2 hours after the induction of LTP

We looked at MAP2, αCAMKII and T26 mRNA levels 2 hours after the induction of LTP. αCAMKII and MAP2 mRNA were found in both the cell bodies of area CA1 and the dendrites, whereas expression of T26 mRNA was restricted to the cell bodies as before.

αCAMKII hybridisation signal over the molecular and dendritic layers of the CA1 region in control (a) and potentiated slices (b) can be seen in Figure 4.2.2.1. Levels of αCAMKII mRNA are clearly increased in both the cell bodies and dendrites of the CA1 region. Figure 4.2.2.2 shows MAP2 hybridisation signal over the CA1 region in the cell bodies and proximal dendrites in control (a) and potentiated slices (b). Levels of MAP2 mRNA also appear to be slightly elevated in both the cell body and dendritic layers. Figure 4.2.2.3 shows the % change in mRNA levels of αCAMKII, MAP2 and T26 two hours after the induction of LTP in the cell bodies of the CA1 region compared to non-potentiated controls. αCAMKII mRNA levels were significantly increased following the induction of LTP (21.4±7.5%; *$p<0.05$, n=7), but MAP2 mRNA levels, although increased, were not significantly different from control levels (14±16.1%; n=3). T26 mRNA levels were unchanged two hours post-tetanus.

In addition mRNA levels of αCAMKII and MAP2 were quantified in the dendritic layers of the CA1 region. Figure 4.2.2.4 shows the % change in mRNA levels in the stratum oriens (s.o.) and stratum radiatum (s.r.). Levels of αCAMKII were dramatically increased in these regions, more so than in the cell bodies. MAP2 mRNA levels were slightly increased, but the changes were not significantly different from those found in non-potentiated controls.

Figure 4.2.1.1

zif/268 hybridisation signal in the cell bodies of the CA1 region in (a) control and (b) potentiated slices 45 minutes after the induction of LTP.

Scale bar =50 μm

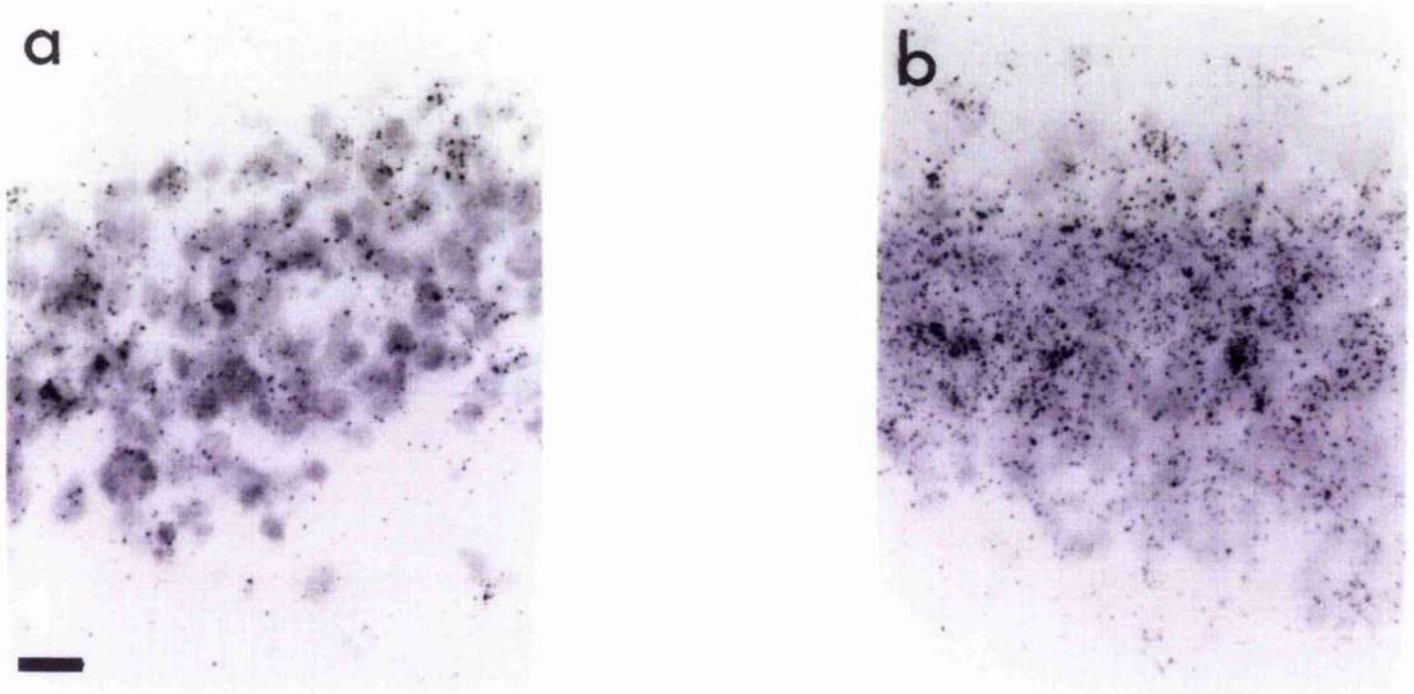


Figure 4.2.1.1
zif/268

Figure 4.2.1.2

c-fos hybridisation signal in the cell bodies of the CA1 region in (a) control and (b) potentiated slices 45 minutes after the induction of LTP.

Scale bar =50 μm

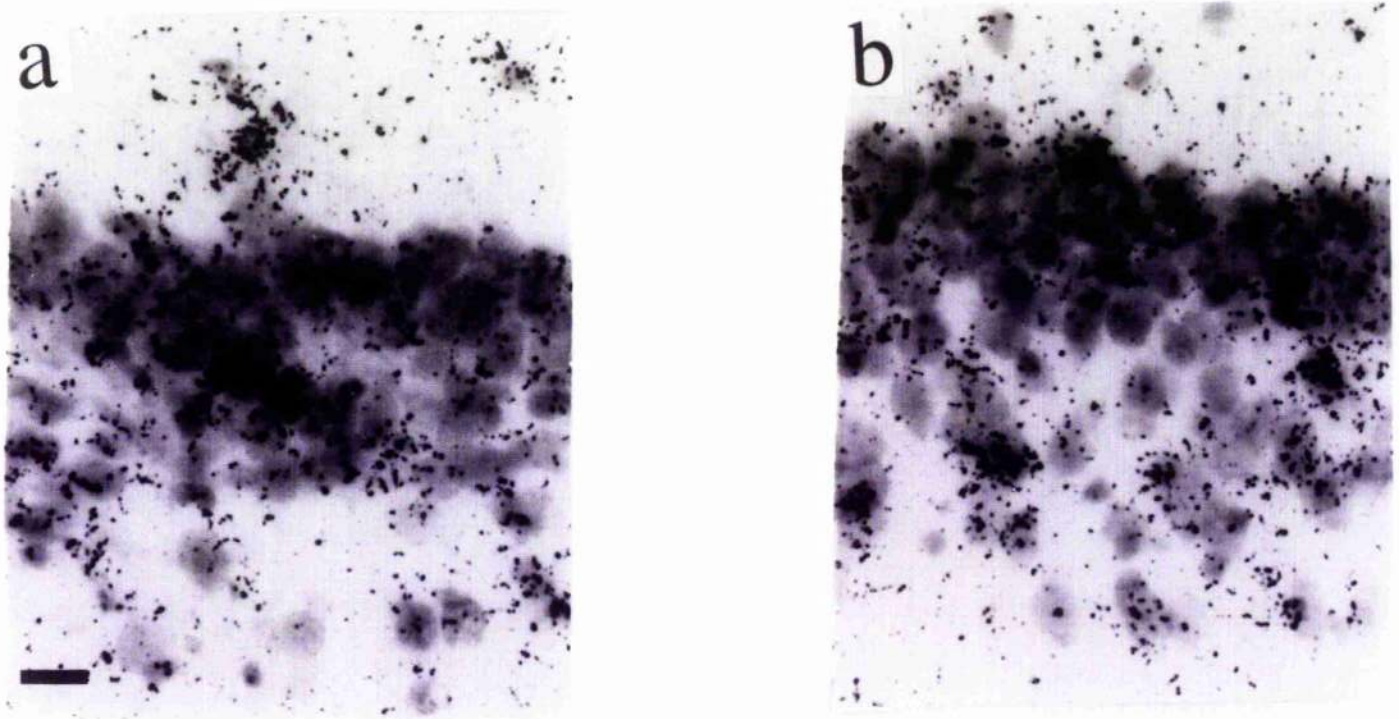


Figure 4.2.1.2
c-fos

Figure 4.2.1.3

% change in zif/268, c-fos and T26 mRNA levels in the cell bodies of the CA1 region 45 minutes after the induction of LTP compared to non-potentiated controls.

* median significantly different from 100%; $p<0.05$, Wilcoxon test

n=3-6

Figure 4.2.1.3

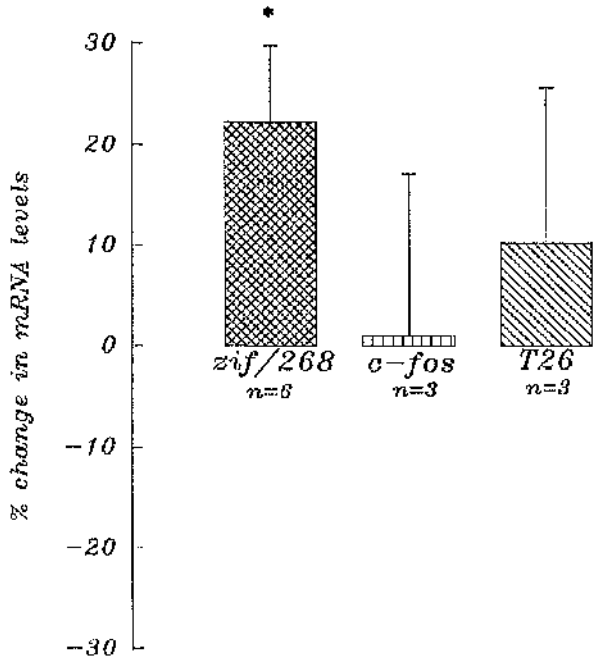

*
30
20
10
0
−10
−20
−30
% change in mRNA levels
zif/268
n=6
c−fos
n=3
T26
n=3

Figure 4.2.2.1

αCAMKII hybridisation signal in the cell bodies and dendrites of the CA1 region in (a) control and (b) potentiated slices 2 hours after the induction of LTP.

Scale bar =50 μm

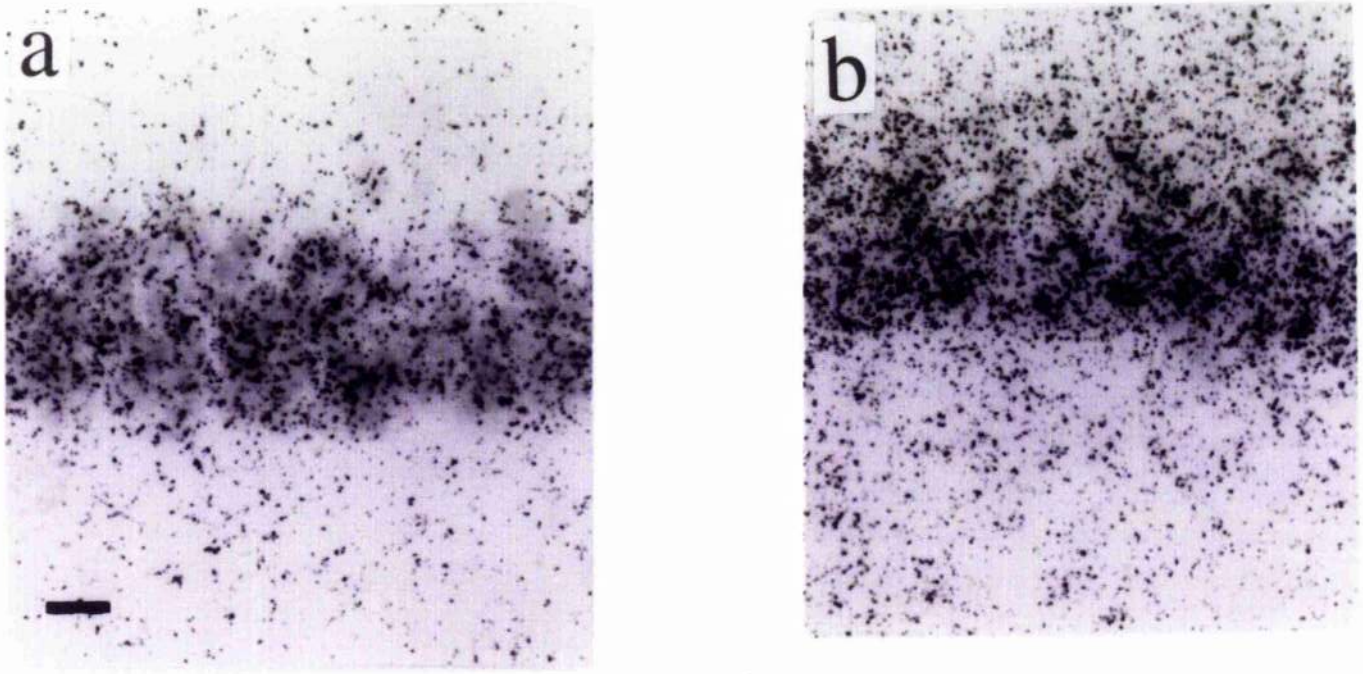


Figure 4.2.2.1
αCAMKII

Figure 4.2.2.2

MAP2 hybridisation signal in the cell bodies and dendrites of the CA1 region in (a) control and (b) potentiated slices 2 hours after the induction of LTP.

Scale bar =50 μm

Figure 4.2.2.2
MAP2

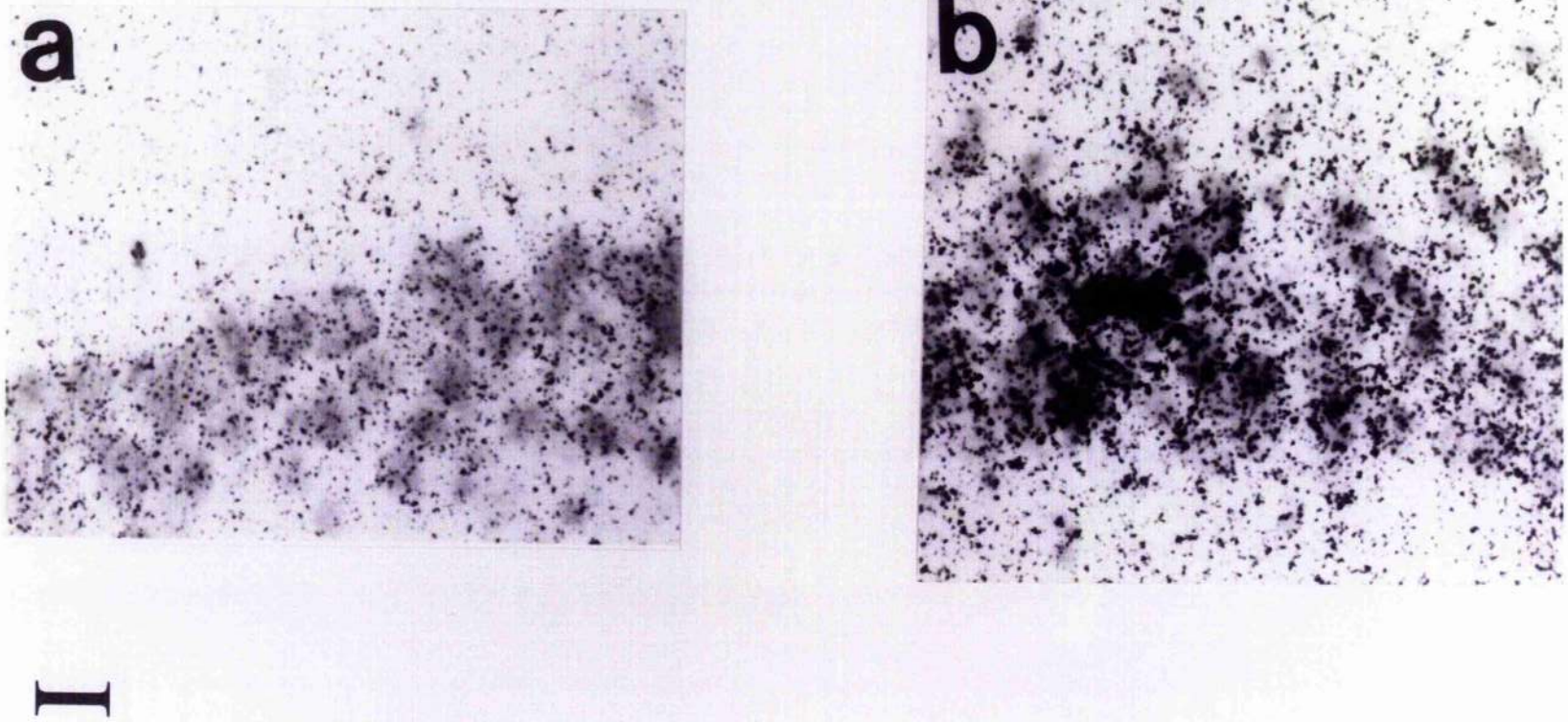


Figure 4.2.2.3

% change in αCAMKII, MAP2 and T26 mRNA levels in the CA1 region cell bodies 2 hours after the induction of LTP compared to non-potentiated controls.

*median significantly different from 100%, $p<0.05$, Wilcoxon test

n=3-7

Figure 4.2.2.3

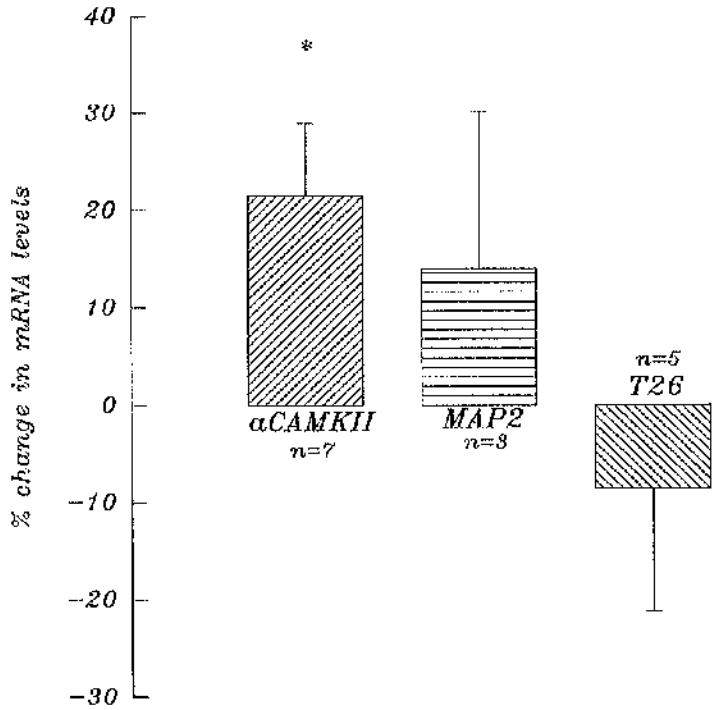
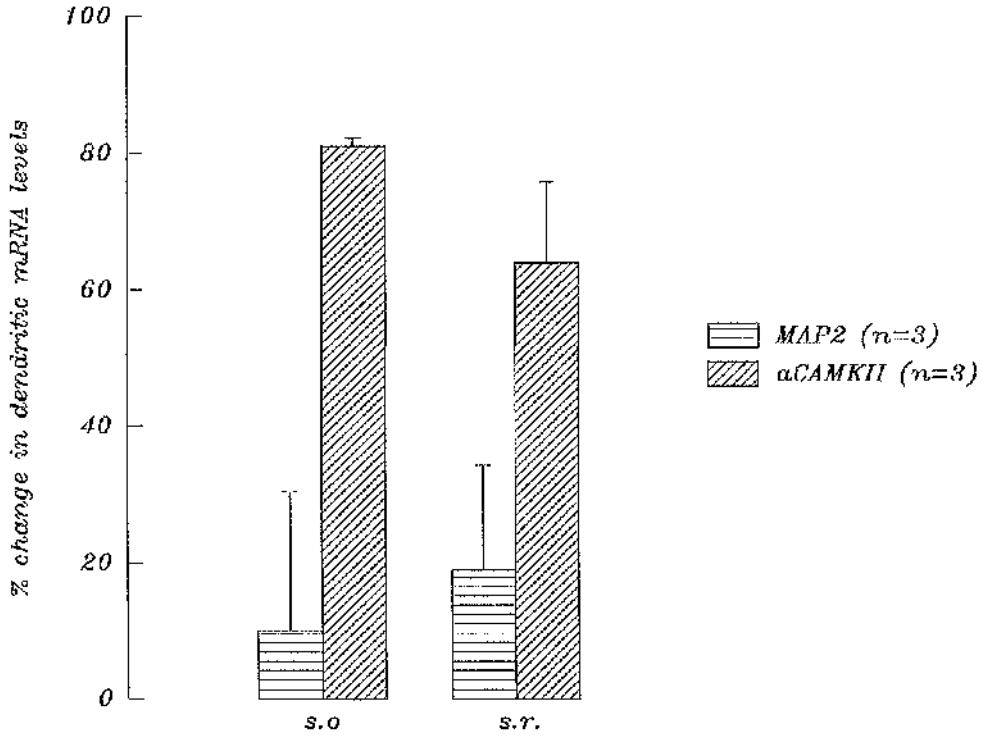

40
30
20
10
0
-10
-20
-30
% change in mRNA levels
*
αCAMKII
n=7
MAP2
n=3
n=5
T26

Figure 4.2.2.4

% change in αCAMKII and MAP2 mRNA levels in the stratum oriens (s.o.) and the stratum radiatum (s.r.) of the CA1 region 2 hours after the induction of LTP compared to non-potentiated controls.
Medians are not significantly different from 100% (Wilcoxon test)
n=3

Figure 4.2.2.4


100
80
60
40
20
0
% change in dendritic mRNA levels
s.o
s.r.
MAP2 (n=3)
αCAMKII (n=3)

4.3 DISCUSSION

4.3.1 Aims of the study

There are very few published reports on the use of *in vitro* preparations, such as brain slices, to study changes in gene expression. It has previously been shown that one hour after the induction of a form of LTP in area CA1 of hippocampal slices, using tetraethylammonium, an increase in zif/268 mRNA levels was observed which returned to basal level within 3 hours (Chinestra *et al.*, 1994). It has also been shown that 4 hours after the electrical induction of LTP in area CA1 of the hippocampus, both brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) mRNA levels are increased (Patterson *et al.*, 1992). Most of the available data about altered gene expression following the induction of LTP have come from *in vivo* studies of perforant path/dentate gyrus LTP. Other work has involved microapplication of NMDA and related compounds to the dendrites of hippocampal granule cells *in vivo* and subsequent study of the alterations in mRNA levels which this produces. The success of the latter approach in producing selective alterations in the mRNA levels of several genes involved in hippocampal plasticity such as prodynorphin, proenkephalin (Johnston & Morris, 1994c; Morris *et al.*, 1988; Morris & Johnston, 1995), MAP2 (Johnston & Morris, 1994b & e) and αCAMKII (Johnston & Morris, 1995), has prompted an extension of these studies to encompass the investigation of LTP *in vitro*, to determine whether the same genes are involved in its expression in area CA1 of the hippocampus.

4.3.2 mRNA distribution

The distribution of five mRNA species was as expected. Levels of αCAMKII and MAP2 mRNA were highest over the cell bodies and were also found in the

dendrites consistent with previous results (Garner *et al.*, 1988; Benson *et al.*, 1992; Johnston & Morris, 1994b & e, 1995). T26 and zif/268 mRNAs were confined to the cell bodies in area CA1, and c-fos mRNA was localised to scattered neurones rather than being expressed by all neurones. Levels of T26 mRNA were measured as an internal control to confirm that any changes in the expression of other genes were due to experimental stimuli, and not due a non-specific artefact of tissue handling. As predicted (Johnston & Morris, 1994b & c; Johnston & Morris, 1995), the levels of T26 mRNA were unaffected by the induction of LTP, both 45 minutes and 2 hours after the stimulus. The counterstaining of the cells showed that there was no obvious damage to the tissue during processing, and that it remained intact when sectioned.

4.3.3 Immediate early gene expression

Increases in zif/268 mRNA levels have been detected following the induction of LTP in the dentate gyrus *in vivo* (Cole *et al.*, 1989; Richardson *et al.*, 1992; Wisden *et al.*, 1990; Worley *et al.*, 1993). It was demonstrated that levels of zif/268 mRNA in animals trained in the Morris' water maze were no greater than levels in untrained rats (Wisden *et al.*, 1990), which suggests that although zif/268 expression may be linked to the induction of LTP, in the dentate gyrus, the nature of its relationship with spatial learning is unclear. In this study, we report that high frequency stimulation of the Schaffer collaterals, sufficient to induce LTP in the synapses onto the pyramidal cells, results in increased zif/268 expression in the post synaptic cells. Our observations are supported by previous work where semi-quantitative analysis of zif/268 mRNA levels in CA1 neurones, suggested an increase following the induction of LTP *in vitro* (Mackler *et al.*, 1992). It seems clear that the induction of zif/268 by LTP is not a response unique to the granule cells of the dentate gyrus, but may also contribute to the late phase of LTP in CA1 neurones.

Interestingly, it has also been reported that when stimulation of the commisural input to the dentate gyrus was performed prior to perforant path stimulation, and the induction of LTP was prevented, bilateral induction of the zif/268 gene still occurred (Wisden *et al.*, 1990). However, this tetanisation of the hippocampal commisural pathway, which would presumably have produced LTP in the commisural/CA1 neurone synapses (Feasey *et al.*, 1986), failed to produce any change in zif/268 mRNA levels in the CA1 pyramidal cell neurones. This observation taken together with our findings that zif/268 mRNA levels are increased following the induction of LTP in Schaffer collateral/CA1 neurone synapses, suggests that there may be differences in maintenance mechanisms of LTP depending on its pathway of origin. Schaffer collateral fibres synapse with the dendrites of CA1 neurones at a more distal site from the nucleus than the commisural fibres do (Raisman *et al.*, 1965). The unaltered levels of zif/268 mRNA in the CA1 neurones following the induction of LTP in commissural/CA1 neurone synapses (Wisden *et al.*, 1990) are, therefore, unlikely to be explained by some local transcription factor taking longer to reach the nucleus and upregulate expression of the zif/268 gene, from the commisural as opposed to the Schaffer collateral synapses. It has been shown that some mRNA species are differentially distributed within axons and dendrites of the hippocampus (Miyashiro *et al.*, 1994), so that certain proteins can be targeted to specific regions. This could mean that some regulatory proteins are localised to the area where the Schaffer collaterals terminate on CA1 dendrites, but are absent from the region where commisural fibres terminate. It is possible that somehow transmission from the dendrites to the cell body is altered depending on the particular neurochemical properties of that synapse. Although it is uncertain whether our *in vitro* model mimics the situation *in vivo*, the contrasting results raise the possibility that regional differences in the synapses formed with the dendrites of CA1 neurones can in some way differentially regulate gene transcription within the cell body. The

exact nature of these differences and how they might affect transmission remains to be determined. It can be concluded that zif/268 plays an important, but as yet undefined role following the induction of LTP.

Alterations in the levels of c-fos mRNA in the dentate gyrus have also been investigated following the induction of LTP. Most work has shown that c-fos mRNA and/or protein levels are only increased when a 50 train stimulus pattern is used (Douglas *et al.*, 1988; Worley *et al.*, 1993), and that LTP can be induced in the dentate gyrus without causing any changes in levels of c-fos protein (Jeffrey *et al.*, 1990). Our data extend these findings by demonstrating that there is no change in c-fos mRNA levels after the induction of LTP in Schaffer collateral/CA1 pyramidal cell synapses using theta rhythm based stimulus parameters. As the induction of c-fos seems to be highly dependent on the strength of stimulation used to produce LTP, the possibility that c-fos mRNA levels might have been increased, if different stimulus parameters had been used, cannot be excluded. Our stimulation paramcters (5 trains at 200Hz) are much weaker than those used by groups which have reported induction of c-fos expression following the induction of LTP (Worley *et al.*, 1993), and are similar to the parameters used by Wisden *et al.* (1990), which failed to induce expression of c-fos, following the induction of LTP. The expression of LTP in Schaffer collateral/CA1 sysnapses using our stimulation parameters does not appear to be dependent on the induction of c-fos expression

The identity of genes whose expression might be regulated by c-fos or zif/268 is unclear. It is thought that the existence of an AP-1 binding site on the promoter region of the proenkephalin gene might be an indication that it is regulated by an immediate early gene of the fos/jun family (Hyman *et al.*, 1988). However, it has also been shown that although proenkephalin mRNA levels are increased 24 hours after the induction of LTP in the dentate gyrus *in*

vivo (Morris *et al.*, 1988), and following NMDA receptor stimulation of the dendrites of the granule cells of the hippocampus (Johnston & Morris, 1994a, b), this increase can not be attributed to either c-fos or zif/268. Our results showing the increase in zif/268 mRNA levels but not c-fos mRNA levels after the induction of LTP in area CA1 following Schaffer collateral stimulation, could well suggest that similar molecular mechanisms, involving as yet unidentified downstream genes, operate in the pyramidal cells of the CA1 region and in the granule cells of the dentate gyrus to sustain LTP.

4.3.4 αCAMKII and MAP2 mRNA levels

Evidence of a potential role for the α subunit of CAMKII in LTP has come from work on transgenic mice (Silva *et al.*, 1992a & b), from *in vitro* studies (Malinow *et al.*, 1989; Pettit *et al.*, 1994) and from observations made following the induction of LTP in the dentate gyrus *in vivo* (Thomas *et al.*, 1994) or following stimulation of NMDA receptors on granule cell dendrites (Johnston & Morris, 1995). Once CAMKII has been activated by, for example, a rise in intracellular calcium ions (as occurs in the induction of LTP), it undergoes autophosphorylation enabling it to function independently of Ca^{2+} ions. There are a large number of substrates for CAMKII including AMPA receptors, microtubule associated proteins, and nitric oxide synthase (Braun & Schulman, 1995). All evidence obtained to date seems to imply that a definite link exists between αCAMKII activation and the induction of LTP although the exact nature of the link has yet to be determined.

Our results have shown that 2 hours after the induction of LTP, αCAMKII mRNA levels in the cell bodies of area CA1 are significantly increased compared to non-potentiated controls. This result strongly correlates with observations made in the dentate gyrus (Thomas *et al.*, 1994a; Chapter 5, this

volume), to the extent that the increase in αCAMKII hybridisation signal in the dentate gyrus *in vivo* is similar in magnitude to the increase observed in CA1 neurones following the induction of LTP *in vitro*, and also correlates with other observations (Johnston & Morris, 1995). In addition potential increases in αCAMKII mRNA levels in individual CA1 neurones have also been reported following the induction of LTP in this area *in vitro* (Mackler *et al.*, 1992). These observations strongly suggest that the induction of LTP in the dentate gyrus and the CA1 region share common mechanisms which involve the regulation of expression of αCAMKII .

MAP2 is one of a family of microtubule associated proteins (MAPs) which are thought to regulate and maintain neuronal morphology and neurite outgrowth (Caceres *et al.*, 1988; Ferriera *et al.*, 1990). MAP2 protein and the mRNA which encodes it are found in the cell bodies and dendrites throughout the CNS, but unlike the MAP tau it is not found in axons. Although the regulation of MAP2 may be a feature of synaptic plasticity very little work has been undertaken to establish whether or not its expression is affected by the induction of LTP and whether it might be responsible for some of the morphological changes associated with the induction of LTP (Chang & Greenough, 1984; Desmond & Levy, 1986a & b; Geinisman *et al.*, 1991; Hosokawa *et al.*, 1995). Meberg *et al.* (1993) showed that three days after the induction of LTP in perforant path/granule cell synapses, levels of MAP2 mRNA were unchanged. Johnston & Morris (1994b) showed MAP2 mRNA levels to be increased 24 hours after treatment with NMDA and nitric oxide releasing agents. These observations suggest that MAP2 may be important for morphological changes occurring up to 24 hours after NMDA receptor stimulation, but that the increase in its expression is not detectable after 72 hours. Although the increases we observed 2 hours after the induction of LTP were not significant, it is possible that the alteration in expression of this gene

begins to take place within a few hours of the induction of LTP, but that its expression peaks at a later time point. It is also conceivable that our small sample number prevented us from finding the increase to be significant from control values. We have also found evidence for an increase in MAP2 mRNA 2 hours after the induction of LTP in perforant path/granule cell synapses *in vivo* (Chapter 5, this volume).

Given that there are a number of different isoforms of MAP2 (a, b & c) which are differentially regulated in the CNS (Tucker, 1990) the lack of a significant increase which we have reported may be due to simultaneous increases and decreases in mRNA levels of the different isoforms. Our oligonucleotide probe does not distinguish between the different isoforms as it hybridises to mRNA encoding all splice variants.

In addition to changes in mRNA levels in the cell bodies of area CA1 following the induction of LTP, we also observed increases in αCAMKII mRNA levels in the proximal dendrites of area CA1. Similar observations have been made in the dendrites of hippocampal granule cells *in vivo* following the application of NMDA (Johnston & Morris, 1995) or induction of LTP (Thomas *et al.*, 1994a). An important feature of this increase is that it occurs not only in the stratum radiatum, but also in the stratum oriens. As the Schaffer collateral fibres terminate primarily on CA1 pyramidal cell dendrites in the stratum radiatum, it is of considerable interest that an increase in dendritic αCAMKII mRNA levels is seen in both the stratum radiatum, and the stratum oriens, the latter of which receives a greater input from commissural fibres compared to Schaffer collaterals (Buzsàki & Eidelberg, 1982). Two possible mechanisms can be proposed for this phenomenon. One explanation could be that some commissural fibres are activated during the induction of LTP leading to potentiation of synapses in the stratum oriens as well as the stratum radiatum,

and that LTP in synapses in the stratum radiatum is also associated with a local increase in αCAMKII mRNA levels. Another possibility is that αCAMKII mRNA is produced in the cell body in response to the induction of LTP in the Schaffer/apical dendrite synapses in the stratum radiatum, and then transported in both directions to the dendrites, regardless of the location of the potentiated synapses. This theory has been previously addressed (Thomas *et al.*, 1994a), when αCAMKII mRNA levels in the granule cells, following the induction of LTP in vivo were shown to be increased at 2 hours and then returned to normal at 24hrs, whereas levels of αCAMKII mRNA in the proximal and then the distal dendrites were increased at 24 and 48hr respectively. It was suggested that this might reflect dendritic transport of mRNA from the cell body to the site of stimulation. It is questionable whether, in our model, the manufacture and transport of mRNA could take place within 2 hr of the induction of LTP One intriguing possibility is that the induction of LTP and/or activation of NMDA receptors somehow triggers a mechanism which results in the stabilisation of mRNA, perhaps by inhibiting enzymes which would normally cause its breakdown. Interestingly MAP2 mRNA levels were also slightly increased in the stratum oriens and stratum radiatum, and were significantly increased in granule cell dendrites following the induction of LTP *in vivo* (Chapter 5, this volume). In addition, dendritic levels of MAP2 have been shown to be regulated by nitric oxide (Johnston & Morris, 1994e). Whatever the underlying mechanisms these results provide further evidence that an increase in αCAMKII (and possibly MAP2) expression of this magnitude may play a fundamental role in LTP expression which is common to all regions of the hippocampus.

4.3.5 Conclusions

These results demonstrate the feasibility of studying changes in gene expression which accompany the induction of LTP in hippocampal slices maintained *in vitro*. Similar intracellular processes appear to be activated following the induction of LTP in the dentate gyrus *in vivo* and in area CA1 *in vitro*. Although αCAMKII plays some important, but as yet incompletely defined role in LTP, the identity of the most important IEG(s) in this model, and the role they might play, remains to be fully resolved. Further work would be needed to determine the effects of the induction of LTP on expression of MAP2 and, in particular, which isoforms are regulated and in what manner.

CHAPTER 5

CHANGES IN HIPPOCAMPAL GENE EXPRESSION ASSOCIATED WITH THE INDUCTION OF LTP IN VIVO

5.1 INTRODUCTION

5.1.1 Long-term potentiation and the induction of gene expression

The induction of long-term potentiation (LTP) in the hippocampus is associated with altered levels of expression of many genes. Within one hour of the induction of LTP the expression of the immediate early genes (IEGs) zif/268, c-fos, jun D and jun B is increased (Cole *et al.*, 1989; Wisden *et al.*, 1990; Jeffrey *et al.*, 1990; Demmer *et al.*, 1993; Worley *et al.*, 1993; Chapter 4, this volume). Over the next few hours the levels of the mRNAs encoding several protein kinases, growth factors, cell adhesion molecules, synaptic vesicle proteins and proteases are altered (see sections 1.52-1.54, this volume). It has been suggested that these are involved in the expression of LTP and in the morphological changes associated with its induction.

5.1.2 Aims of this study

We have already examined the expression of 5 genes following the induction of LTP in the Schaffer collateral/CA1 synapses (Chapter 4, this volume) and we now extend these studies to examine changes in gene expression associated with the induction of LTP in perforant path/granule cell synapses *in vivo*.

Previously we looked at levels of the IEGs zif/268 and c-fos, but in this model attention will be centred on potential downstream genes which may be affected by IEGs. Changes occurring two hours after the induction of LTP have been investigated, which is later than the optimum time for detection of changes in IEG expression.

We have chosen to examine mRNA levels of αCAMKII, MAP2 and T26 which have been previously examined following the induction of LTP *in vitro* (Chapter 4, this volume). In addition, mRNA levels of candidate plasticity gene (CPG), tissue inhibitor of metalloproteinases (TIMP) and activity-regulated cytoskeleton-associated protein (Arc), have been investigated. The increased expression of these genes has been reported following the induction of LTP (Nedivi *et al.*, 1993; Link *et al.*, 1995; Lyford *et al.*, 1995). To see whether the induction of LTP in perforant path/granule cell synapses is associated with the induction of any previously unreported genes levels of proenkephalin (pENK) and 25kD-synaptosomal associated proteins A & B (SNAP A & B) have also been examined in this model.

5.2 RESULTS

5.2.1 Electrophysiology

When recording from the hilus of the dentate gyrus, the measurement of the EPSP slope is often compromised by the superimposition of the population spike over the EPSP response, particularly following the induction of LTP. Consequently the more accurate measure of potentiation is the amplitude of the population spike (C.H. Large, personal communication). Using the parameters of stimulation outlined in the methods (Chapter 2) population spike amplitude was increased 326±64% (n=6) and the slope of the EPSP was increased 21.8±4.5% (n=6) following the induction of LTP. Figure 5.2.1.1 shows population spike recordings from the hilus of the dentate gyrus before (A) and immediately after (B) high frequency stimulation of the medial perforant path. Note the increase in population spike and slope of the EPSP. Figures 5.2.1.2 and 5.2.1.3 show typical examples of how the amplitude of the population spike and EPSP slope recorded in the hilus are increased following high frequency stimulation of the medial perforant path.

5.2.2 mRNA species whose levels are unaffected by the induction of LTP

Photomicrographs showing T26 hybridisation signal over the granule cells of the dentate gyrus two hours after (a) low frequency stimulation and (b) tetanic stimulation can be found in figure 5.2.2.1. It is clear that T26 mRNA levels are not altered following the induction of LTP.

Figure 5.2.2.2 shows TIMP (a, b), CPG (c, d) and arc (e, f) hybridisation signal in the granule cells two hours after low frequency (control) stimulation (a, c, e)

and two hours after high frequency potentiating stimulation (b, d, f). Levels of TIMP and CPG mRNA are not increased following the induction of LTP. Arc mRNA was not found to be present two hours after low frequency or high frequency stimulation.

5.2.3 mRNA species whose levels appear to be altered following the induction of LTP

Figure 5.2.3.1 shows αCAMKII hybridisation signal in the granule cells and dendrites of the dentate gyrus two hours after low frequency (control) stimulation (a) and after tetanic stimulation (b). It is clear from this that levels of αCAMKII mRNA are increased in the cell bodies and proximal dendrites two hours after the induction of LTP.

MAP2 hybridisation signal over the granule cells and proximal dendrites of the dentate gyrus is shown in Figure 5.2.3.2, (a) two hours after control stimulation and (b) two hours after the induction of LTP in the perforant path granule cell synapses. Levels of MAP2 mRNA appear to be increased in the cell bodies and dendrites following the induction of LTP.

Figure 5.2.3.3 shows SNAP A (a, b) and SNAP B (c, d) hybridisation signal in the granule cells of the dentate gyrus two hours after low frequency (control) stimulation (a, c) and two hours after high frequency stimulation (b, d). Levels of both appear to be increased following the induction of LTP.

Figure 5.2.3.4 shows pENK hybridisation signal in the granule cells of the dentate gyrus (a) two hours after low frequency (control) stimulation and (b) two hours after the induction of LTP. There appears to be an increase in the number of cells expressing pENK following the induction of LTP.

5.2.4 *Quantitative results*

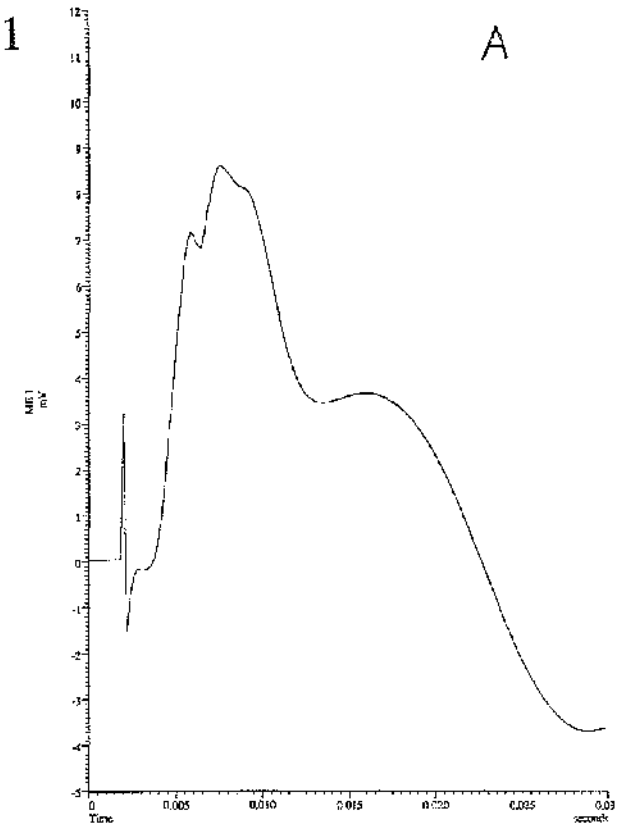
Figure 5.2.4.1 shows the percentage change in mRNA levels in the granule cell bodies of T26, MAP2, αCAMKII, SNAP A, SNAP B, TIMP and CPG two hours after the induction of LTP compared to control levels in the low frequency stimulated contralateral granule cells. Levels of T26, CPG and TIMP were not significantly increased, however levels of αCAMKII (18±6%) SNAP A (45±13%), SNAP B (18±4%) were significantly increased (*$p<0.05$, Wilcoxon test; n=6). Levels of MAP2 mRNA, although increased in the granule cells of the majority of animals, were not significantly increased above control values. The number of cells expressing pENK was found to be increased by 123±31.1% two hours after the induction of LTP. This result was significant ($p<0.05$, Wilcoxon test; n=6).

In addition to the cell body mRNA levels, the levels of mRNA for αCAMKII and MAP2 were also quantified in the proximal dendrites. Figure 5.2.4.2 shows the % change in mRNA levels of αCAMKII and MAP 2 in this area two hours after the induction of LTP compared to non-potentiated contralateral granule cell dendrites. Levels of dendritic αCAMKII (18±6%) and MAP2 (24±11%) mRNA were significantly increased following the induction of LTP (*$p<0.05$, Wilcoxon test; n=6).

Figure 5.2.1.1

Population spike recordings from the hilus of the dentate gyrus (A) before and (B) immediately after high frequency stimulation of the medial perforant path.

Figure 5.2.1.1



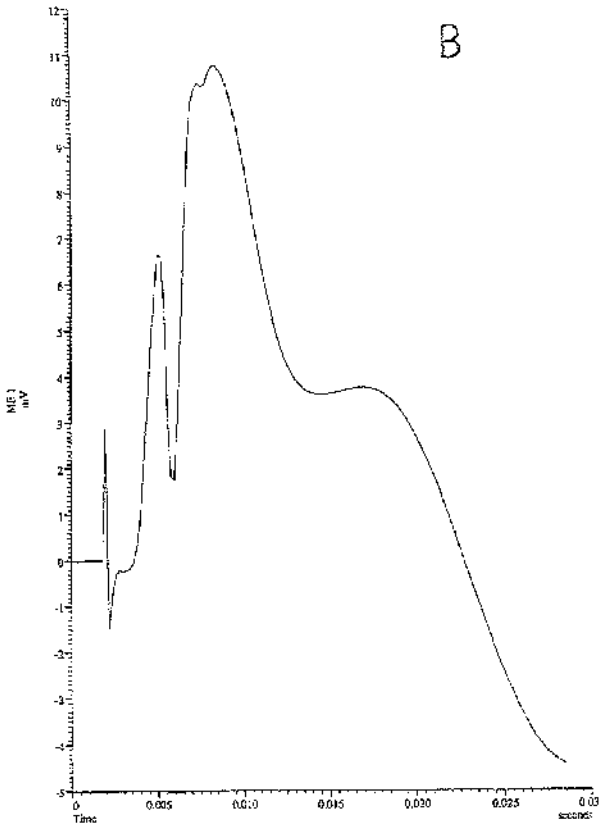


Figure 5.2.1.2

Typical example of how the amplitude of the amplitude of the population spike recorded in the hilus is increased following high frequency stimulation of the medial perforant path at 30 minutes.

Figure 5.2.1.2

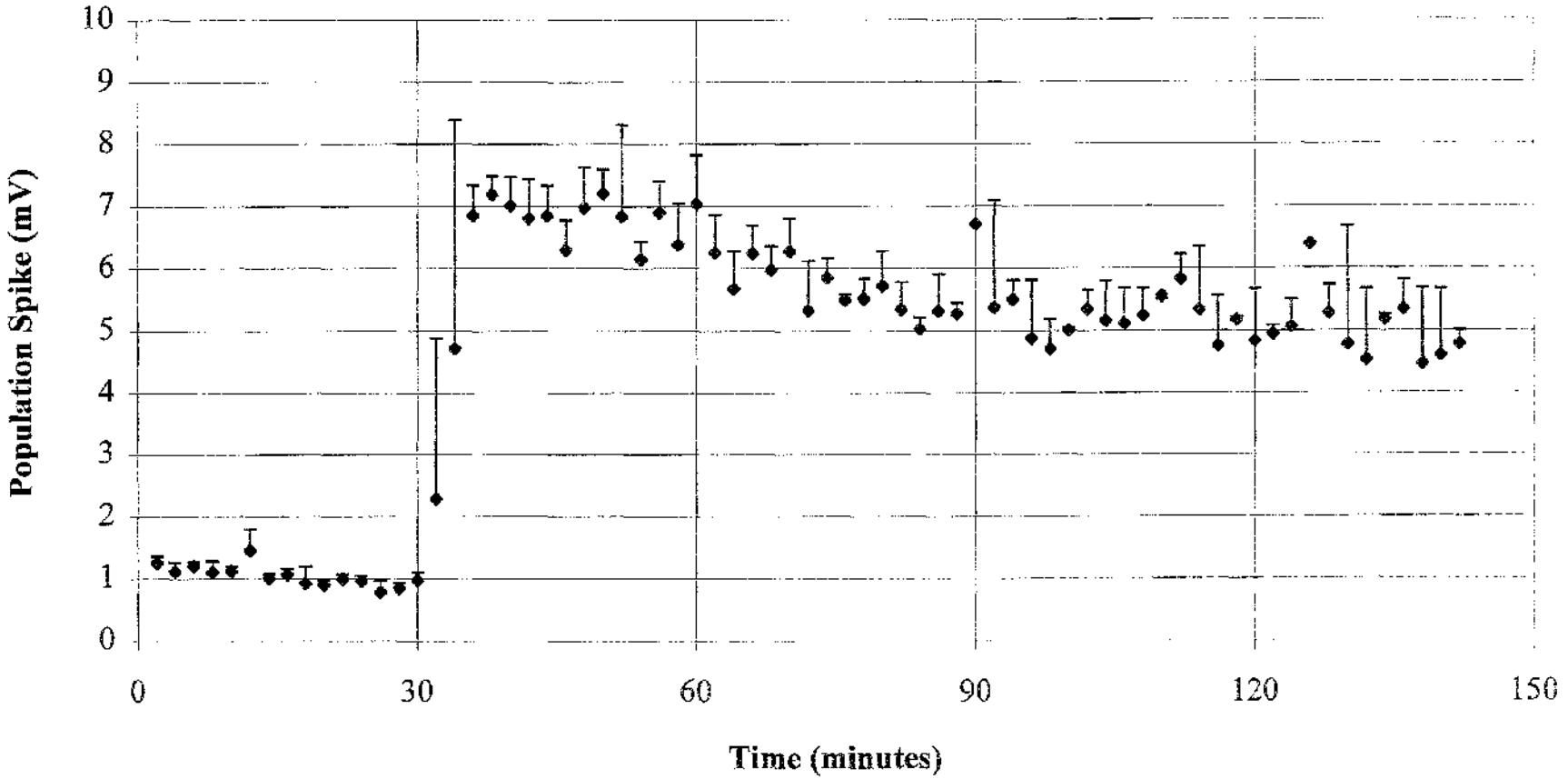

Population Spike (mV)
10
9
8
7
6
5
4
3
2
1
0
0
30
60
90
120
150
Time (minutes)

Figure 5.2.1.3

Typical example of how the slope of the EPSP is increased following high frequency stimulation of the medial perforant path at 30 minutes

Figure 5.2.1.3

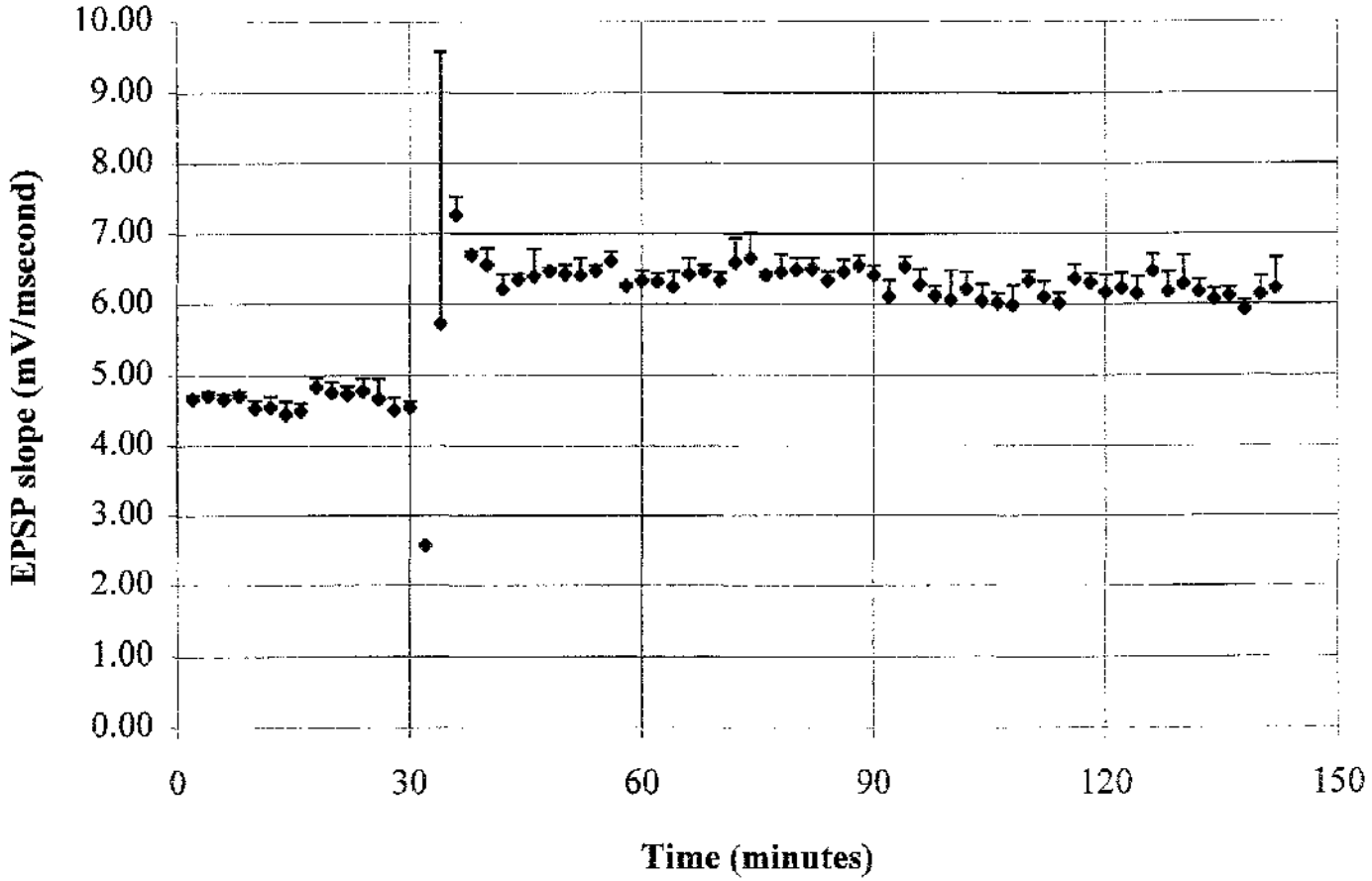

10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
EPSP slope (mV/msecond)
0
30
60
90
120
150
Time (minutes)

Figure 5.2.2.1

T26 hybridisation signal in the granule cells of the dentate gyrus 2 hours after (a) low frequency stimulation and (b) tetanic stimulation of the medial perforant path.

Scale bar = 25μm

Figure 5.2.2.1

Figure 5.2.2.2

Hybridisation signal of TIMP (a, b), CPG (c, d) and arc (e, f) mRNA in non potentiated granule cells (a, c, e) and in granule cells which have received high frequency stimulation producing LTP (b, d, f) at a 2 hour time point.

Scale bar = 25μm

Figure 5.2.2.2

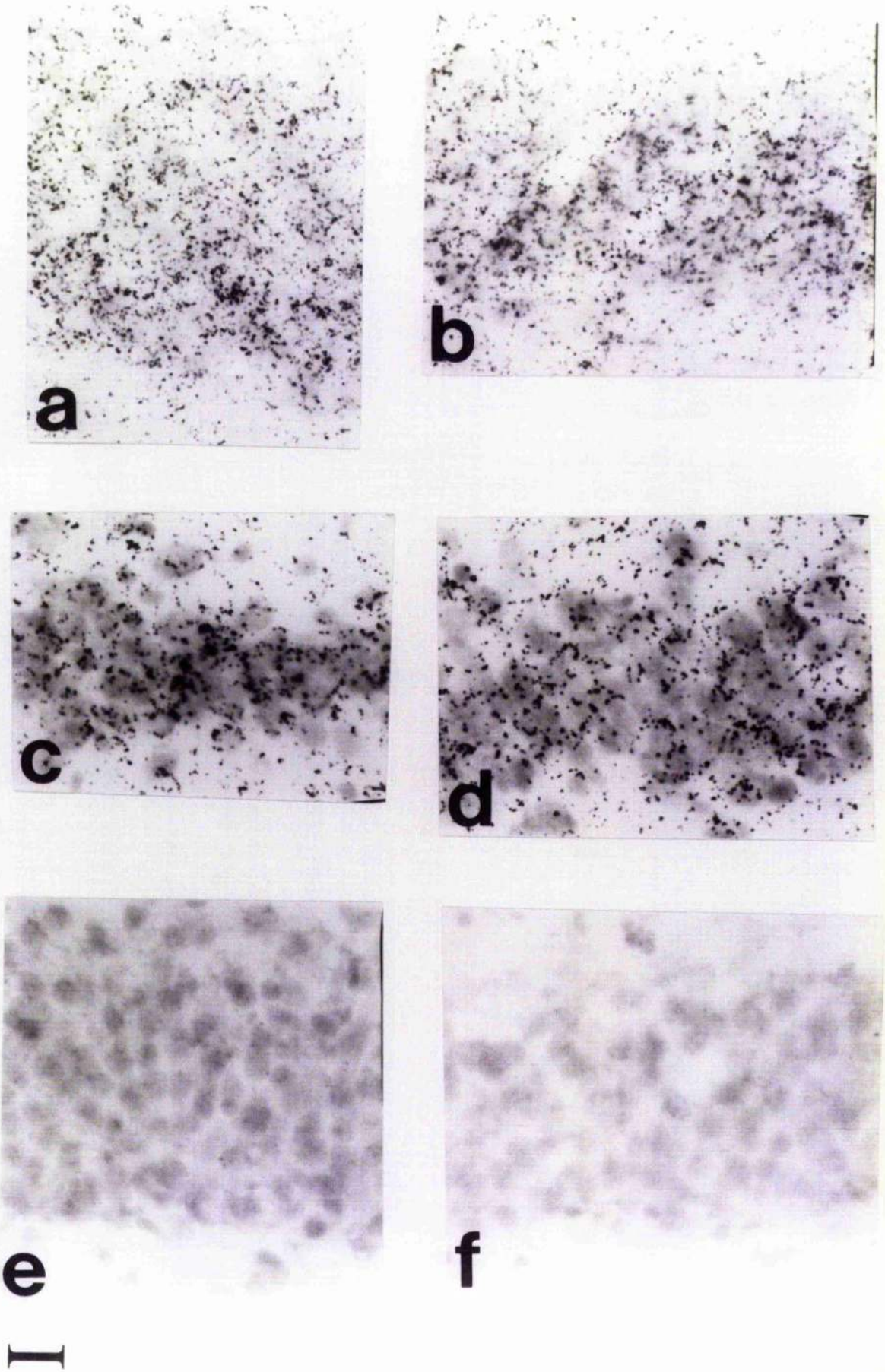


Figure 5.2.3.1

αCAMKII hybridisation signal in the granule cells of the dentate gyrus 2 hours after (a) low frequency stimulation and (b) tetanic stimulation of the medial perforant path.

Scale bar = 25μm

Figure 5.2.3.1

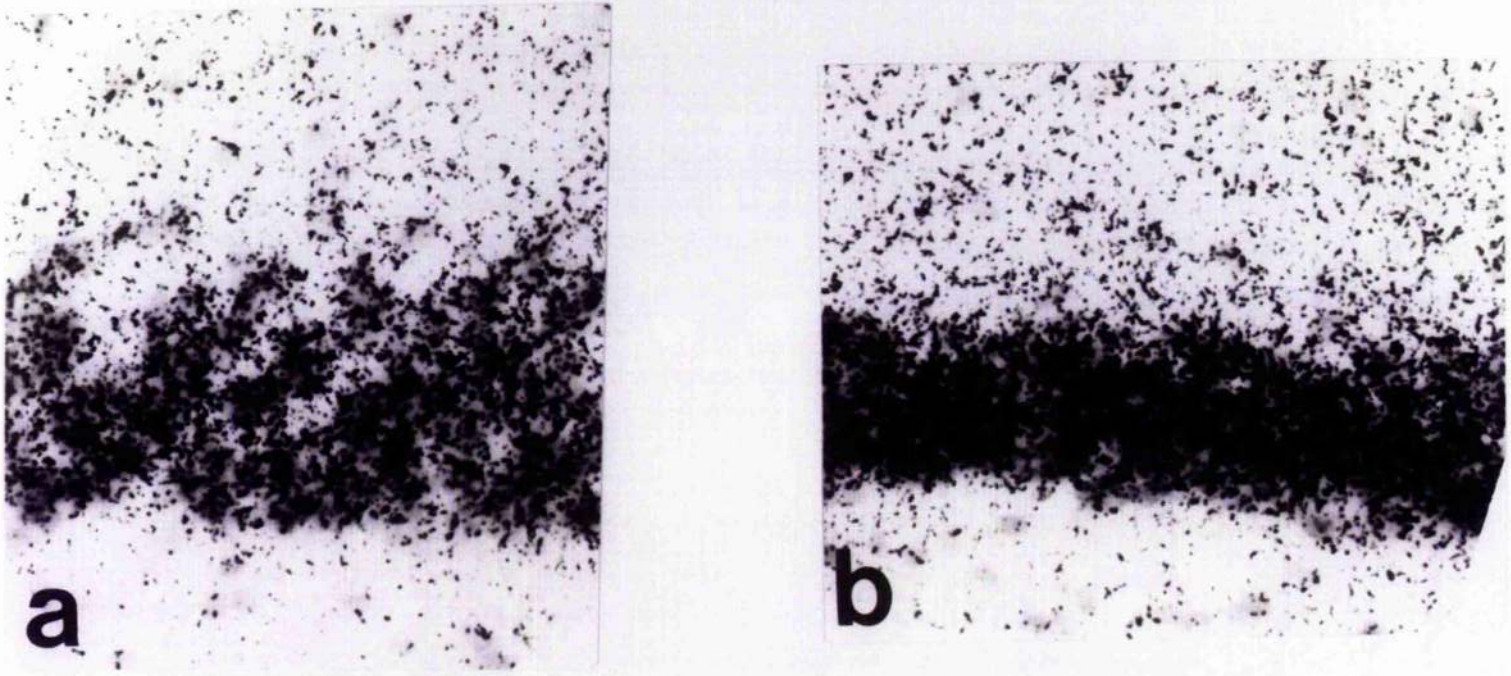


Figure 5.2.3.2

MAP2 hybridisation signal in the granule cells of the dentate gyrus 2 hours after (a) low frequency stimulation and (b) tetanic stimulation of the medial perforant path.

Scale bar = 25µm

Figure 5.2.3.2

I

Figure 5.2.3.3

Hybridisation signal of SNAP A (a, b) and SNAP B (c, d) mRNA in the granule cells of the dentate gyrus 2 hours after low frequency stimulation (a, c) and high frequency stimulation (b, d) of the medial perforant path.

Scale bar = 25μm

Figure 5.2.3.3

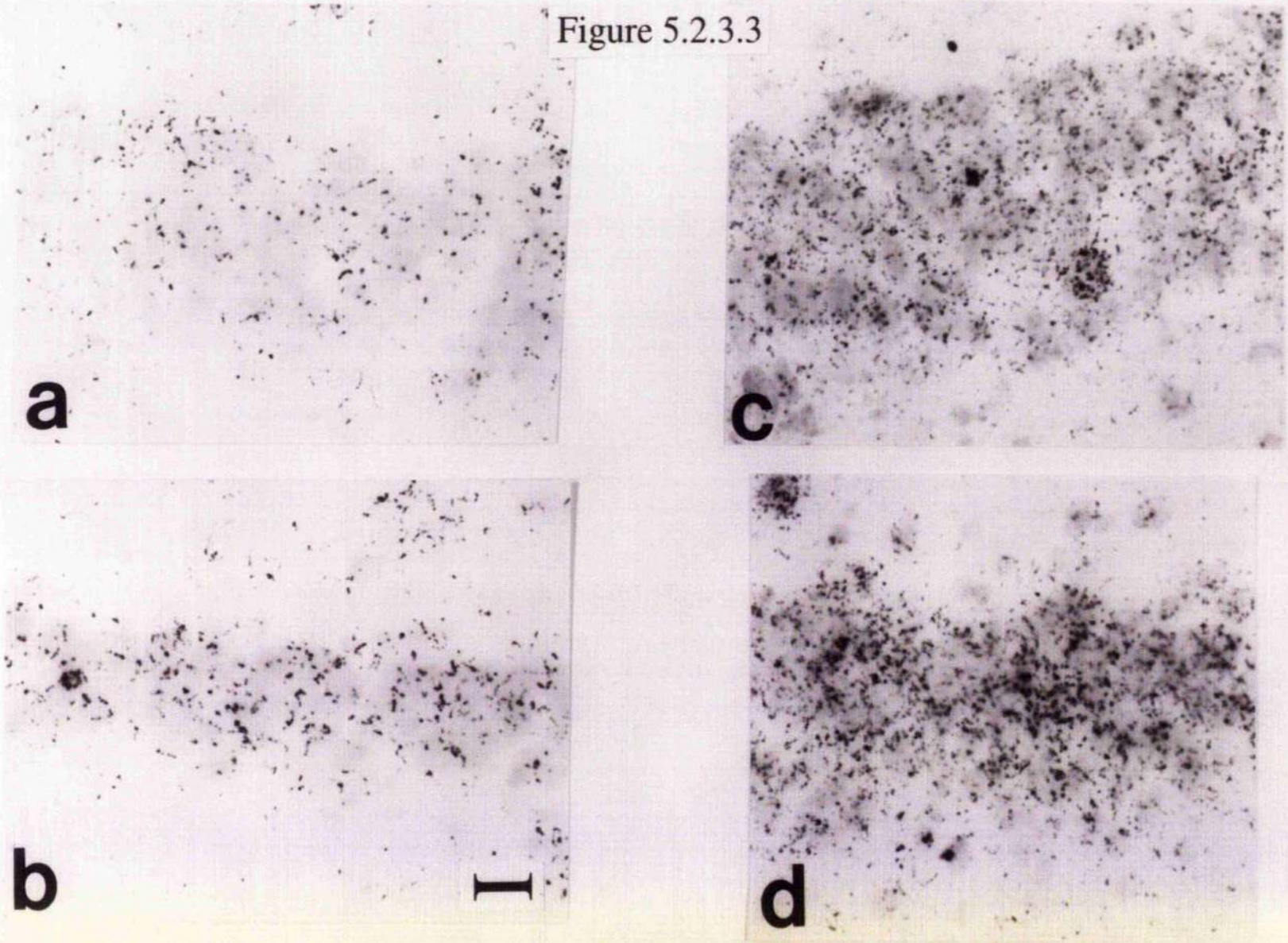


Figure 5.2.3.4

pENK hybridisation signal in the granule cells of the dentate gyrus 2 hours after (a) low frequency stimulation and (b) tetanic stimulation of the medial perforant path.

Scale bar = 50μm

a
b

Figure 5.2.4.1

% change in mRNA levels of T26, MAP2, αCAMKII, SNAP A, SNAP B, TIMP and CPG 2 hours after the induction of LTP compared to non potentiated controls.

* median significantly different from 100%; $p<0.05$, Wilcoxon test

n=3 or 6

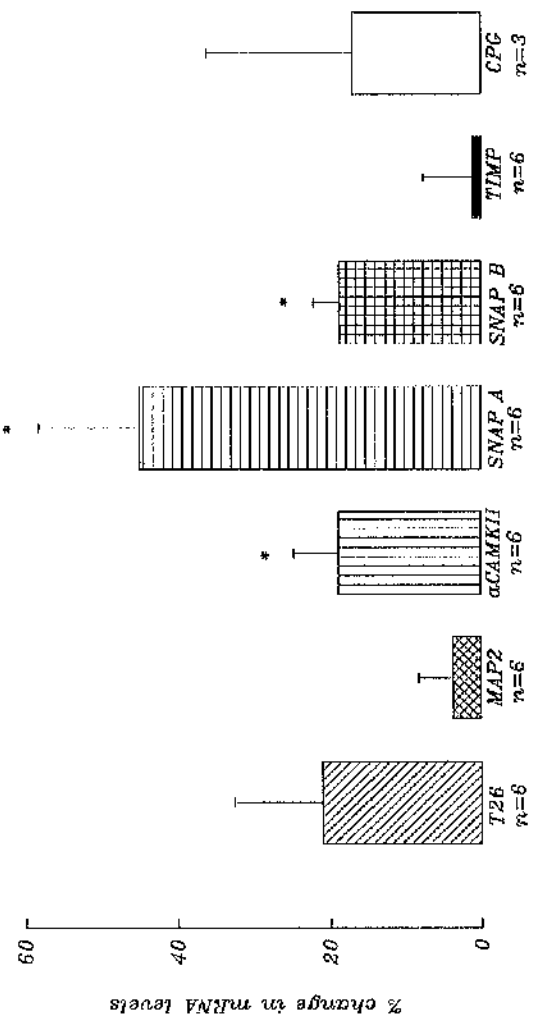

% change in mRNA levels
60
40
20
0
T26 n=6
MAP2 n=6
αCAMKII n=6
SNAP A n=6
SNAP B n=6
TIMP n=6
CPC n=3
*
*

Figure 5.2.4.1

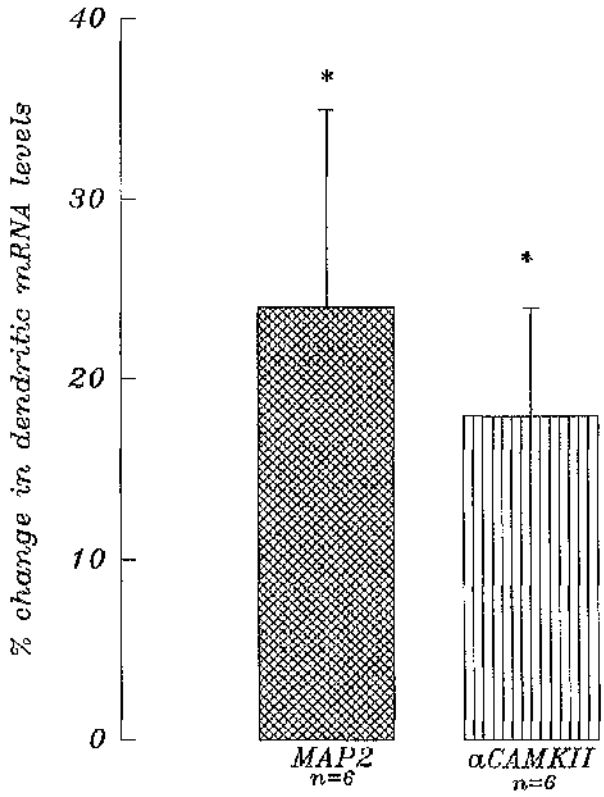
Figure 5.2.4.2

% change in MAP2 and αCAMKII mRNA levels in the dendrites of the granule cells two hours after the induction of LTP compared to non potentiated controls.

*median significantly different from 100%; $p<0.05$, Wilcoxon test

n=6

Figure 5.2.4.2


40
30
20
10
0
% change in dendritic mRNA levels
*
*
MAP2
n=6
αCAMKII
n=6

5.3 DISCUSSION

5.3.1 Aims of the study

The induction of LTP in the hippocampus is associated with the induction of several different classes of gene. We have previously looked at the effects of the induction of LTP in Schaffer collateral/CA1 synapses on the expression of MAP2, αCAMKII, T26, zif/268 and c-fos (Chapter 4, this volume) and wished to extend these studies to look at changes in gene expression associated with the induction of LTP in the dentate gyrus following stimulation of the medial perforant path. We chose to look at three genes which were studied *in vitro*, αCAMKII, MAP2 and T26 (Chapter 4, this volume) and three genes, CPG, TIMP and arc, which have previously been shown to be induced in parallel with the expression of LTP (Nedivi *et al.*, 1993; Link *et al.*, 1995; Lyford *et al.*, 1995). In addition, to investigate the role of enkephalins in the expression of LTP, we examined pENK mRNA levels, and to look at the role of vesicle associated proteins we looked at mRNA levels of SNAP A and SNAP B, both of which are may be involved in synaptic plasticity.

5.3.2 T26 mRNA levels

T26 mRNA was confined to the cell bodies of the dentate gyrus as previously described (Johnston & Morris, 1994b, c & d, 1995) and its levels were not significantly altered following the induction of LTP. This finding is consistent with previous studies which have shown T26 mRNA levels to be unaffected by the induction of LTP in Schaffer collateral/CA1 synapses (Chapter 4, this volume) and the application of glutamate receptor agonists to granule cell dendrites (Chapter 6, this volume; Johnston & Morris 1994b, c & d, 1995)

This observation provides evidence that non-specific changes in gene expression are not occurring.

5.3.3 CPG and TIMP mRNA levels

CPG and TIMP were found to be induced following the induction of LTP in perforant path/granule cell synapses (Nedivi *et al.*, 1993) and it was suggested that these genes played a role in synaptic plasticity. We did not find mRNA levels of either gene to be significantly increased two hours after the induction of LTP, however increases observed by Nedivi *et al.* (1993) were seen six hours after the induction of LTP so it is possible that increases in mRNA levels for these genes cannot be detected as early as two hours after the induction of LTP.

5.3.4 Arc mRNA levels

Using differential cloning techniques Lyford *et al.* (1995) and Link *et al.* (1995) identified a novel gene which the former group named arc (activity-regulated cytoskeleton associated protein). The mRNA and protein of this gene are found in the granule cell bodies and dendrites when its expression is induced and both groups demonstrated rapid increases in mRNA levels following the induction of LTP, electrically induced seizures (Lyford *et al.*, 1995) and pentylenetetrazol induced seizures (Link *et al.*, 1995). Link *et al.* (1995) used similar stimulation parameters to ours to induce LTP and saw increases in arc mRNA levels after one hour with expression returning to basal levels after 4 hours. Lyford *et al.* (1995) used higher frequency stimulation (400Hz) to induce LTP and observed increases in arc mRNA after 30 minutes. Our failure to observe any induction of arc expression following the induction of LTP is not likely to be due to the time point we chose, because basal levels

are so low that even if a peak in its expression was seen after one hour at least some detection of arc mRNA would be expected after two hours even if levels were declining. It is conceivable, however, that the use of urethane induced anaesthesia in our study has prevented the induction of arc, as both Lyford *et al.* (1995) and Link *et al.* (1995) used conscious rats in their studies. The blockade of expression of c-fos following the induction of LTP has also been demonstrated in anaesthetised rats. It is thought that increases in c-fos mRNA levels can only be seen if the conditions for the induction of LTP lead to a potentiation which lasts several weeks (LTP3) (Douglas *et al.*, 1988; Jeffrey *et al.*, 1990; Abraham *et al.*, 1993; Worley *et al.*, 1993). This can only be achieved in conscious animals, and it is possible that LTP-induced expression of arc only occurs when this form of LTP has been induced.

5.3.5 αCAMKII mRNA levels (cell bodies)

Levels of αCAMKII mRNA have previously been shown to be increased following the induction of LTP in perforant path/granule cell synapses (Thomas *et al.*, 1994a) and Schaffer collateral/CA1 synapses (Mackler *et al.*, 1992; Chapter 4, this volume). In addition, expression of αCAMKII is regulated by NMDA receptor activation (Johnston & Morris, 1995). We found significant increases in αCAMKII mRNA levels in the granule cell bodies of the dentate gyrus consistent with observations made by Thomas *et al.* (1994a). The increased levels of αCAMKII most probably reflect the induction of a mechanism which allows the cell to sustain the effects of a transient increase in intracellular calcium levels so that calcium-regulated processes can be activated. In addition, the phosphorylation of glutamate receptors subunits by CAMKII (McGlade-McCulloh *et al.*, 1993; Tan *et al.*, 1994; Yakel *et al.*, 1995; Moon *et al.*, 1995) suggests that increased αCAMKII expression contributes to the increase in postsynaptic sensitivity of glutamate receptors

which occurs following the induction of LTP (Reymann *et al.*, 1990). Also the phosphorylation of CREB by CAMKII (Dash *et al.*, 1991; Sheng *et al.*, 1991) indicates that increased expression of αCAMKII may lead to raised activity of CREB-related transcription factors following the induction of LTP.

5.3.6 MAP2 mRNA levels (cell bodies)

MAP2 is a cytoskeletal protein whose expression has been shown to promote neurite outgrowth (Caceres *et al.*, 1988; Ferriera *et al.*, 1990). It is possible that the structural changes which occur following the induction of LTP (Chang & Greenough, 1984; Desmond & Levy, 1986a & b; Geinisman *et al.*, 1991) may be due to altered expression of microtubule associated proteins such as MAP2. We found that two hours after the induction of LTP, MAP2 mRNA levels were slightly increased, but not significantly so, in the granule cell bodies. Similar observations were made following the induction of LTP in Schaffer collateral/CA1 synapses (Chapter 4, this volume) where levels of MAP2 mRNA were increased in the pyramidal cell bodies two hours after the induction of LTP, but not significantly so. If an increase in MAP2 expression occurs following the induction of LTP it is possible that it peaks at a later time point, as observations made about MAP2 expression following NMDA receptor stimulation (Johnston & Morris, 1994b & e) show that mRNA levels are increased after 24 hours.

There are a number of different isoforms of MAP2 which are differentially regulated in the CNS (Tucker, 1990) and it is possible that differential regulation of their expression takes place following the induction of LTP. As our oligonucleotide probe detects all splice variants it is possible that a decrease in one isoform of MAP2 masks an increase in another. This could

explain why the increases in MAP2 mRNA levels which we observed were not of the same magnitude as the increases observed for other genes.

5.3.7 SNAP A and SNAP B mRNA levels

25kD synaptosomal-associated protein (SNAP) is highly expressed in the hippocampus, particularly in the mossy fibre nerve terminals and inner molecular layer of the dentate gyrus (Oyler *et al.*, 1989). Its expression is increased during synaptogenesis (Catsicas *et al.*, 1991) and its localisation shifts from axons and cell bodies to presynaptic nerve terminals during development (Oyler *et al.*, 1991). In addition, it has been shown that SNAP is involved in transmitter release (Schiavo *et al.*, 1993) and that it may participate in membrane fusion (Blasi *et al.*, 1993).

Recently two different isoforms of this protein (SNAP A & B) were reported (Bark, 1993; Bark *et al.*, 1995). Bark *et al.* (1995) demonstrated that these isoforms are differentially regulated during development. The expression of SNAP A remains constant from early developmental ages through to adulthood whereas levels of SNAP B, which are low in embryonic tissue rise dramatically between postnatal days 1 and 21. It is possible, therefore, that each isoform plays a distinct role in early development. Localisation of each isoform to distinct populations of synapses in the brain may reflect the ability of some, but not all, synapses to undergo plasticity related changes.

The increase in presynaptic glutamate release associated with the induction of LTP (Bliss *et al.*, 1986; Errington *et al.*, 1987) and the alterations in levels of synaptic vesicle proteins and syntaxin following the induction of LTP (Smirnova *et al.*, 1993; Lynch *et al.*, 1994) suggests that presynaptic changes are important for the expression of LTP. We looked at mRNA levels of both

SNAP A and SNAP B following the induction of LTP in perforant path/granule cell synapses to see whether levels were altered and in what manner.

The distribution of both isoforms was as previously reported (Boschert *et al.*, 1996). SNAP B was more highly expressed throughout the adult hippocampus than SNAP A. Levels of mRNA for both isoforms were highest in the CA3 region and lowest in the CA1 region. SNAP B was more highly expressed in the granule cells than SNAP A. Neither isoform mRNA was found in the hippocampal dendrites. We used oligonucleotide probes which have previously been shown to be specific for each isoform (Boschert *et al.*, 1996). Two hours after the induction of LTP, mRNA levels of both isoforms were increased in granule cell bodies. SNAP A mRNA levels were increased by a greater percentage than SNAP B mRNA levels (compared to low frequency stimulated contralateral granule cells), but this may be a reflection of the lower starting levels of this transcript.

The increased expression of SNAP A and B following the induction of LTP has not previously been reported, but studies by Steffensen *et al.* (1996) have shown that mice which have a contiguous gene defect, encompassing the deletion of the gene encoding SNAP, display reduced potentiation of transmission in the hippocampus following high frequency stimulation of the perforant path. Boschert *et al.* (1996) demonstrated that two days after systemic administration of kainate, levels of SNAP A (but not SNAP B) were significantly increased in the granule cells of the dentate gyrus, suggesting that SNAP A may be important for axonal growth and synaptic remodelling which occur following kainate-induced neurotoxicity.

Developmental regulation of the two isoforms suggests that SNAP B is involved in the functioning of mature synapses, and that SNAP A plays a role

in axonal growth and synaptogenesis occurring up to 21 days after birth. Our observations support the hypothesis that SNAP plays a functional role in synaptic plasticity and that both isoforms appear to be involved in the expression of LTP. It would appear that, if the hypothesis about the relative roles of the two isoforms is correct, as expression of both isoforms is increased after the induction of LTP, synaptogenesis and the maintenance of existing synapses are equally important for this form of synaptic plasticity. It could also mean that SNAP A plays an important role in neurotransmitter release in mature synapses.

6.3.8 pENK mRNA levels

It has previously been shown that opioid peptides derived from the proenkephalin gene enhance neurotransmission in mossy fibre/CA3 synapses (Derrick *et al.*, 1992; Derrick & Martinez, 1994) while peptides derived from the prodynorphin gene have opposite effects at the same synapses (Weisskopf *et al.*, 1993). The generation of LTP in this region of the hippocampus occurs independently of NMDA receptor activation (Harris & Cotman, 1986) and is thought to be mediated, at least partially, by opioid peptides (Williams & Johnston, 1996). Previous work has shown that pENK mRNA levels are increased in the granule cells of the dentate gyrus twenty four hours after NMDA receptor stimulation (Johnston & Morris, 1994c) and that levels may be altered within a few hours of this stimulus (Johnston & Morris, personal communication). In addition it has been shown that high frequency stimulation of the granule cells, which produces LTP in mossy fibre/CA3 synapses is associated with increased expression of pENK mRNA (Morris *et al.*, 1988). Basal levels of pENK mRNA in the granule cells of the dentate gyrus are low. It is usually only expressed by about 15 % of the cells present. We have found that two hours after the induction of LTP in perforant path/granule cell

synapses the number of cells expressing pENK is significantly increased. The peptides derived from pENK will be transported from the granule cells along the mossy fibre axons to be released from the mossy fibre nerve terminals. In this way excitation of one group of synapses in the hippocampus can lead to effects on downstream synapses, a phenomenon referred to as "domino plasticity" (Morris & Johnston, 1995). It is possible, therefore, that electrical induction of LTP in perforant path/granule cell synapses could lead to enkephalin mediated potentiation of mossy fibre/CA3 synapses and possible effects on transmission in Schaffer collateral/CA1 synapses. Smirnova *et al.* (1993) made similar observations when they found levels of syntaxin protein were increased in the mossy fibre nerve terminals following the induction of LTP in perforant path/granule cell synapses. Our observations strengthen the theory that opioid peptides modulate transmission in the hippocampus and are involved in functional plasticity in the structure (Morris & Johnston, 1995).

5.3.9 Dendritic mRNA levels

In addition to changes which we observed in the granule cell bodies, levels of αCAMKII mRNAwere also significantly increased in the dendrites of the granule cells two hours after the induction of LTP. Dendritic increases in αCAMKII mRNA levels have previously been reported twenty four hours after the induction of LTP (Thomas *et al.*, 1994a) and two hours after NMDA receptor stimulation (Johnston & Morris, 1995). Thomas *et al.* (1994a) did not report increases in dendritic αCAMKII mRNA levels two hours after the induction of LTP and suggested that the increases which they observed after twenty four hours were due to transport of mRNA from the cell bodies to the dendrites. However, it is unlikely that two hours would be sufficient time for such transport to occur (Davis *et al.*, 1990) and it is possible that the induction of LTP in these synapses may lead to stabilisation of mRNA levels and

localised increases in protein synthesis. Our observations are consistent with those made following the induction of LTP in Schaffer collateral/CA1 synapses (Chapter 4, this volume) although the increases in levels of αCAMKII did not reach statistical significance due to low sample number. Other work in this lab has demonstrated that the release of nitric oxide can stabilise dendritic mRNA levels of αCAMKII and MAP2 in hippocampal cultures (B.J. Morris, personal communication). We also found that levels of MAP2 mRNA were significantly increased in the granule cell dendrites two hours after the induction of LTP. This result is consistent with observations made two hours after the induction of LTP in Schaffer collateral/CA1 synapses (Chapter 4, this volume) when MAP2 mRNA levels were shown to be increased (although not significantly so). In addition, Johnston & Morris (1994e) demonstrated that dendritic MAP2 levels could be increased following NMDA receptor stimulation or the actions of nitric oxide, and that it may be a reflection of LTP-related structural changes. The observation that MAP2 mRNA levels are significantly increased in the granule cell dendrites, but not the cell bodies, could indicate that mechanisms which bring about the stabilisation of all dendritic mRNA species are activated following the induction of LTP, but that the mechanism does not extend to mRNAs confined to the cell body. These observations confirm that the expression of LTP in both CA1 pyramidal cells and granule cells of the dentate gyrus appears to involve increases in levels of dendritic mRNA species. In addition we may have uncovered a mechanism which allows localised changes in protein synthesis triggered by the induction of LTP.

5.3.10 Conclusions

We have shown that synthesis of enkcphalins in the dentate gyrus may contribute to enhancement of transmission in downstream synapses and support the hypothesis for a role for opioid peptide in synaptic plasticity. Increases in

mRNA levels of SNAP A and B suggests that both these isoforms are involved in plasticity, although their functions (not yet fully understood) may be different. We have also shown that the induction of LTP in both perforant path/granule cell synapses and Schaffer collateral/CA1 synapses may involve similar mechanisms. One of these mechanisms may involve localised stabilisation of dendritic mRNA species.

CHAPTER 6

PHARMACOLOGICAL MANIPULATION OF GENE EXPRESSION IN HIPPOCAMPAL GRANULE CELLS

6.1 INTRODUCTION

6.1.1 Pharmacology of neurotransmission in the hippocampus

Several subtypes of glutamate receptor are found in the hippocampus. *N*-methyl-D-aspartate (NMDA) receptors have been shown to be highly expressed in the hippocampus, particularly in the granule cells of the dentate gyrus where the medial perforant path terminates (Cotman *et al.*, 1987), and their distribution overlaps that of amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA) receptors (Monaghan *et al.*, 1984). AMPA receptors are sometimes classed alongside kainate receptors as non-NMDA receptors. This group of receptors are made up from different subunits, the majority of which are derived from the same gene, and display similar properties although they can be distinguished by their pharmacological profiles. In addition, there are thought to be at least eight subtypes of metabotropic glutamate receptor (mGluR1-8) all of which have been shown to be expressed in the hippocampus to varying degrees (Shigemoto *et al.*, 1992; Ohishi *et al.*, 1993a & b; Shigemoto *et al.*, 1993; Kinzie *et al.*, 1995; Ohishi *et al.*, 1995). NMDA and AMPA/kainate receptors are ligand gated and composed of a variety of subunits. The functional properties of these receptors varies according to which subunits they comprise. AMPA/kainate receptors receptors mediate fast excitatory synaptic transmission in the hippocampus and their activation results in a postsynaptic depolarisation caused by sodium entry into the cell. In its resting state the channel formed by NMDA receptor subunits is impermeable to other ions due to the presence of magnesium ions. For NMDA receptor activation to occur the membrane must be sufficiently depolarised to expel the magnesium ions form the channel and allow influx of sodium and calcium ions into the cell. This means that the activation state of NMDA receptors is critically dependent on the electric potential of the cell membrane and the ionic

permeability of these channels to sodium and calcium ions is usually only increased following the activation of AMPA/kainate receptors. In contrast mGluRs, are G-protein coupled receptors which, when activated, regulate several second messenger cascades and intracellular signalling pathways. The roles played by mGluRs in the induction of LTP have been previously addressed (Chapter 1, this volume). It has been suggested that their properties may be suited to translating transient activity such as learning into long lasting memory (Riedel, 1996).

NMDA receptor activation has been shown to mediate the release of nitric oxide (Garthwaite *et al.*, 1988), which activates guanylate cyclase, leading to a rise in intracellular levels of cGMP and activation of cGMP-dependent protein kinase (PKG). Activation of AMPA receptors produces a short lasting excitatory post synaptic potential (EPSP) by allowing the passage of sodium and potassium ions. Some isoforms are also calcium permeable. mGluRs have been subdivided into three classes. Class I include mGluR1 and mGluR5, and their activation results in stimulation of phospholipase C, leading to formation of inositol (1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mediates the release of Ca^{2+} from intracellular stores and DAG activates protein kinase C (PKC). Class II and class III comprise mGluR2,3 and mGluR4,6,7,8 respectively. Both these classes of mGluR are negatively coupled to adenylate cyclase and their activation results in a reduction in cAMP levels and a decrease in activity of cAMP dependent protein kinase (PKA).

6.1.2 Aims of the study

Previous work has demonstrated that 24 hours after the application of NMDA, a nitric oxide releasing agent and PKG activators to granule cell dendrites *in vivo*, changes in proenkephalin (pENK), prodynorphin (pDYN), microtubule

associated protein 2 (MAP2) and αCalcium/calmodulin-dependent protein kinase II (αCAMKII) mRNA are observed in the granule cell bodies (Johnston & Morris, 1994b & c; Johnston & Morris, 1995). The changes seen were similar to those observed following the induction of LTP in perforant path/granule cell synapses. As activation of mGluRs has been shown to induce protein synthesis (Weiler & Greenough, 1993), we decided to see whether mGluR activation or blockade could produce changes in mRNA levels in the same model. In addition we also looked at what effect AMPA receptor stimulation might have on gene expression, and whether protein kinase A might be involved in intracellular signalling and regulation of protein synthesis in the same way that PKG appears to be. To carry out this work we used the selective mGluR agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), the class I mGluR antagonist (+)-alpha-methyl-4carboxyphenyl-glycine (MCPG), (S)-AMPA and the protein kinase A activator, Sp-adenosine-3',5'-cyclic monophosphothioate triethylamine (Sp-cAMPS). To control for any changes which might be due to the technique itself we also looked at the effects of injecting 0.9% saline solution.

6.2 RESULTS

We looked at the levels of the mRNAs encoding αCAMKII, T26, candidate plasticity gene (CPG) and tissue inhibitor of metalloproteinases (TIMP) two hours after treatment and at the levels of the mRNAs encoding αCAMKII, T26, pENK, pDYN and MAP2 twenty four hours after treatment.

6.2.1 mRNA levels at 2 hours

Figure 6.2.1a-d show % change in mRNA levels of αCAMKII (a), T26 (b), CPG (c) and TIMP (d) in the granule cells of the dentate gyrus two hours after application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS (n=2-6). No significant changes in any mRNA levels were seen following any of the treatments (ANOVA).

6.2.2 mRNA levels after 24 hours

Figure 6.2.2a shows % change in levels of αCAMKII mRNA in the granule cell bodies 24 hours after microapplication of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS (n=3-6). mRNA levels were significantly increased following treatment with 1mM ACPD and 1mM Sp-cAMPS (16±3.5% and 9+9.8% respectively) compared to saline. ($F(4,19)=5.15$; $p=0.006$, ANOVA; $^*p<0.05$ by Fishers test for multiple pairwise comparison).

Figure 6.2.2b shows mRNA levels of αCAMKII in the granule cells of the dentate gyrus in a region unaffected by the injection of 80nl of 1mM ACPD, but ipsilateral to the site of injection (i) and directly under the needle tip 24 hours after the injection of 80nl of 1mM ACPD (ii). Levels of αCAMKII are

clearly increased 24 hours after the application of 1mM ACPD to the dendrites of these cells.

Figure 6.2.2c shows mRNA levels of αCAMKII in the granule cells of the dentate gyrus in a region unaffected by the injection of 80nl of 1mM Sp-cAMPS, but ipsilateral to the site of injection (i) and directly under the needle tip 24 hours after the injection of 80nl of 1mM Sp-cAMPS (ii). Levels of αCAMKII are clearly increased 24 hours after the application of 1mM Sp-cAMPS to the dendrites of these cells.

Figure 6.2.2d shows mRNA levels of αCAMKII in the granule cells of the dentate gyrus in a region ipsilateral to the site of injection of 80nl of 0.9% saline, and directly under the needle tip 24 hours after the injection of 80nl of 0.9% saline (ii). Levels of αCAMKII are unchanged 24 hours after the application of 0.9% saline to the dendrites of these cells.

Figure 6.2.2e shows mRNA levels of T26 in the granule cells of the dentate gyrus in a region ipsilateral to the site of injection of 80nl of 1mM ACPD, and directly under the needle tip 24 hours after the injection (ii). Levels of T26 are unchanged 24 hours after the application of 1mM ACPD to the dendrites of these cells.

Figure 6.2.3a-d show % change in mRNA levels of T26 (a), pENK (b), pDYN (c) and MAP2 (d) in the granule cells of the dentate gyrus 24 hours after application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS (n=2-6). No significant changes in any mRNA levels were seen following any of the treatments (ANOVA).

Figure 6.2.1a

% change in mRNA levels of αCAMKII in the cell bodies of the dentate gyrus 2 hours after a single application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS to the dendrites of the granule cells.

Changes were not significant (ANOVA)

n=3-5

Figure 6.2.1a

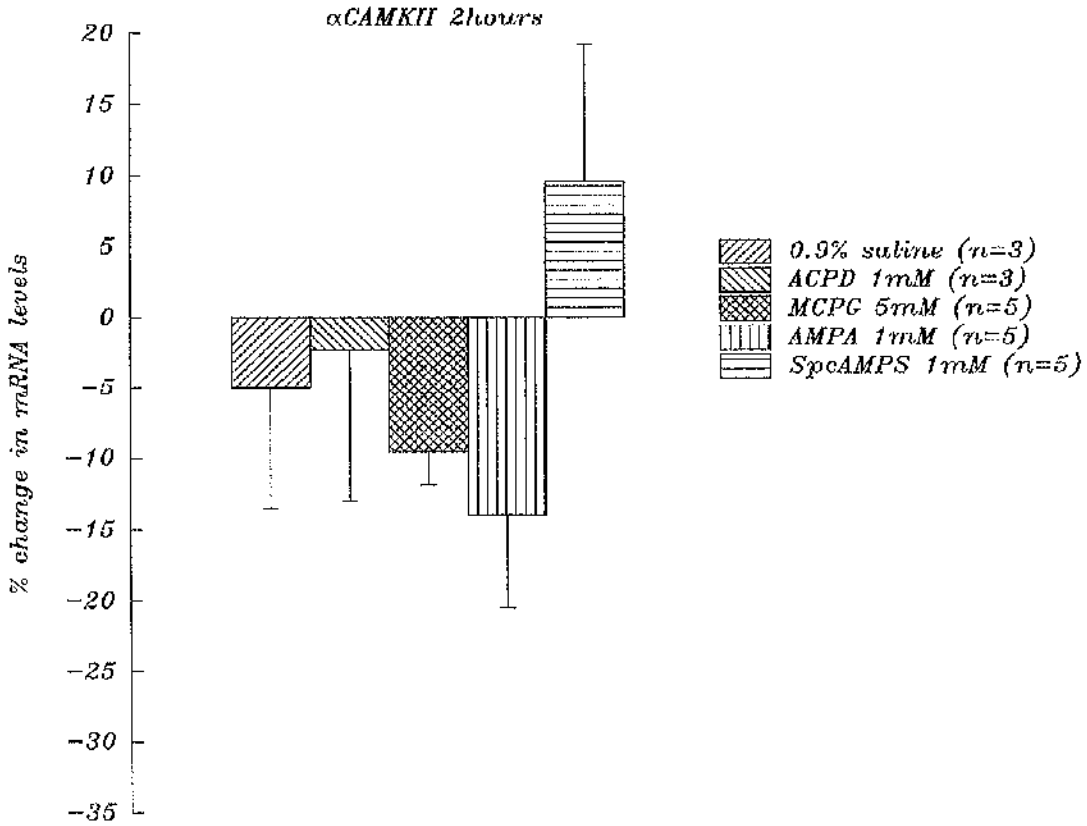

αCAMKII 2hours
% change in mRNA levels
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
0.9% saline (n=3)
ACPD 1mM (n=3)
MCPG 5mM (n=5)
AMPA 1mM (n=5)
SpcAMPS 1mM (n=5)

Figure 6.2.1b

% change in mRNA levels of T26 in the cell bodies of the dentate gyrus 2 hours after a single application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS to the dendrites of the granule cells.

Changes were not significant (ANOVA)

n=2-6

Figure 6.2.1b

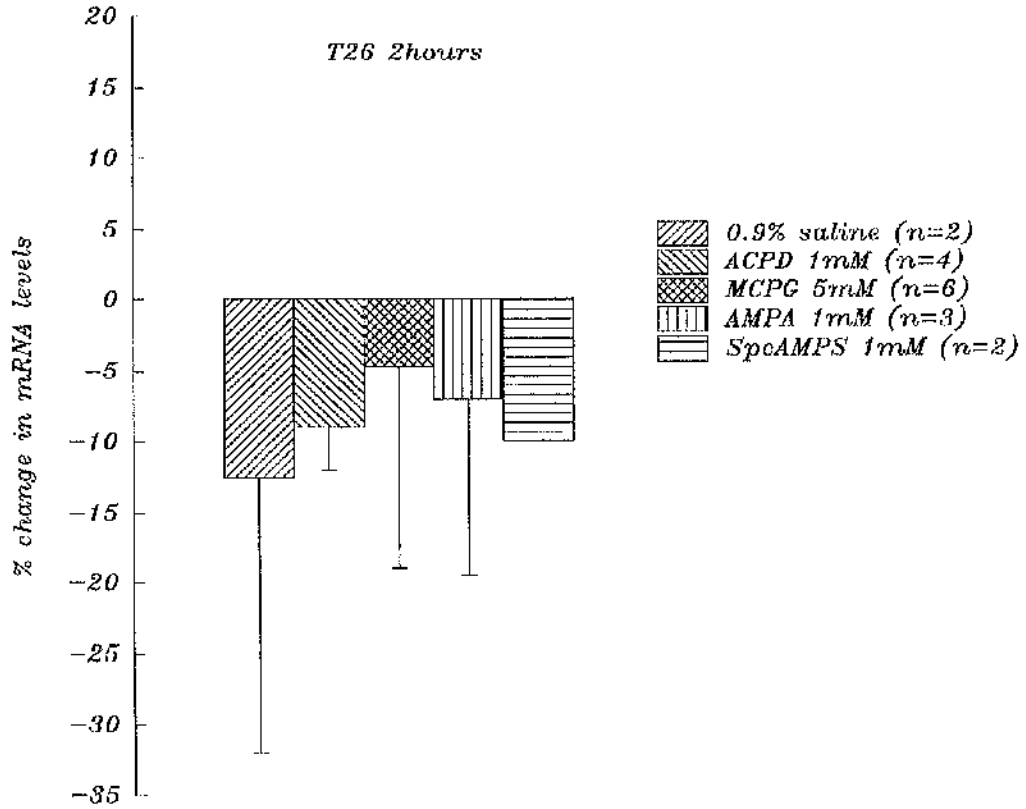

T26 2hours
% change in mRNA levels
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
0.9% saline (n=2)
ACPD 1mM (n=4)
MCPG 5mM (n=6)
AMPA 1mM (n=3)
SpcAMPS 1mM (n=2)

Figure 6.2.1c

% change in mRNA levels of CPG in the cell bodies of the dentate gyrus 2 hours after a single application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS to the dendrites of the granule cells.

Changes were not significant (ANOVA)

n=2-5

Figure 6.2.1c

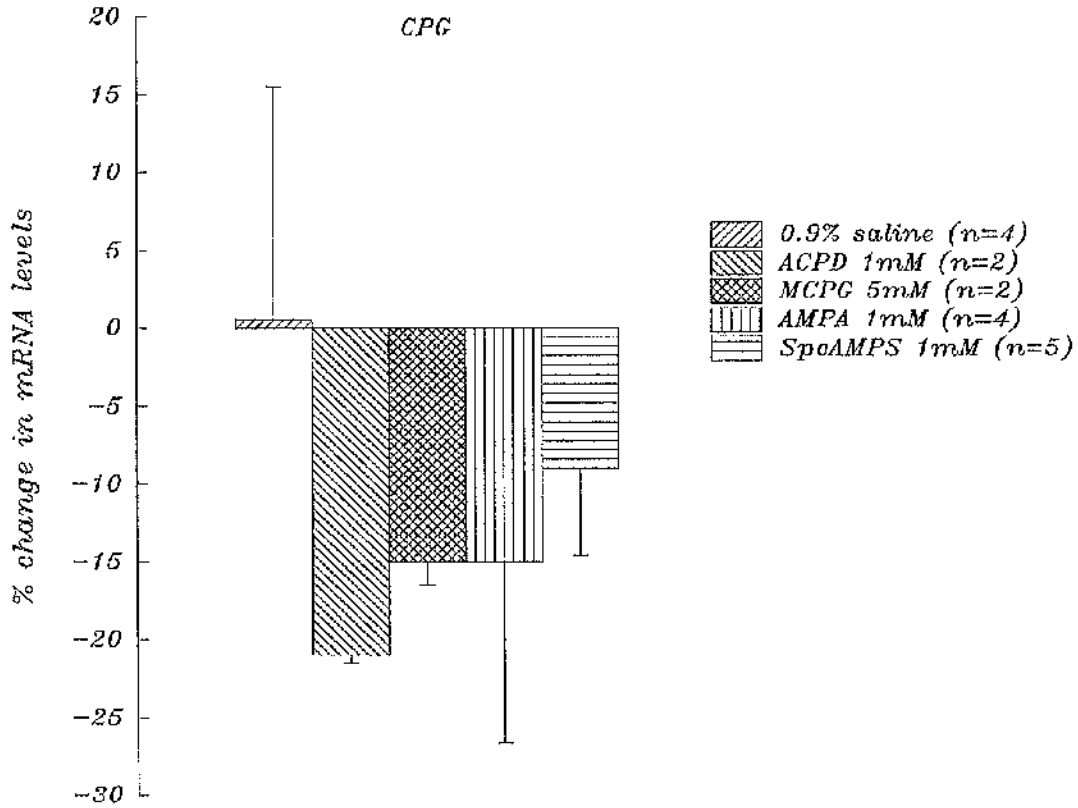

CPG
% change in mRNA levels
20
15
10
5
0
-5
-10
-15
-20
-25
-30
0.9% saline (n=4)
ACPD 1mM (n=2)
MCPG 5mM (n=2)
AMPA 1mM (n=4)
SpoAMPS 1mM (n=5)

Figure 6.2.1d

% change in mRNA levels of TIMP in the cell bodies of the dentate gyrus 2 hours after a single application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS to the dendrites of the granule cells.

Changes were not significant (ANOVA)

n=3-4

Figure 6.2.1d

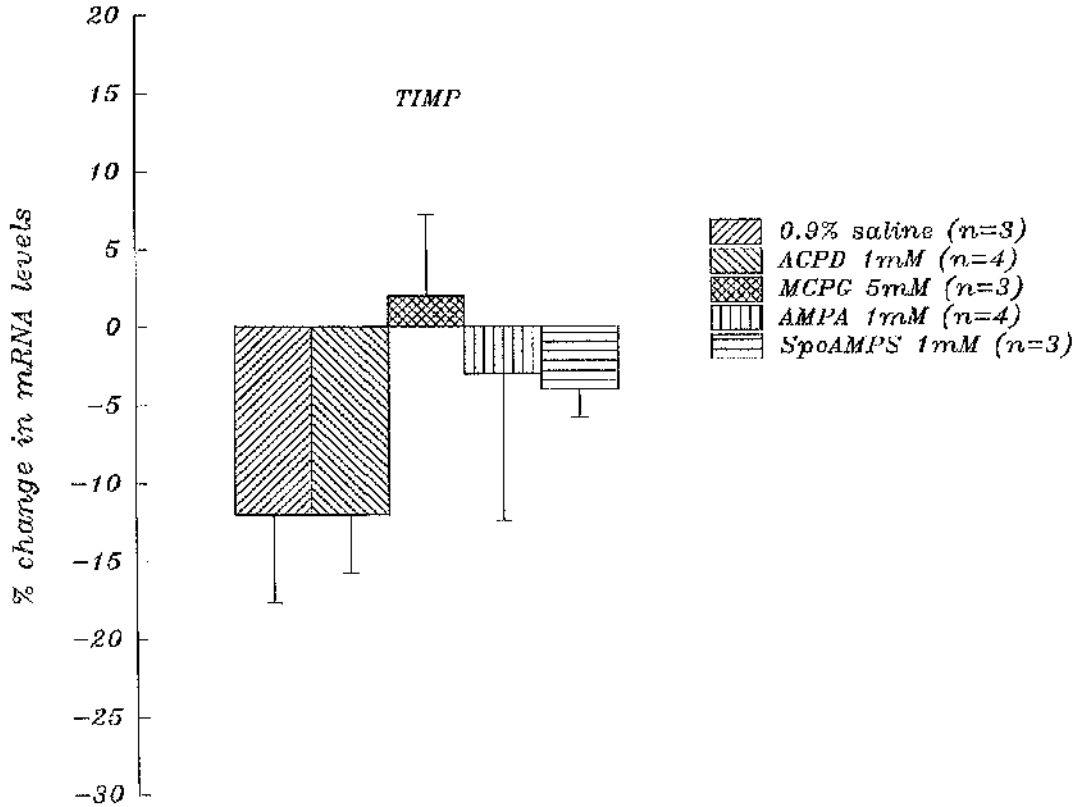

TIMP
% change in mRNA levels
20
15
10
5
0
-5
-10
-15
-20
-25
-30
0.9% saline (n=3)
ACPD 1mM (n=4)
MCPG 5mM (n=3)
AMPA 1mM (n=4)
SpoAMPS 1mM (n=3)

Figure 6.2.2a

% change in mRNA levels of αCAMKII in the cell bodies of the dentate gyrus 24 hours after a single application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS to the dendrites of the granule cells.

Levels were significantly increased compared to saline following treatment with 1mM ACPD and 1mM Sp-cAMPS ($F(4,19)=5.15$; $p=0.006$, ANOVA; *$p<0.05$ by Fishers test for multiple pairwise comparisons).

n=3-6

Figure 6.2.2a

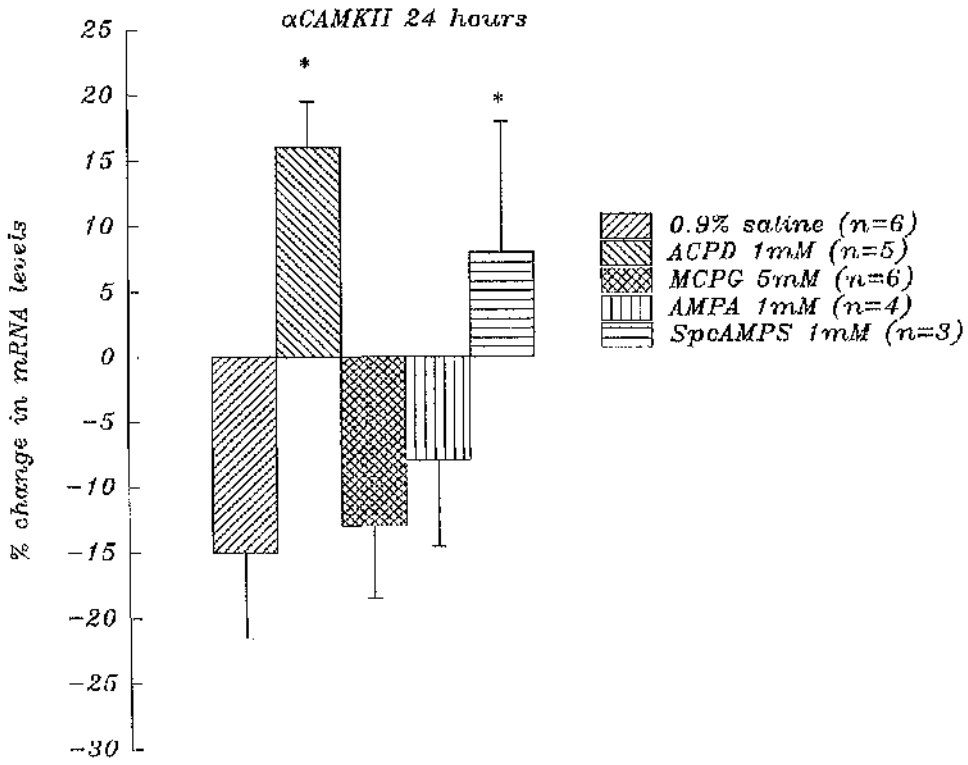
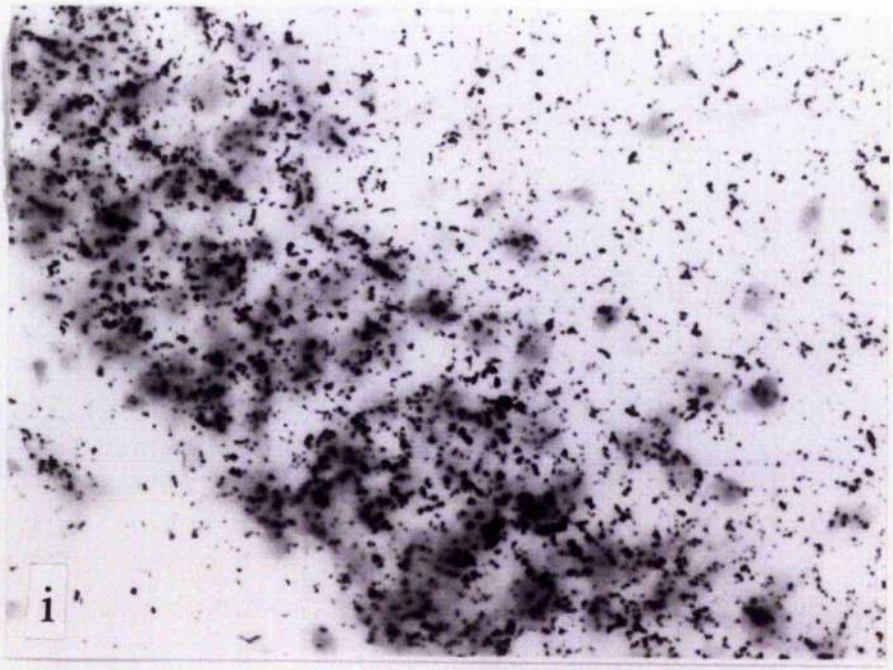

αCAMKII 24 hours
% change in mRNA levels
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
*
*
0.9% saline (n=6)
ACPD 1mM (n=5)
MCPG 5mM (n=6)
AMPA 1mM (n=4)
SpcAMPS 1mM (n=3)

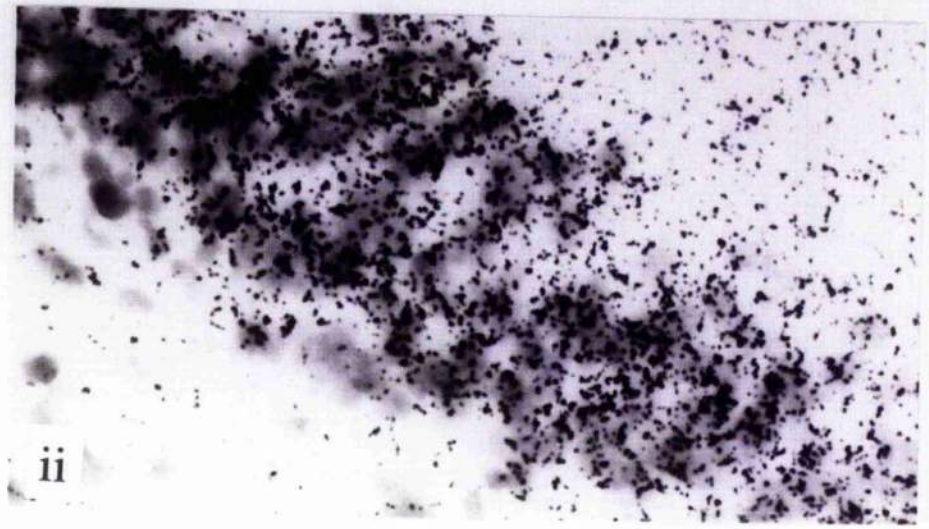
Figure 6.2.2b

Photomicrograph showing αCAMKII hybridisation in (i) untreated granule cells and in granule cells 24 hours after dendritic microapplication of 80nl of 1mM ACPD (ii)

Scale bar = 25μm

Figure 6.2.2b





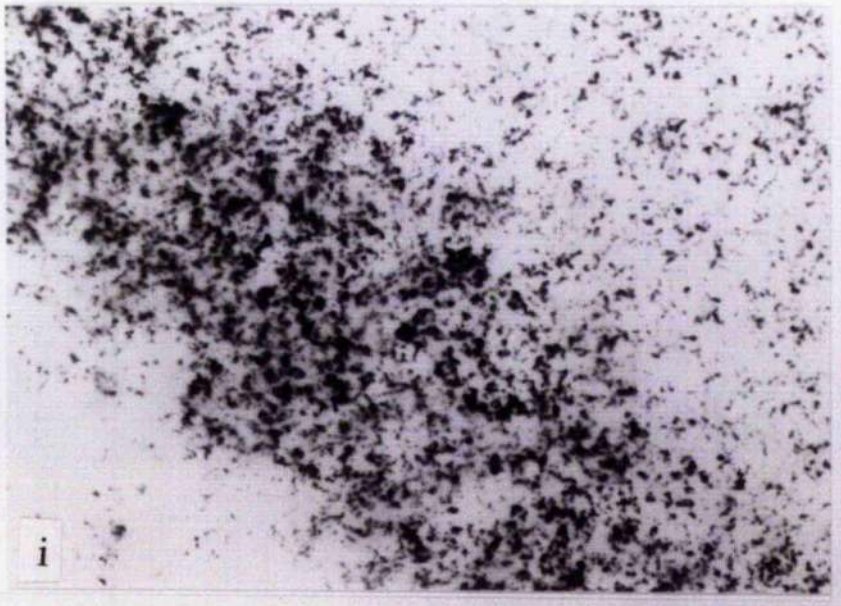
I

Figure 6.2.2c

Photomicrograph showing αCAMKII mRNA levels in (i) untreated granule cells and in granule cells 24 hours after dendritic microapplication of 80nl of 1mM Sp-cAMPS (ii)

Scale bar = 25μm

Figure 6.2.2c



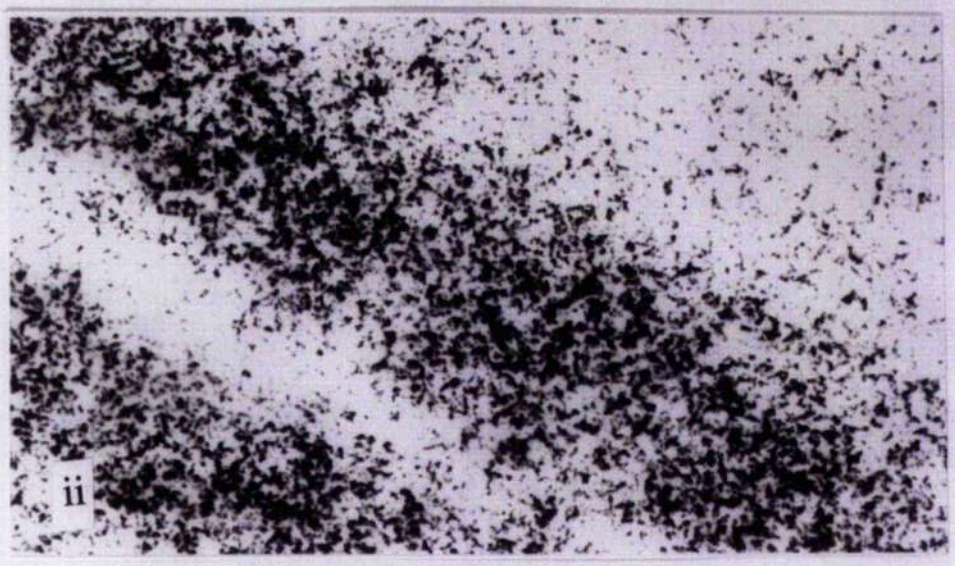
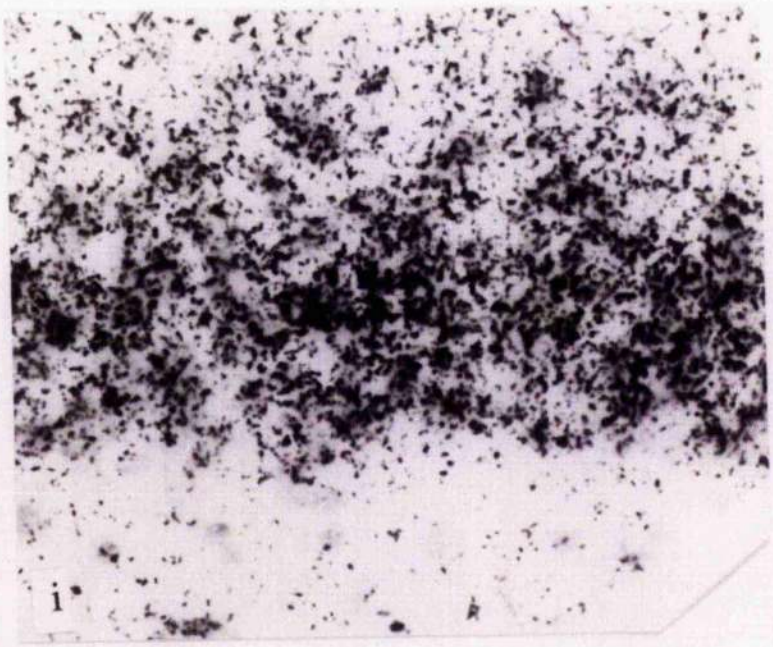


Figure 6.2.2d

Photomicrograph showing αCAMKII mRNA levels in (i) untreated granule cells and in granule cells 24 hours after dendritic microapplication of 80nl of 0.9% saline (ii)

Scale bar = 25μm

Figure 6.2.2d



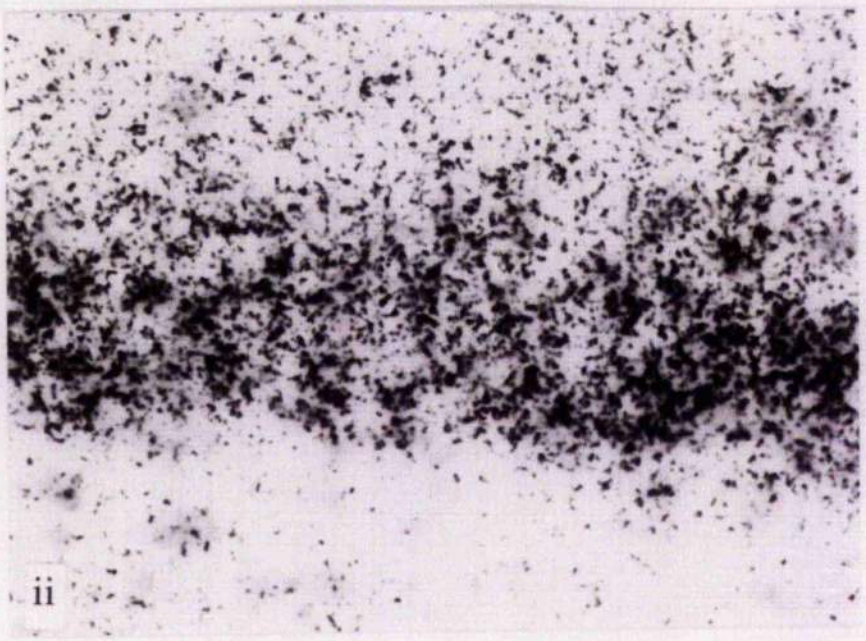
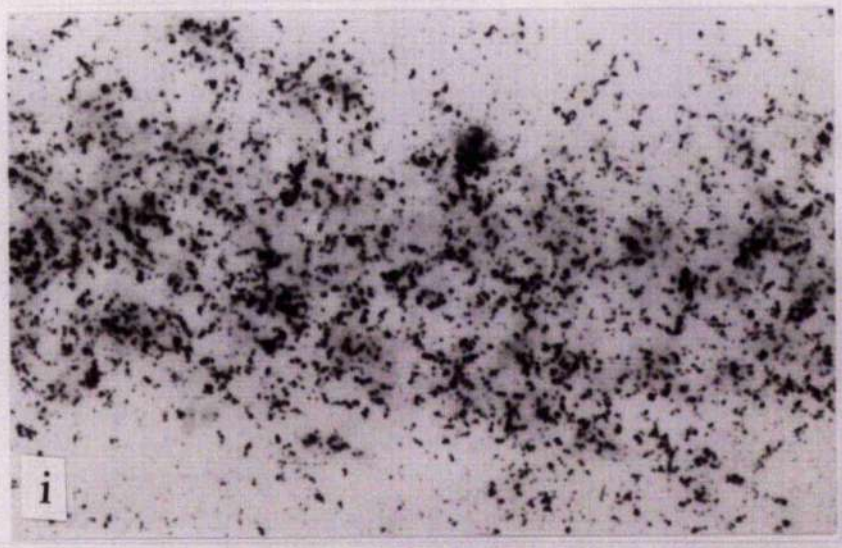


Figure 6.2.2e

Photomicrograph showing T26 mRNA levels in (i) untreated granule cells and in granule cells 24 hours after dendritic microapplication of 80nl of 1mM ACPD (ii)

Scale bar = 25μm

Figure 6.2.2e



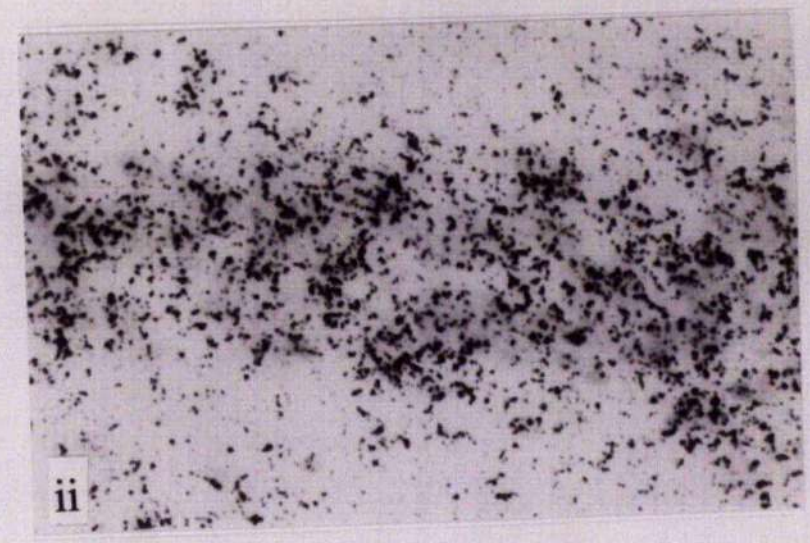


Figure 6.2.3a

% change in mRNA levels of T26 in the cell bodies of the dentate gyrus 24 hours after a single application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS to the dendrites of the granule cells.

Changes were not significant (ANOVA)

n=2-6

Figure 6.2.3a

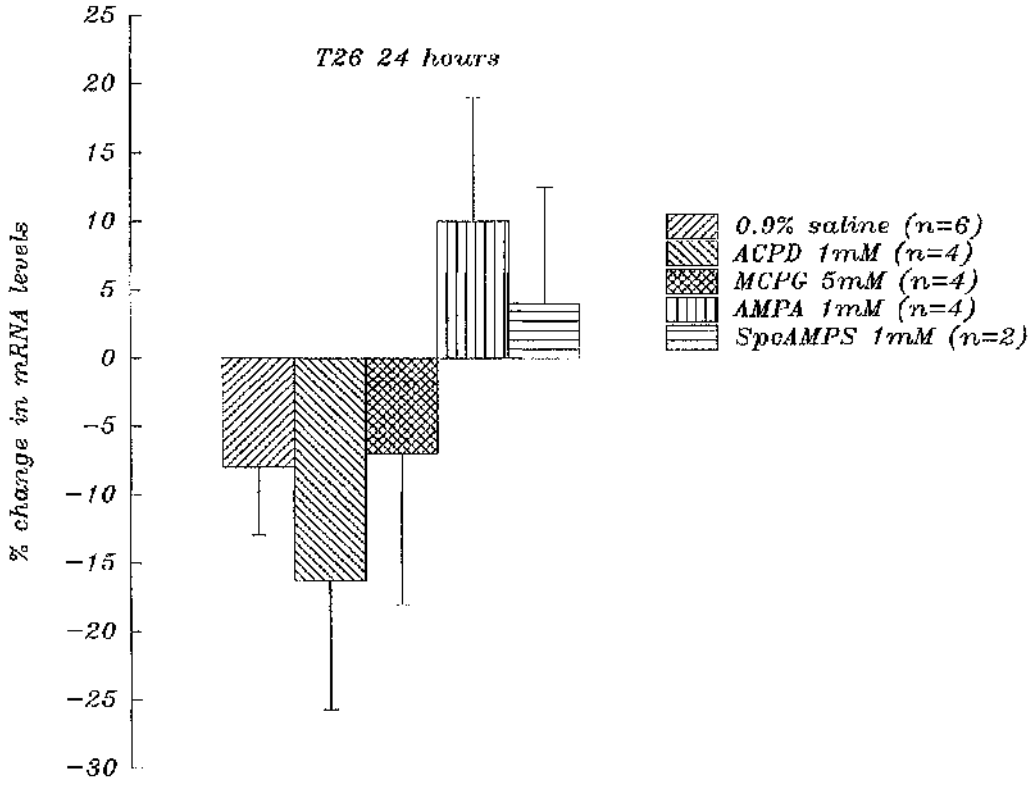

T26 24 hours
% change in mRNA levels
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
0.9% saline (n=6)
ACPD 1mM (n=4)
MCPG 5mM (n=4)
AMPA 1mM (n=4)
SpcAMPS 1mM (n=2)

Figure 6.2.3b

% change in mRNA levels of pENK in the cell bodies of the dentate gyrus 24 hours after a single application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS to the dendrites of the granule cells.

Changes were not significant (ANOVA)

n=2-6

Figure 6.2.3b

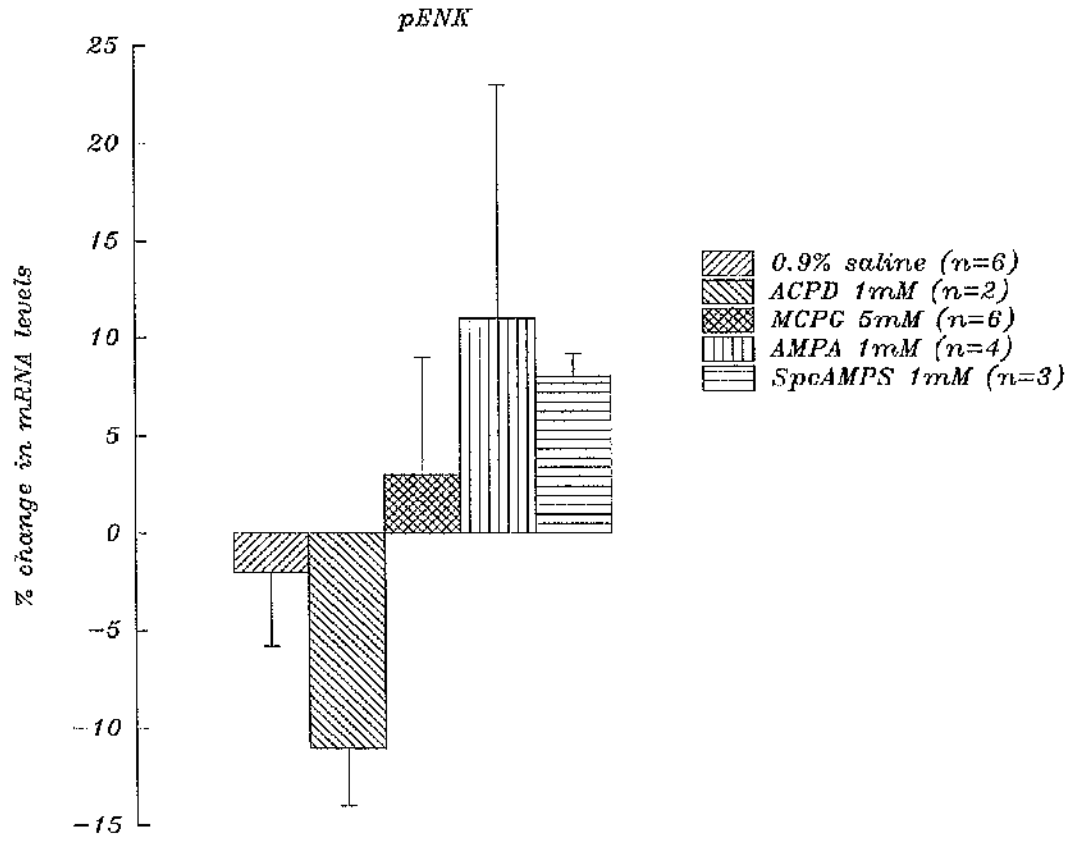

pENK
% change in mRNA levels
25
20
15
10
5
0
-5
-10
-15
0.9% saline (n=6)
ACPD 1mM (n=2)
MCPG 5mM (n=6)
AMPA 1mM (n=4)
SpcAMPS 1mM (n=3)

Figure 6.2.3c

% change in mRNA levels of pDYN in the cell bodies of the dentate gyrus 24 hours after a single application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS to the dendrites of the granule cells.

Changes were not significant (ANOVA)

n=3-4

Figure 6.2.3c

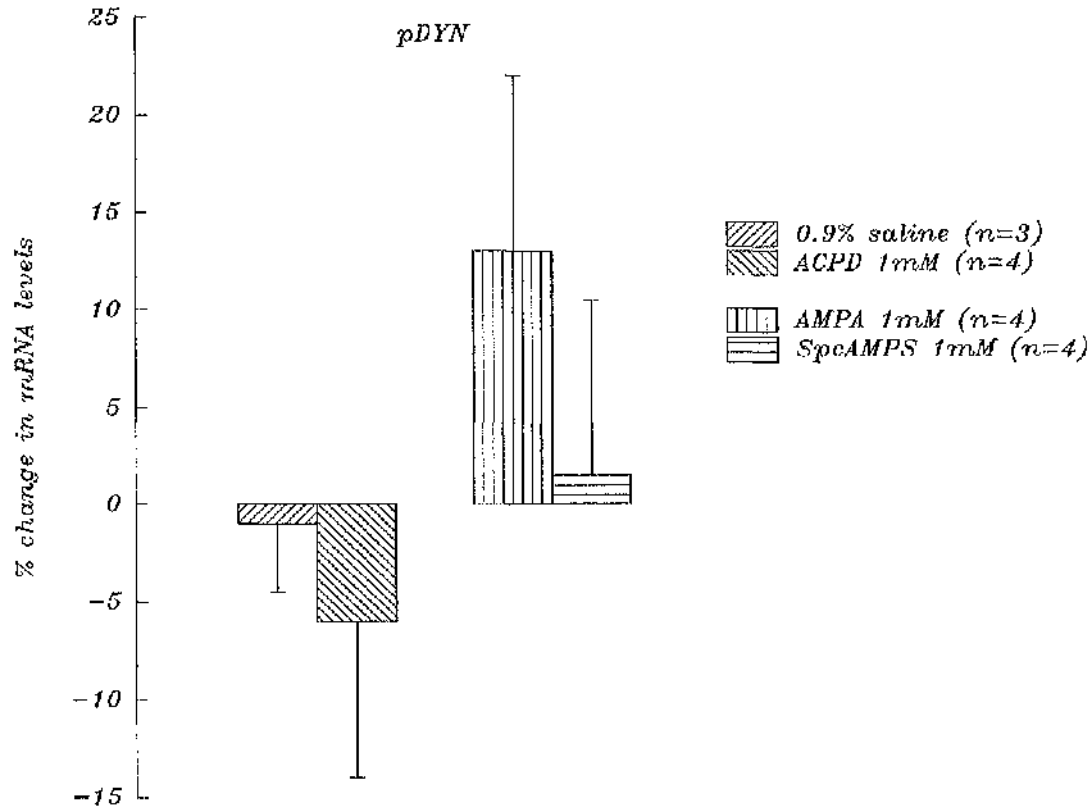

pDYN
% change in mRNA levels
25
20
15
10
5
0
-5
-10
-15
0.9% saline (n=3)
ACPD 1mM (n=4)
AMPA 1mM (n=4)
SpcAMPS 1mM (n=4)

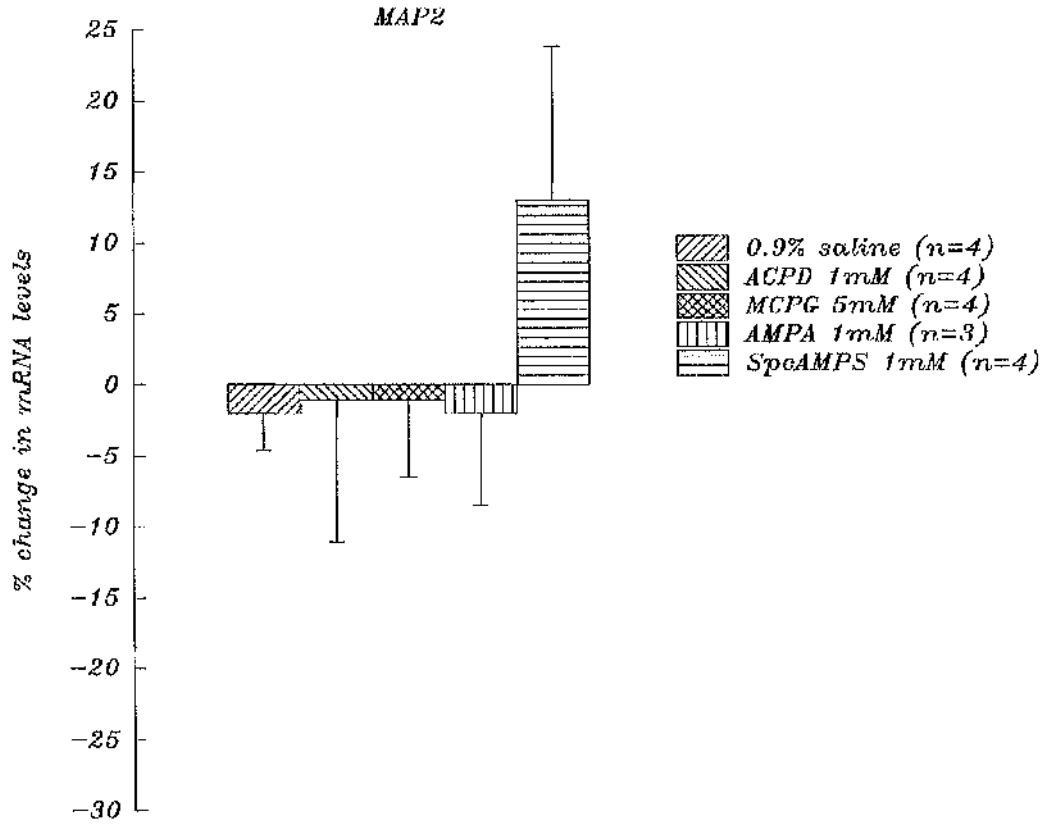
Figure 6.2.3d

% change in mRNA levels of MAP2 in the cell bodies of the dentate gyrus 24 hours after a single application of 80nl of 0.9% saline, 1mM ACPD, 5mM MCPG, 1mM AMPA and 1mM Sp-cAMPS to the dendrites of the granule cells.

Changes were not significant (ANOVA)

n=3-4

Figure 6.2.3d


MAP2
% change in mRNA levels
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
0.9% saline (n=4)
ACPD 1mM (n=4)
MCPG 5mM (n=4)
AMPA 1mM (n=3)
SpcAMPS 1mM (n=4)

6.3 DISCUSSION

6.3.1 Aims of the study

This work was undertaken to extend the findings of Johnston & Morris (1994a, b, c, d & e 1995). They showed that the expression of several genes could be manipulated by NMDA receptor activation, nitric oxide and cGMP. We were interested to see whether activation of other intracellular second messenger systems and glutamate receptors could affect the expression of these and other genes, and how these changes might be related to synaptic plasticity and LTP.

6.3.2 Choice of compound

AMPA receptors mediate fast excitatory synaptic transmission in the hippocampus and it is thought that LTP is induced following coactivation of NMDA and AMPA receptors. Stimulation of AMPA receptors causes transient post synaptic membrane depolarisation unless the stimulation is repetitive, when a summation of AMPA mediated EPSPs occurs leading to sustained membrane depolarisation and the relief of the Mg^{2+} blockade of NMDA receptors. It is possible, therefore that AMPA receptor stimulation using (S)-AMPA may lead to potentiation of transmission in the dentate gyrus and perhaps changes in gene expression, depending on its duration. mGluR activation has been shown to induce protein synthesis (Weiler & Greenough, 1993). In addition, the development of a slow-onset potentiation is seen following mGluR activation (Borlotto & Collingridge, 1993) and it is thought that the activation of mGluRs may be necessary for sustaining NMDA receptor-dependent LTP (Bashir *et al.*, 1993). To examine mGluR function in this study we used ACPD, an agonist which is specific for mGluRs compared to AMPA and NMDA receptors, but shows similar levels of activity at all the

mGluR subtypes. To see whether mGluR activity might have a tonic effect on neurotransmission in the hippocampus we looked at the effects of MCPG, a class I mGluR antagonist, on gene expression. The importance of adenylate cyclase/cAMP/PKA in invertebrate learning is well established. Long-term facilitation in *Aplysia* leads to activation of PKA and CREB related transcription factors (Bacskai *et al.*, 1993; Kaang *et al.*, 1993), and mutations in genes involved in the cAMP second messenger system impair learning in *Drosophila* (Byers *et al.*, 1981; Levin *et al.*, 1992; Yin *et al.*, 1994). In mammals activation of receptors which are positively coupled to adenylate cyclase can induce a protein synthesis-dependent slowly developing potentiation in the CA1 region of the hippocampus (Huang & Kandel, 1995) and that, during the protein synthesis-independent phase of LTP, the cAMP signalling pathway gates the induction of LTP through protein phosphatases (Blitzer *et al.*, 1995). NMDA receptor activation increases cAMP levels in the hippocampus (Chetkovich *et al.*, 1994) and cAMP analogues stimulate the induction of LTP in the CA1 region of the hippocampus (Frey *et al.*, 1993; Slack & Walsh, 1995). It has also been shown that mRNA for calmodulin sensitive adenylate cyclase is found in regions of the brain associated with long-term memory (Xia *et al.*, 1991). We chose the protein kinase A activator/cAMP analogue Sp-cAMPS to investigate the effects of increased cAMP levels on gene expression.

6.3.3 Choice of mRNA

We looked at αCAMKII, pENK, pDYN and MAP2 mRNA, levels of which have been shown to be altered following NMDA receptor stimulation and release of nitric oxide (Johnston & Morris, 1994b, c & e, 1995). Enkephalins (derived from pENK) are thought to have net excitatory effects on synaptic transmission in the hippocampus, and dynorphins derived from pDYN are

thought to have inhibitory effects on transmission in the hippocampus (Wagner *et al.*, 1993). It was demonstrated that induction of LTP in mossy fibre synapses *in vivo* results in a rise in pENK levels and a concurrent decrease in pDYN levels (Morris *et al.*, 1988). MAP2 is a cytoskeletal protein which is capable of producing structural changes as its expression promotes neurite outgrowth (Caceres *et al.*, 1988; Ferreira *et al.*, 1990). CAMKII has been shown to be necessary and sufficient for the induction of LTP (Pettit *et al.*, 1994) and the α subunit is expressed at higher levels following the induction of LTP in the hippocampus (Mackler *et al.*, 1992; Thomas *et al.*, 1994a; Chapter 4 & 5, this volume). We also looked at T26 mRNA levels to give an indication of whether any non-specific changes in gene expression were occurring. In addition, we looked at mRNA levels of CPG and TIMP, two putative plasticity-related genes which have been shown to be expressed at higher levels following the induction of LTP (Nedivi *et al.*, 1993). The nature of the technique involved in these experiments makes it impractical to look at the expression of c-fos and zif/268 as both are induced by the act of lowering the needle tip into the brain (Johnston & Morris, 1994a & d).

6.3.4 Methodology

The technique used in these experiments has been previously shown to produce no damage to the granule cells of the dentate gyrus (Johnston & Morris, 1994a, b, c, d, e, 1995). Although other studies have shown that focal damage to the hippocampus produced by saline infusion leads to non-specific increases in expression of immediate early genes (Dragunow & Hughes, 1993), neurotrophins (Hughes *et al.*, 1993) and neurotrophin receptors (Mudó *et al.*, 1993) these studies have used larger gauge needles than we have (30G compared to 50G) and have infused much larger volumes of solutions (5μl compared to 0.08μl) over a shorter period of time. Although the expression of

zif/268 and c-fos has been shown to be increased non-specifically using this technique (Johnston & Morris, 1994a & d), the changes in levels of expression of all other genes studied are specific to the treatment received as all results were compared to the effects of saline.

As with most *in vivo* work it is not possible to determine the exact concentrations of a compound at its site of action and assumptions about its dilution have to be made. For this reason we decided to apply a constant volume of solution for each compound directly to the dendrites of the cells which we wanted to affect. The concentrations of solutions used were ten times greater than their pharmacologically active concentrations as we assumed that approximately a ten-fold dilution of each would occur in the extracellular fluid.

The results which we have presented are based on the assumption that the compounds used are acting specifically in the manner expected from information available. Studies using the appropriate antagonists would be needed to support this assumption. There are, however, few selective antagonists for the subtypes of mGluR which are known. MCPG is a selective antagonist at mGluR 1 and 2 but most other compounds fail to distinguish between the different subtypes and some even display a complex mix of excitatory and inhibitory actions from subtype to subtype. Ideally the effects of Sp-cAMPS on PKA activation should have been tested using the PKA inhibitors H-89 or KT5720.

6.3.5 mRNA levels two hours post injection

Expression of αCAMKII, T26, CPG and TIMP were examined two hours post-injection, because previous work has shown that changes in mRNA levels

for these genes can be seen as early as 2 hours post-injection. None of the compounds which we looked at produced any significant changes in mRNA levels of any of the four genes studied compared to saline. Levels of T26 mRNA were not expected to change, but levels of αCAMKII mRNA have been shown to be increased two hours after NMDA treatment (Johnston & Morris, 1995). This may indicate that this increase is specific to NMDA receptor activation or that the compounds we used affect gene expression at a later time point than two hours. If ACPD application were producing a slow-onset potentiation in this model then it would take at least two hours to develop, and its maintenance might depend on the expression of genes at a later time point than NMDA receptor-dependent LTP. Intracerebroventricular injection of ACPD has been shown to increase levels of expression of BDNF and NGF mRNA, and decrease NT-3 mRNA (Murray *et al.*, 1996) in under six hours. However similar observations have been made about neurotrophin expression following direct injection of saline into the hippocampus (Hughes & Dragunow, 1993) so the significance of the previous observation is unclear. Levels of CPG and TIMP mRNA have been shown to be increased six hour after the induction of LTP (Nedivi *et al.*, 1993). Neither were increased two hours after the induction of LTP in perforant path/granule cell synapses (Chapter 5, this volume) which suggests that two hours may be too soon for optimum detection of altered levels of expression of these genes. It may also simply mean that their levels of expression are not affected by AMPA- and mGlu-receptor stimulation or PKA activation.

6.3.6 mRNA levels 24 hours post-injection

Previous work has shown that pENK and MAP2 mRNA levels are increased in the granule cells twenty four hours after NMDA receptor stimulation and nitric oxide release (Johnston & Morris, 1994b & c, 1995) while αCAMKII mRNA

levels are decreased. In addition, pDYN mRNA levels are decreased (Johnston & Morris, 1994c), and T26 mRNA levels are unchanged (Johnston & Morris, 1994b & c, 1995). Our work has shown that mGluR activation and blockade, AMPA receptor and PKA activation have no significant effect on mRNA levels of pENK, pDYN, MAP2 and T26. These results suggest that the observations made in previous studies are NMDA receptor/PKG-specific. Although levels of pENK were not significantly increased following administration of Sp-cAMPS other studies have suggested that a CRE binding site is present on the promoter region of the pENK gene as cAMP can induce pENK expression in striatal neurones (Giraud *et al.*, 1991; Konradi *et al.*, 1995), the human neuroblastoma cell line SK-N-MC (Folkesson *et al.*, 1989) and in bovine chromaffin cells (Kley *et al.*, 1987). It is possible that the increase observed here following Sp-cAMPS injection may have proved to be significant with further experiments as, interestingly, pENK levels were also decreased (although not significantly) by ACPD application, an effect which may be due to the reduction in cAMP this can produce.

In contrast to this levels of αCAMKII mRNA were found to be increased 24 hours after treatment with ACPD and Sp-cAMPS compared to saline. Johnston & Morris (1995) showed that 24 hours after NMDA receptor stimulation and release of nitric oxide levels of αCAMKII mRNA were decreased in the cell bodies and proximal dendrites of the affected granule cells, suggesting that NMDA and NO/cGMP mediate a reduction of αCAMKII mRNA to a sub-basal level of expression following the initial increase seen at two hours. The apparent decrease in expression of αCAMKII we observed following saline treatment, although not significant, may be due to endogenous release of small amount of glutamate which leads to NMDA receptor activation. Following the induction of LTP αCAMKII mRNA levels were found to be increased in the granule cell dendrites, but not the cell bodies 24

hours after the induction of LTP, however, no reduction in αCAMKII below control levels was seen (Thomas *et al.*, 1994a). Taken together these observations suggest that slightly different intracellular second messenger pathways may be involved in the expression of LTP compared to NMDA receptor activation or events mediated by nitric oxide such as cGMP synthesis. It may be that concurrent activation of NMDA, AMPA and mGlu receptors, which would occur during LTP, somehow shifts the expression of αCAMKII in favour of its synthesis rather than its down regulation after 24 hours. Our observations which show increased levels of αCAMKII 24 hours after mGluR activation with ACPD support the theory that mGluR activation rather than AMPA receptor activation may be involved in the changes observed by Thomas *et al* (1994a). The lack of effect of AMPA on αCAMKII mRNA levels may indicate that activation of these receptors by an exogenous compound is not sufficient to produce the same changes as endogenous glutamate does following high frequency afferent stimulation.

All the mGluRs which are present on the dendrites of the granule cells are activated by ACPD, producing a reduction in cAMP levels and/or activation of PLC, leading to increased synthesis of IP_3 and DAG. IP_3 mediates the release of Ca^{2+} from intracellular Ca^{2+} store and DAG activates PKC. Although no inhibitor studies were carried out it seems more likely that the effects on αCAMKII expression produced by ACPD are due to activation of phospholipase C. IP_3 and release of Ca^{2+} from intracellular stores has been shown to activate CAMKII in PC12 cells (MacNicol *et al.*, 1990), and autophosphorylation of CAMKII is able to prolong the effects of a transient rise in intracellular calcium and phosphorylate (and thus activate) several intracellular proteins over a more prolonged period. The increased expression of αCAMKII may be triggered in response to increased activity of CAMKII which is already present. It is also possible that activated CAMKII may

phosphorylate a transcription factor which can bind to the promoter region of the αCAMKII gene thus inducing its expression.

The suggestion that activation of mGluRs, which are negatively coupled to adenylate cyclase is not likely to be responsible for this increased expression of αCAMKII is supported by the observation that Sp-cAMPS also increases αCAMKII expression after 24 hours. An inhibition of cAMP formation would therefore be predicted to reduce αCAMKII expression. Activation of PKA leads to phosphorylation of several proteins and transcription factors including CREB, and, although the promoter region of the αCAMKII gene has not been found to contain a CRE site (Sunyer & Sahyoun, 1990), it is possible that one of the genes which is activated by CREB may activate the αCAMKII expression. If such a mechanism were in operation then it may explain why changes in αCAMKII mRNA levels are not seen for 24 hours. αCAMKII has also been shown to phosphorylate CREB (Dash *et al.*, 1991; Sheng *et al.*, 1991) and it is possible that this activation of CREB and CREB-related transcription factors leads to the generation of a positive feedback mechanism whereby CAMKII activates CREB, CREB induces expression of CREB-related transcription factors which, in turn, induce expression of CAMKII. The failure of MCPG to affect αCAMKII expression demonstrates that class I mGluRs (at least) are not constitutively active.

6.3.7 Conclusions

The expression of MAP2, pENK and pDYN appears to be specific to NMDA receptor activation and activation of the guanylate cyclase pathway (Johnston & Morris, 1994b & c). Levels of αCAMKII mRNA can be altered by activation of both NMDA and mGluRs which are coupled to PLC and protein kinases A and G. However these receptors and kinases appear to have

differential effects on αCAMKII mRNA levels, suggesting that NMDA receptor/PKG activation involves different transcriptional changes from PKA/mGluR activation. These data reinforce the importance of PKA in plasticity and illustrate that long-lasting changes in gene expression produced following mGluR stimulation could be one of the mechanisms by which a transient activity such as learning is converted into a more permanent memory. Further work would have to be carried out to determine whether the effects seen are due to mechanisms which have been described.

CHAPTER 7

MEASUREMENT OF CHANGES IN mRNA LEVELS USING REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) AND ASSOCIATED TECHNIQUES

7.1 INTRODUCTION

7.1.1 Principles of RT-PCR

RT-PCR was first described by Saiki *et al.* (1985). Reverse transcription (RT) involves the synthesis of a length of nucleotides complementary to the RNA present in the reaction mixture (from this point onwards known as cDNA), and PCR describes the method whereby particular regions of that cDNA are amplified to give a large number of copies. The region of cDNA which is amplified is determined by the PCR "primers" used in the reaction. For RT and amplification of a known sequence of mRNA, one sense and one antisense primer are used (usually 18-25mer), and the region of cDNA between the primers (the size of which is known if the mRNA has been sequenced) becomes the target in the amplification reaction. During PCR the cDNA is initially heated to separate the two strands. The temperature is then lowered to allow the primers to anneal to the two strands of cDNA (this temperature will vary from primer to primer). Raising the temperature at this stage activates DNA polymerase which extends the 3' end of each primer by adding nucleotides complementary to the cDNA template strand. Each time this cycle is repeated the amount of target cDNA increases exponentially until the reaction is terminated, either deliberately or because of an exhaustion of reagents in the reaction mixture (e.g. nucleotide shortage, lowered enzyme specific activity).

RT-PCR is a more sensitive method for detecting mRNA than in-situ hybridisation. In theory it should be possible to detect a single molecule of mRNA in a sample, however, as amplification of sequences is only exponential over a certain range of cycles, quantitative analysis of products has proved to be difficult. This study aims to see how PCR compares with in-situ hybridisation and to see how quantitative PCR can be.

7.1.2 mRNA studied using RT-PCR

As it was intended to examine changes in mRNA levels following the induction of LTP in the dentate gyrus in vivo mRNA species were chosen accordingly.

Tubulin T_{26} was used as a control, because its level of expression has been shown to be unaltered following a variety of pharmacological manipulations (Johnston & Morris, 1994b & c, 1995).

αCAMKII was used so that results could be compared with in-situ hybridisation studies which have shown it to be increased 2 hours after the induction of LTP in the dentate gyrus in vivo (Thomas *et al.*, 1994a; Chapter 5 this volume).

Egr3 was first described by Patwardhan *et al.* (1991) and has been shown to be induced by neuronal activity in the hippocampus (Yamagata *et al.*, 1994). Belonging to the same family as the transcription factor zif/268, its expression peaks at 2 hours which is ideal for studying in this model of LTP.

Neural Cell Adhesion Molecules (NCAM) are membrane bound glycoproteins which play a role in cell-cell adhesion. Their adhesive properties are dependent on exon composition and post-translational modifications. NCAM are thought to play a role in LTP (Rønn *et al.*, 1995; Lüthi *et al.*, 1994), but the vast number of isoforms makes studying them difficult. We have chosen to investigate the use of the variable alternatively spliced exon (VASE) in all isoforms of NCAM (Small & Ackeson, 1990) as it has been shown to be involved in neurite outgrowth (Doherty *et al.*, 1992a; Walsh *et al.*, 1992; Saffell *et al.*, 1994) and might, therefore, be involved in structural changes which accompany LTP.

7.1.3 Experimental Design

As reagents in the PCR buffer mixture become depleted a point is reached where no further cycling of temperature will achieve synthesis of more product. This point depends to some extent on the amount of initial template and on the specific activity of the DNA polymerase, which gradually decreases as the number of cycles increases. It is important, therefore, to make the number of cycles used for amplification below the level where the plateau of mRNA levels occurs, and to ensure that the reaction remains within the range where amplification is exponential. To begin with a range of cycle numbers was used before an optimal number of cycles for subsequent experiments was chosen.

It is extremely difficult to guarantee that the starting template for each PCR is identical to the next. Quantitation of initial RNA extracted from tissue is not always accurate, due to protein contamination, and from this point onwards more variables become involved. Some of these errors can be minimised by using a common buffer mixture for each set of reactions, which reduces pipetting errors, but every PCR set up will always be slightly different. Although with all experiments there is a margin of error any errors which are introduced in this work become, by the nature of PCR itself, amplified during the reaction. In an attempt to combat this we introduced a second set of primers for the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) into the PCR to act as an endogenous control. A similar approach was first used by Chelly *et al.*, (1988).

Before attempting to measure mRNA levels in the rat hippocampus following the induction of LTP we carried out a study to see whether developmental regulation of these genes could be detected in whole brain, and if the changes

agreed with observations in the literature. The optimum number of cycles for PCR of whole brain and hippocampus had to be determined for each study.

7.2 RESULTS

7.2.1 Validation of methods

The integrity of 1μg RNA is shown in Figure 7.2.1 for 4 different samples of tissue using an ethidium bromide TBE/DEPC gel under UV light. Both the 18S and 28S bands are clearly visible, but note that although the amount of RNA should be equal according to results obtained by spectrophotometric analysis, it is clear that some samples contain more RNA than others.

As expected GAPDH primers produced a single PCR product 492bp in length and NCAM primers produced 2 PCR products 118 and 88bp in length (with and without VASE respectively). T_{26}, αCAMKII and Egr3 produced single PCR products 127, 134 and 232bp in length respectively.

All the PCR products obtained were purified and sequenced. They were shown to contain the correct sequences for the mRNA species which they represented.

The measurements produced by the phosphor imager are referred to as optical density (O.D.). These are arbitrary units which reflect the amount of PCR product present on the Southern blot and hence the amount of mRNA in the original sample. As previously mentioned (Section 2.4.7) some results are expressed as a percentage of GAPDH optical density to control for experimental inaccuracies.

7.2.2 *Developmental Study*

7.2.2.1 Determination of optimal cycle number

Figure 7.2.2.1 shows how the amount of PCR product for each mRNA increases as the number of cycles increases (log scale). The results used for this figure are from whole brain of postnatal day 8 (P8) rats, although a similar pattern was observed in all ages. After 28 cycles of PCR GAPDH and αCAMKII mRNA levels have reached a maximum, but levels of T_{26} and Egr3 have not yet reached a plateau. For subsequent experiments 25 cycles was used for each PCR as it was a submaximal cycle number for GAPDH and αCAMKII, and just enough cycles for Egr3 and T_{26} to be detectable under UV light when examining the PCR products after gel separation.

7.2.2.2 Developmental regulation of each mRNA species

Using 25 cycles of PCR the relative mRNA levels of αCAMKII, T_{26} and Egr3 at each developmental stage studied was obtained by expressing the optical density values as a percentage of the optical density values for GAPDH (Figure 7.2.2.2a). From these results it appears that levels of αCAMKII and Egr3 increase steadily postnatally then rise more sharply when the brain reaches maturity. T_{26} mRNA levels do not appear to be altered noticeably either postnatally or on reaching adulthood.

As the 2 NCAM fragments obtained from PCR were very close in size they had to be separated using NuSieve agarose. Unfortunately blotting of these gels in the same way as agarose did not result in transfer of the DNA to the nylon membrane. An attempt was made to separate the bands using a 4% agarose gel, which successfully produced 2 bands which could be separated by eye

under UV light. However when these gels were blotted and the membranes hybridised it was not possible to distinguish the bands from one another so accurate quantification could not be made. Figure 7.2.2.2 shows a photograph of a NuSieve gel with PCR products using NCAM and GAPDH primers after 30 cycles. The far left hand lane is a 100bp DNA ladder and the next 5 lanes are whole brain from E19, P4, P8, P12 and adult Wistar rats. The largest fragments (top) are GAPDH, and the 2 fragments either side of the 100bp marker are the NCAM fragments (the larger fragment being the VASE containing isoforms). It is clear from the photograph that the amount of NCAM in the brain which contains this exon tends to increase towards adulthood and, relatively speaking, the amount of non-VASE containing isoforms of NCAM tend to decrease in abundance towards adulthood. The actual amount by which levels of total NCAM vary developmentally can not be estimated because of the uneven amounts of control (GAPDH) mRNA in each lane.

7.2.3 LTP study

7.2.3.1 Determination of optimal cycle number

As it could not be assumed that the optimum number of cycles used for whole brain in the developmental study would be suitable for just hippocampal tissue, the same procedure of varying the number of PCR cycles had to be carried out. Figure 7.2.3.1 shows the amount of PCR product for each mRNA species with increasing cycle number in control hippocampus. After 30 cycles it appears that the maximum level of product has been achieved so subsequent experiments used 25 cycles of PCR.

7.2.3.2 Alterations in mRNA levels 2 hours after the induction of LTP

Figures 7.2.3.2a, b & c show T_{26}, αCAMKII and Egr3 mRNA levels as a percentage of GAPDH mRNA levels in control and potentiated hippocampi of 3 different animals. T_{26} levels appear to be increased by the induction of LTP in each animal, αCAMKII levels appear to be decreased following the induction of LTP and Egr3 levels are both increased and decreased following the induction of LTP. These results are variable and do not agree with observations made using in-situ hybridisation.

Figure 7.2.1

Agarose gel (1.2%) separation of 4 different RNA samples (1µg each lane) showing both 18S and 28S fragments under UV light

Figure 7.2.1

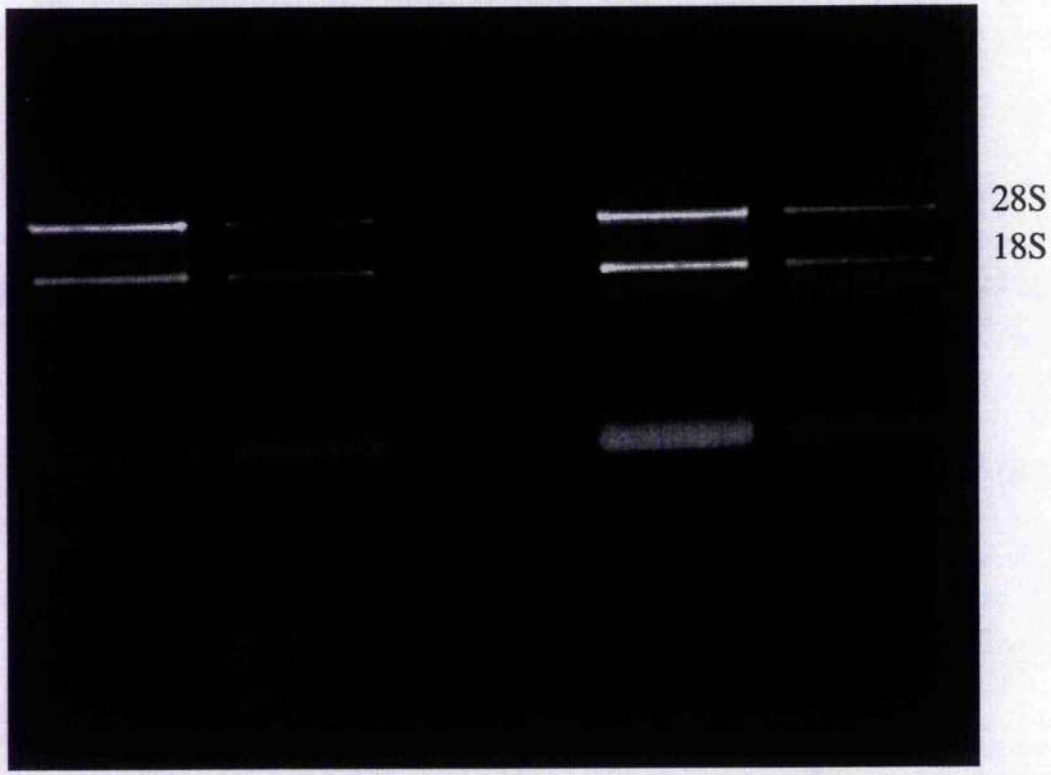


Figure 7.2.2.1

Variation in the amount of PCR product for each mRNA species (log scale) with increasing numbers of cycles using cDNA synthesised from P8 rat brain RNA. Results were obtained from optical density readings of ^{32}P labelled Southern blots.

Figure 7.2.2.1

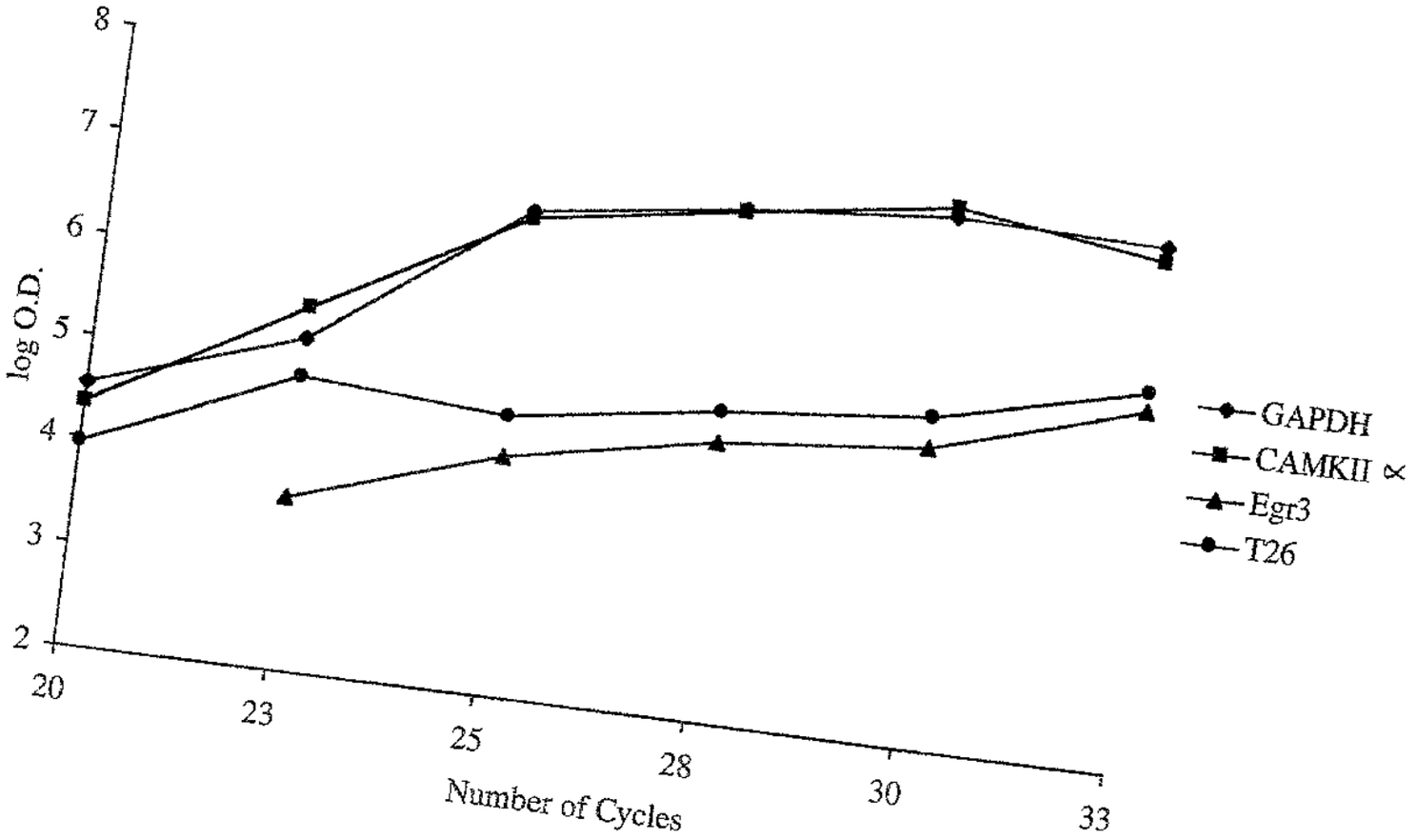

8
7
6
5
4
3
2
log O.D.
20
23
25
28
30
33
Number of Cycles
GAPDH
CAMKII ∝
Egr3
T26

Figure 7.2.2.2a

Developmental regulation of αCAMKII, T_{26} and Egr3 mRNA levels in rat brain from E19 to adult ages. Results are expressed as a percentage of GAPDH mRNA levels, and were obtained using 25 cycles of PCR.

Figure 7.2.2.2a

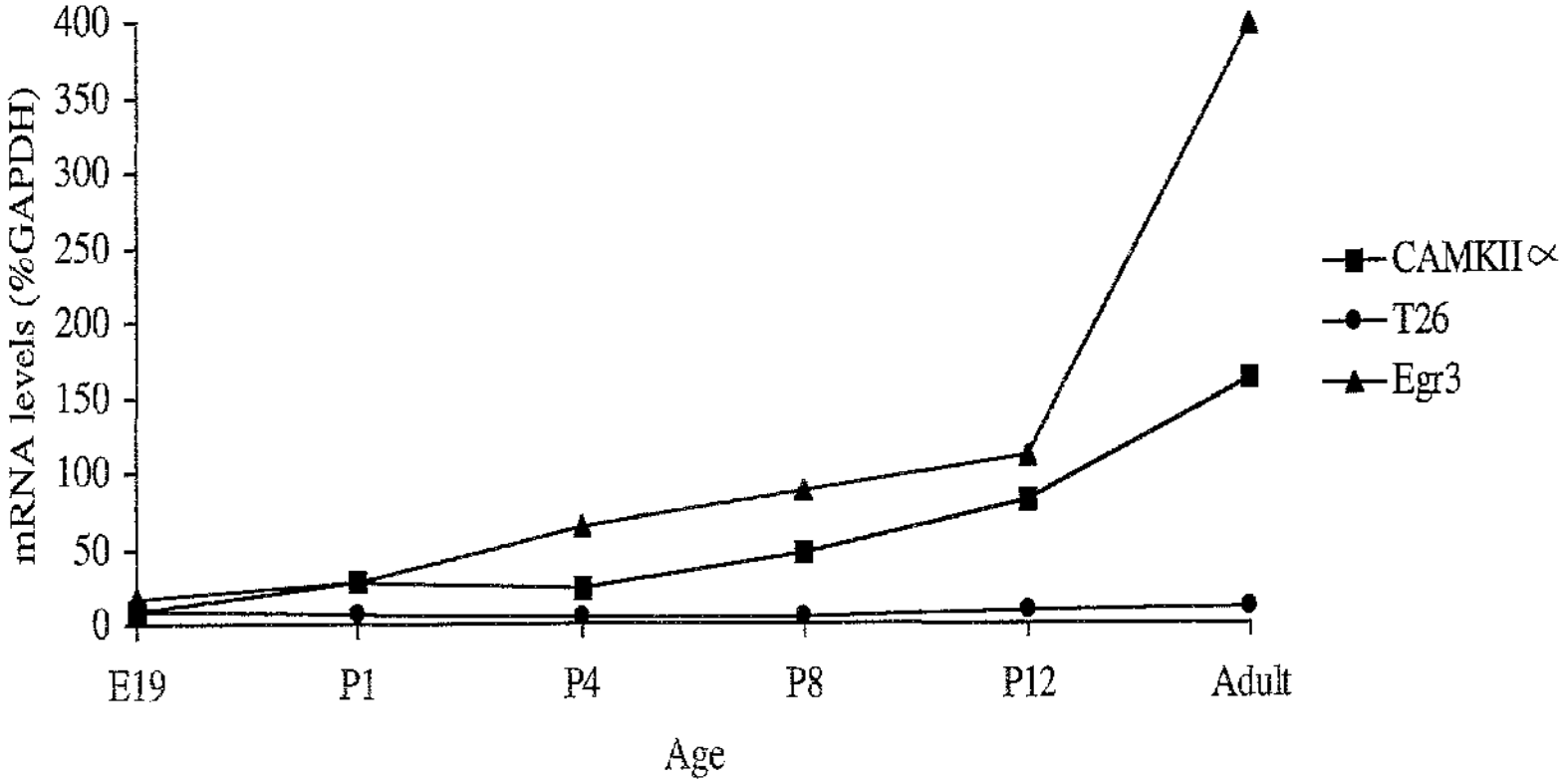


Figure 7.2.2.2b

Photograph of NuSieve agarose gel (4%) separation of GAPDH and NCAM PCR fragments under UV light. The first lane contains a 100bp DNA ladder and the following 5 lanes were obtained from amplification of cDNA synthesised from RNA extracted from E19, P4, P8, P12 and adult rat brain respectively.

Figure 7.2.2.2b

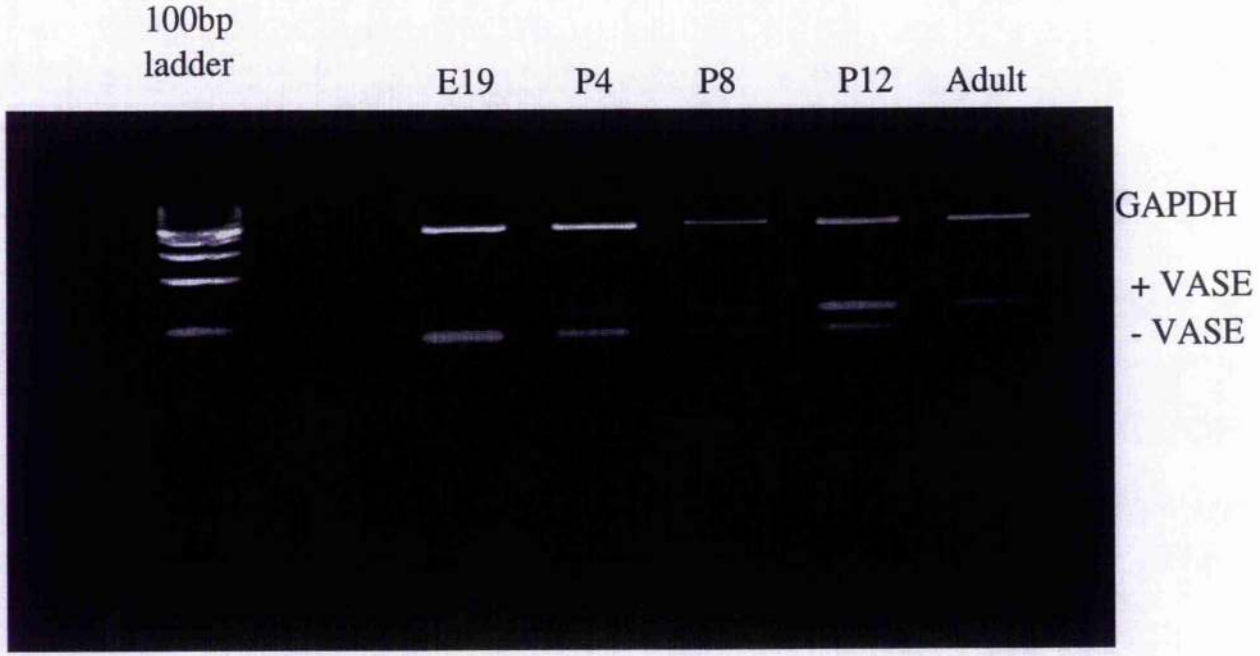


Figure 7.2.3.1

Variation in the amount of PCR product for each mRNA species (log scale) with increasing numbers of cycles using cDNA synthesised from whole hippocampus RNA (adult). Results were obtained from optical density readings of ^{32}P labelled Southern blots.

Figure 7.2.3.1

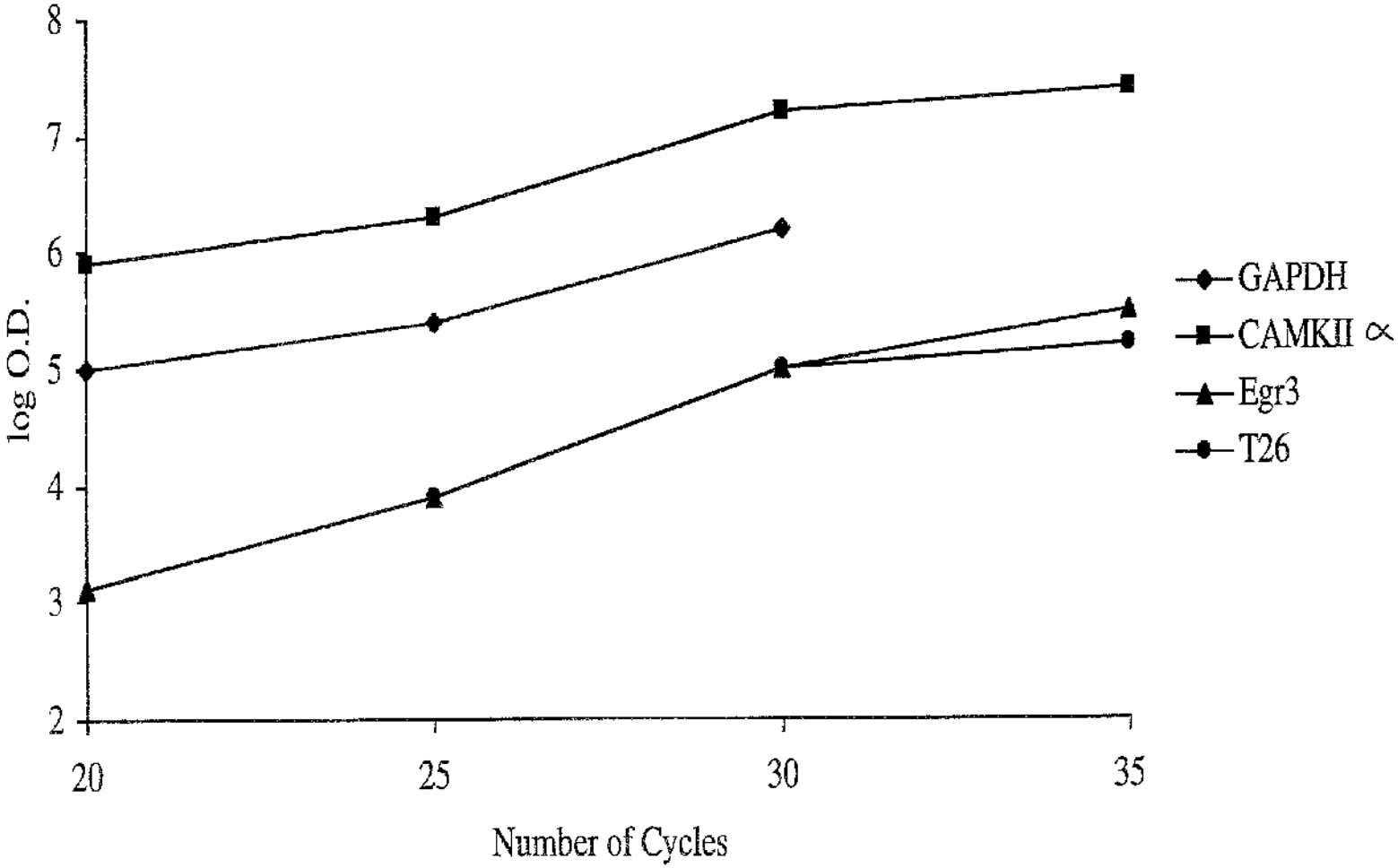

8
7
6
5
4
3
2
log O.D.
20
25
30
35
Number of Cycles
GAPDH
CAMKII ∝
Egr3
T26

Figure 7.2.3.2a

T_{26} mRNA levels in the expressed as a percentage of GAPDH mRNA levels in control (low frequency stimulated) and LTP induced hippocampi (2hr time point) in 3 different animals.

Figure 7.2.3.2a

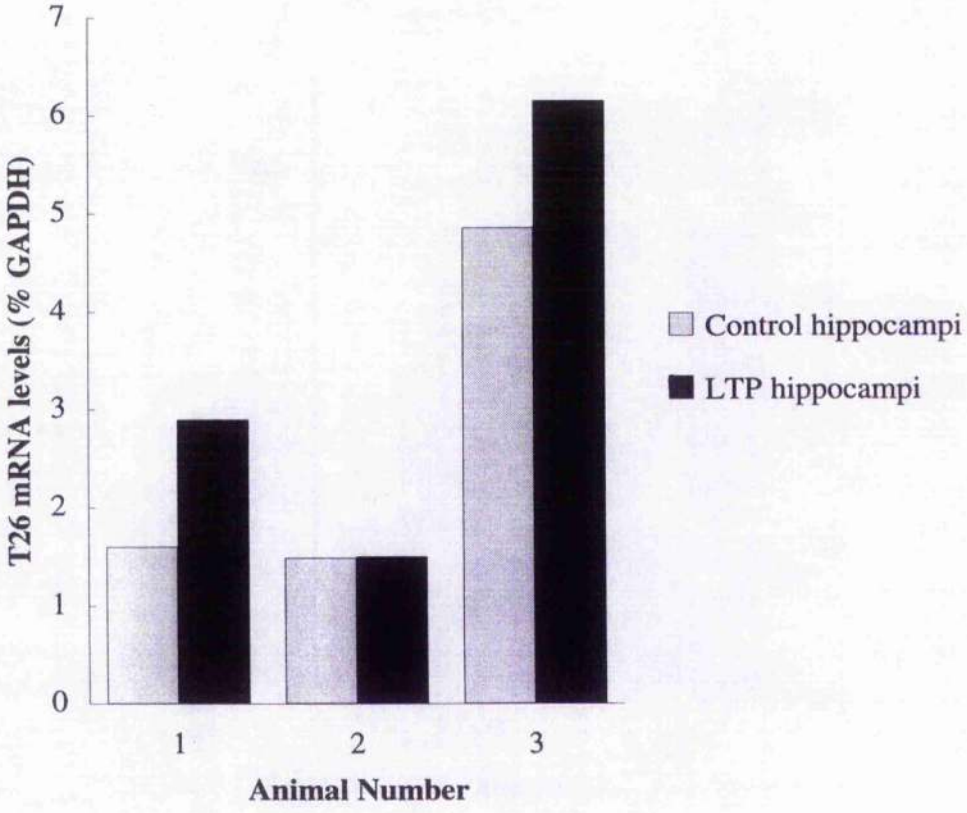


Figure 7.2.3.2b

αCAMKII mRNA levels in the expressed as a percentage of GAPDH mRNA levels in control (low frequency stimulated) and LTP induced hippocampi (2hr time point) in 3 different animals.

Figure 7.2.3.2b

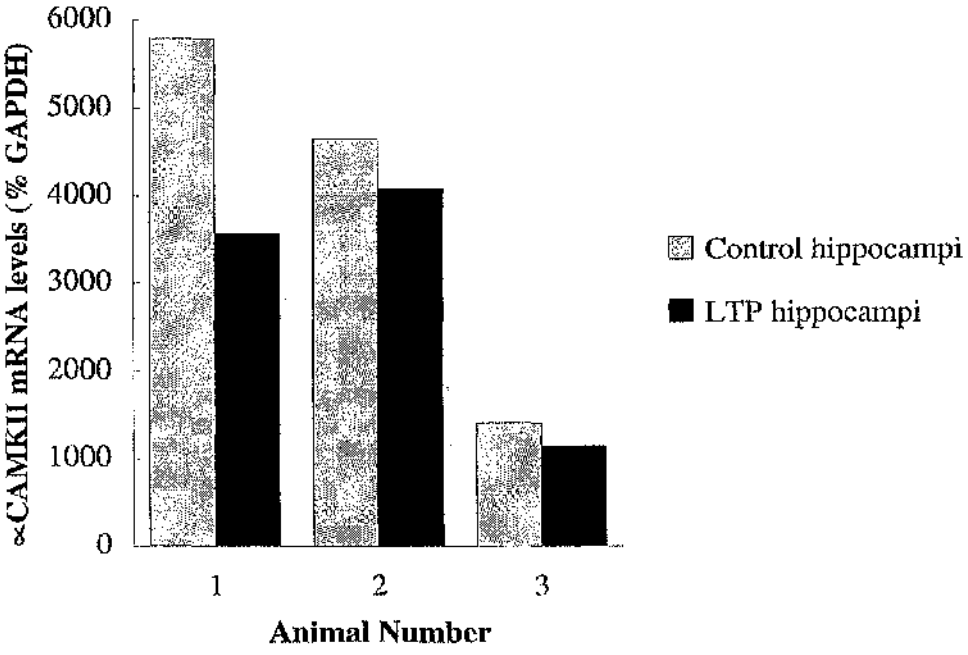
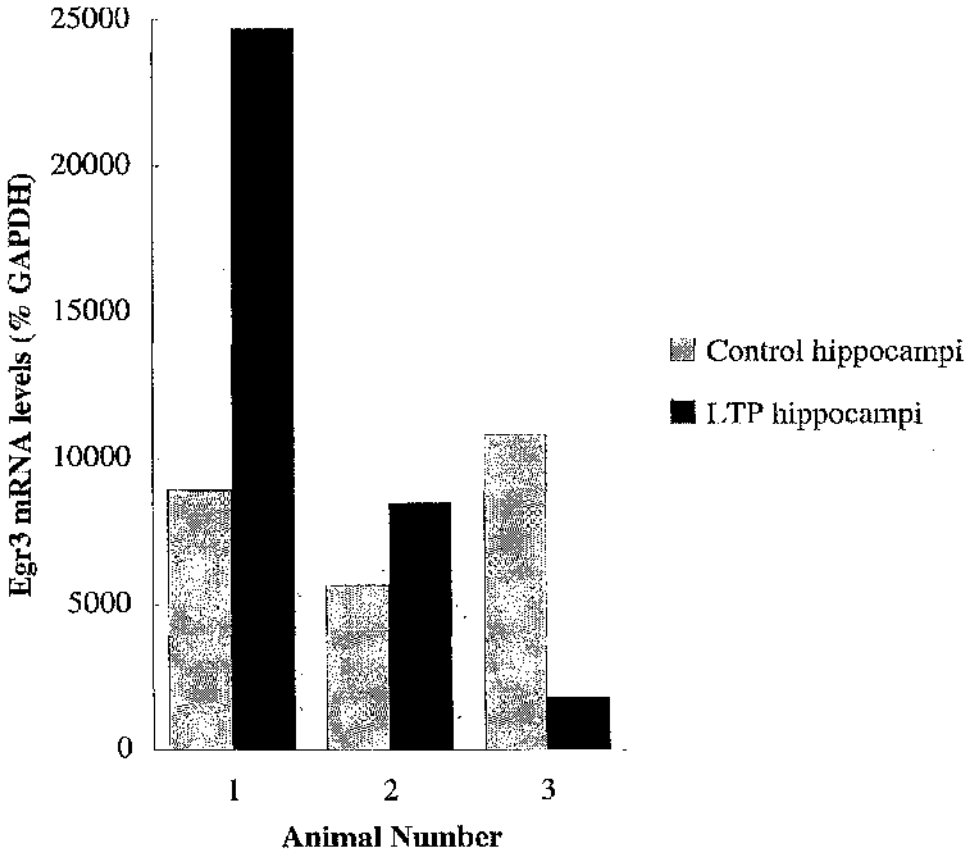


Figure 7.2.3.2c

Egr3 mRNA levels in the expressed as a percentage of GAPDH mRNA levels in control (low frequency stimulated) and LTP induced hippocampi (2hr time point) in 3 different animals.

Figure 7.2.3.2c



7.3 DISCUSSION

7.3.1 Developmental Study

This work was undertaken to give an initial idea of how successfully PCR could be used to detect changes in gene expression by comparing the results to previous reports in the literature.

The following observations rely on the assumption that GAPDH is not developmentally regulated.

αCAMKII expression was shown to be increased most noticeably between P4 and P12 and then rose sharply in adult tissue. In-situ hybridisation studies of whole rat brain are in agreement with these observations and show that at P4 αCAMKII mRNA is barely detectable, but then rises dramatically until, at P16, levels are of a similar magnitude to that observed in adult brain (Burgin *et al.*, 1990).

Our results showed that $\mathbf{T_{26}}$ mRNA levels were not altered developmentally. Miller *et al.* (1987) also found that expression of T_{26} mRNA was not regulated in the developing CNS either temporally or spatially.

Egr3 mRNA levels increased steadily from P1 to P12 and then rose sharply between P12 and adult. A small developmental study carried out by Yamagata *et al.* (1994) using in-situ hybridisation showed that the expression of Egr3 increased from P1 until at P12 the pattern of expression was qualitatively similar to the adult. This study did not evaluate developmental changes quantitively.

Quantitative data for the developmental regualtion of the two isoforms of **NCAM** could not be obtained because of the small difference in sizes of the 2 PCR fragments and the failure to successfully transfer DNA from the NuSieve gels onto

the nylon membrane. Qualitative data obtained from gel separation of the 2 fragments show that the isoform containing VASE increases in abundance as the animal reaches maturity. This is in agreement with the findings of Small & Ackeson (1990), but the apparent reciprocal decrease in the isoform lacking this exon which we observed was not reported. This makes it difficult to judge whether or not this effect is genuine.

The results obtained from the developmental study appear to correlate closely with reported findings, most of which have used in-situ hybridisation. The success of this work prompted an attempt to use this approach in the study of hippocampal LTP.

7.3.2 LTP study

In-situ hybridisation has been used successfully to detect changes in mRNA levels of many genes following the induction of LTP in vivo. Levels of αCAMKII mRNA have been shown to be increased 2 hours after the induction of LTP (Thomas *et al.*, 1994a; Chapter 3 this volume), as have levels of Egr3 (Yamagata *et al.*, 1994). Tubulin T_{26} mRNA levels are unaffected following the induction of LTP (Chapter 3 this volume), but as it is not known exactly what role NCAM plays in the induction and/or maintenance of LTP it was difficult to predict whether levels would increase or decrease and if one isoform would predominate over the other.

Although the developmental results agreed closely with other similar studies the results produced by Southern blot analysis of corresponding PCR fragments for each mRNA species from control and stimulated hippocampus were inconsistent

and did not agree with published findings. αCAMKII and T_{26} mRNA levels appeared decreased and increased respectively following the induction of LTP and Egr3 mRNA levels were both increased and decreased over the group of animals following the induction of LTP. As mentioned previously it was not possible to measure the levels of the two NCAM isoforms because of the difficulty in extracting the PCR fragments from the NuSieve agarose gel.

7.3.3 Shortcomings in these methods

Although we were able to show some interesting data from these experiments the methodology was not ideal and could certainly have been improved.

A big problem facing the use of PCR to quantify changes in gene expression is that one has very little idea how much starting template is initially available, because of the difficulty in obtaining accurate measurements of RNA concentration after extracting it from a tissue sample. One method which attempts to control for this uncertainty is competitive PCR. This involves spiking each PCR mixture with a known amount of synthetic RNA which has been designed to contain the same primer sequences as the sample of RNA, but with an addition/subtraction of nucleotides between the two primer binding sites so that the competitive PCR product can be separated from the true PCR product by size (Gilliland *et al.*, 1990). By making serial dilutions of the synthetic RNA it eventually becomes possible to detect, using gel electrphoresis, when the amount of target produced corresponds to the amount of competitor produced (a known starting concentration), and therefore how much target template is in the original sample. The use of only one primer set in this method has the additional advantage in that it rules out any differences which might be due to unequal efficiency of

amplification due, for example, to the annealing temperature of the cycle being optimum for one primer set, but not the other.

The use of GAPDH in our study attempted to control for this uncertainty over starting template concentration to an extent, but it had to be assumed that GAPDH was not developmentally regulated and unaffected by the induction of LTP. Burgin *et al.* (1990) have shown that GAPDH mRNA levels decreased slightly from P4 to adult, but it is not known whether it is affected by the induction of LTP. This could mean that the increases observed in mRNA levels of the other genes may not be genuine depending on the magnitude of the decrease in GAPDH mRNA over this developmental period.

The data presented in this study can be described as semi quantitative or relatively quantitative, as it is not possible to determine absolute amounts of mRNA using these methods.

7.3.4 Conclusions

In comparison to in-situ hybridisation PCR-related methods have the disadvantage of not providing informations about localised changes within a particular region. In addition the use of a structure containing mRNA from areas which are not being directly manipulated can result in small localised changes being obscured by changes occuring elsewhere in the structure. Although it might have been possible to dissect out the dentate gyrus from the hippocampus, it would have been difficult to extract a measurable amount of RNA from such a small sample, and it could not be guaranteed that it would not be contaminated with RNA from elsewhere.

Although neither PCR nor in-situ hybridisation can give information about absolute amount of mRNA present, in-situ hybridisation is a more simple method with less

variables affecting its outcome, provided each step of the method proceeds without any problems. PCR would be a more appropriate method for studying mRNA levels which fall below the threshold for detection with in-situ hybridisation, as it should, in theory, be able to amplify up a sequence from just one mRNA molecule. In some cases where oligonucleotide probes used in in-situ hybridisation cross react PCR can provide another method for studying the gene of interest (see Chapter 8 for an example).

Overall the detection of localised changes in mRNA levels can be achieved more efficiently using in-situ hybridsation as the variability inherent in RT-PCR makes it a far less reliable method for detecting alterations in mRNA levels in a small group of neurones. This brings reports of the use of RT-PCR to measure such changes under scrutiny for the use of appropriate controls.

CHAPTER 8

DISTRIBUTION OF 2 ISOFORMS OF THE NEURAL CELL ADHESION MOLECULE (NCAM) IN RAT BRAIN

8.1 INTRODUCTION

8.1.1 Diversity of NCAM

The neural cell adhesion molecule (NCAM) belongs to a family of membrane bound glycoproteins which are involved in cell-cell interactions. There are many distinct isoforms of NCAM, generated by alternative splicing of a single gene, which can be grouped into 3 main classes according to size. The 180kD and 140kD isoforms both have extracellular regions, transmembrane domains and intracellular regions (larger for the 180kD isoform), whereas the 120kD isoform is anchored to the cell membrane and has no cytoplasmic domains. Post-translational modification of the different isoforms of NCAM gives rise to a larger number of molecules with unique properties, so that interactions between cells can be tightly regulated in many potentially different ways by varying the expression of a single gene.

8.1.2 Structure/Function relationships of NCAM

The properties of each isoform of NCAM are defined by the exons it comprises and by any post-translational modifications which it has undergone. Unmodified NCAM can bind polymers of α2-8 linked sialic acid (PSA) which makes it less adhesive (Sadoul *et al.*, 1983) and more able to induce increases in neurite outgrowth (Doherty *et al.*, 1990). Highly polysialylated NCAM (NCAM-H) predominates in late embryonic and early postnatal animals and becomes progressively reduced as the brain reaches maturity. However, in the adult brain, NCAM-H levels remain elevated in areas associated with neurogenesis, such as the olfactory bulb and the hippocampal dentate gyrus (Seki & Arai, 1993a & b), which strongly implies a plasticity related role for this form.

The length of the cytoplasmic domain of NCAM affects its properties. Doherty *et al.* (1992b) demonstrated that the 180kD isoform was less able to act as a substrate for neurite outgrowth than the 140kD isoform. This suggests that the removal of some cytoplasmic exons must encourage NCAM-induced neurite outgrowth.

Several different extracellular domains of NCAM have been shown to be involved in different functions. Frei *et al.* (1992) investigated the functional properties of each of the 5 Ig-like domains of the molecule, using antibodies directed at particular domains either alone or in combination. The properties investigated included the ability to influence cell adhesion and promote neurite outgrowth, and the results revealed complex interactions between the domains demonstrating that combinations of particular domains were more functionally efficient at some tasks than others.

Perhaps one of the most interesting and most studied extracellular regions of NCAM is the variable alternatively spliced exon or VASE. Utilisation of this 30 base exon results in an insertion of a 10 amino acid sequence into the fourth extracellular Ig domain and was first described by Small *et al.* (1988). Subsequent wok has shown that incorporation of this sequence into NCAM is more commonly found in the adult CNS (Small & Ackeson, 1990) and results in a down-regulation of NCAM-dependent neurite outgrowth (Doherty *et al.*, 1992a; Walsh *et al.*, 1992; Saffell *et al.*, 1994). As expected NCAM lacking this sequence have greater growth promoting capabilities (Liu *et al.*, 1993). The absence of this sequence in NCAM found in the periphery may partially explain why CNS tissue does not have the power of regeneration that peripheral tissue possesses.

8.1.3 Aims of this study

In the passive avoidance task in chicks the use of antibodies against NCAM has revealed a possible role for the molecule in the stabilisation of long-term memory (Scholey *et al.*, 1993), and it has also been demonstrated, using the Morris' water maze, that spatial learning in NCAM deficient mice is impaired (Cremer *et al.*, 1994). Sensitisation of the gill withdrawal reflex in Aplysia has been shown to be accompanied by an internalisation and decreased synthesis of membrane bound apCAM, the Aplysia variant of NCAM (Bailey *et al.*, 1992; Mayford *et al.*, 1992). Antibodies to NCAM have also been used to reveal a role for NCAM in the induction of LTP in area CA1 of hippocampal slices maintained in vitro (Luthi *et al.*, 1994; Rønn *et al.*, 1995), although this work implied that NCAM was involved in the initial phase of LTP. In addition Fazeli *et al.* (1994) showed that extracellular concentrations of NCAM in the dentate gyrus were increased following the induction of LTP in vivo. These observations, both behavioural and non-behavioural strongly suggest that NCAM is involved in learning, memory and hippocampal synaptic plasticity, and make the regulation of its expression an interesting phenomenon to study using the experimental models in this volume. However, the existence of so many different isoforms of NCAM (some of which are not yet fully characterised) makes the decision of which particular regions of which isoform to study extremely difficult. As the usage of VASE has been shown to be related to neurite outgrowth, and is probably involved in synaptic plasticity, we decided to see whether it was possible, using in-situ hybridisation, to detect developmental changes in the expression of each isoform, link the distribution of the two isoforms to discrete areas of the brain, and, if this proved successful, see whether the expression of either isoform was altered following the induction of LTP.

The nucleotide sequence of this exon and its relationship with the rest of the NCAM sequence has been published (Small & Ackeson, 1990). The oligonucleotide probes were designed so that N-VASE detected all NCAM mRNA containing the VASE (+VASE) and that N-FLANK detected all NCAM mRNA without the VASE (-VASE). To begin with, the pattern of expression of the two isoforms in embryonic and adult brain was determined and then displacement experiments carried out to ensure each oligonucleotide probe was binding to different mRNA species.

8.2 RESULTS

Figure 8.2.1 shows N-FLANK and N-VASE distribution in embryonic day 17 (E17) and adult whole brain. Both isoforms are found in most structures at each age although it is clear that in adult tissue the isoform of NCAM containing VASE predominates, and in embryonic tissue the isoform which lacks VASE is more abundant. The hippocampus is heavily labelled in the adult brain along with the cortex and cerebellum. For the VASE containing isoform of NCAM the distribution of mRNA appears to be specific with little binding to white matter in the adult brain. In the embryonic brain the distribution of N-FLANK mRNA appears to be specific to cell body layers, but in the adult brain the binding to white matter is increased.

The specificity of each oligonucleotide was investigated by carrying out displacement experiments with an unlabelled excess amount of the other oligonucleotide.

Figure 8.2.2A shows N-VASE distribution in adult brain and Figure 8.2.2B shows how this is altered when a 25-fold excess of unlabelled N-FLANK oligonucleotide is added to the hybridisation reaction.

Figure 8.2.3A shows N-FLANK distribution in adult brain and Figure 8.2.3B shows how this is affected when a 25-fold excess of unlabelled N-VASE oligonucleotide is added to the hybridisation reaction.

From these images it is clear that the different oligonucleotide probes are binding to a common sequence, which may or may not be an isoform of NCAM, and displacing one another from it. The N-FLANK oligonucleotide probe also displays a considerable amount of non-specific binding to white matter in the brain.

Figure 8.2.1

Images captured from film showing distribution of the oligonucleotides N-FLANK and N-VASE in both embryonic day 17 (E17) and adult whole brain. The oligonucleotide represent mRNA for two isoforms of NCAM with (N-VASE) and without VASE (N-FLANK).

Figure 8.2.1

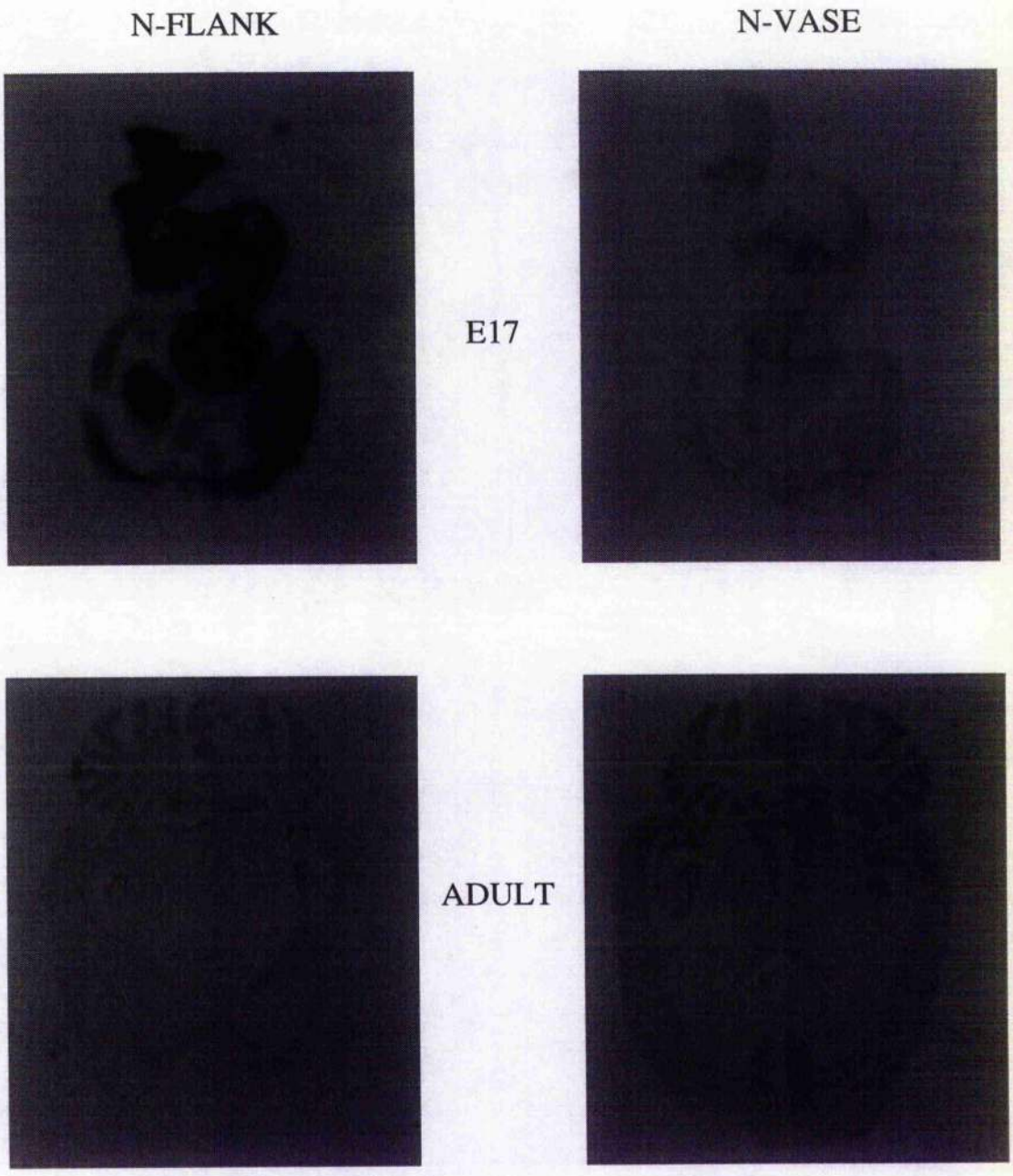


Figure 8.2.2

Image captured from film showing distribution of the oligonucleotide N-VASE (designed to bind to NCAM isoforms containing VASE) in adult rat brain (**A**), and how this pattern is altered when a 25-fold excess of the oligonucleotide N-FLANK (designed to bind to isoforms of NCAM which do not contain VASE) (**B**).

Figure 8.2.2

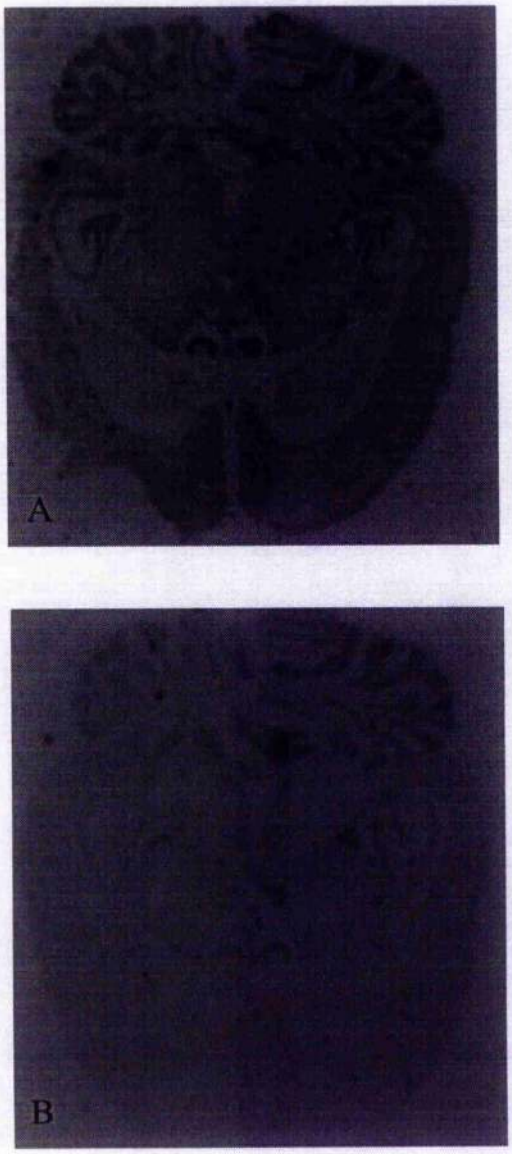
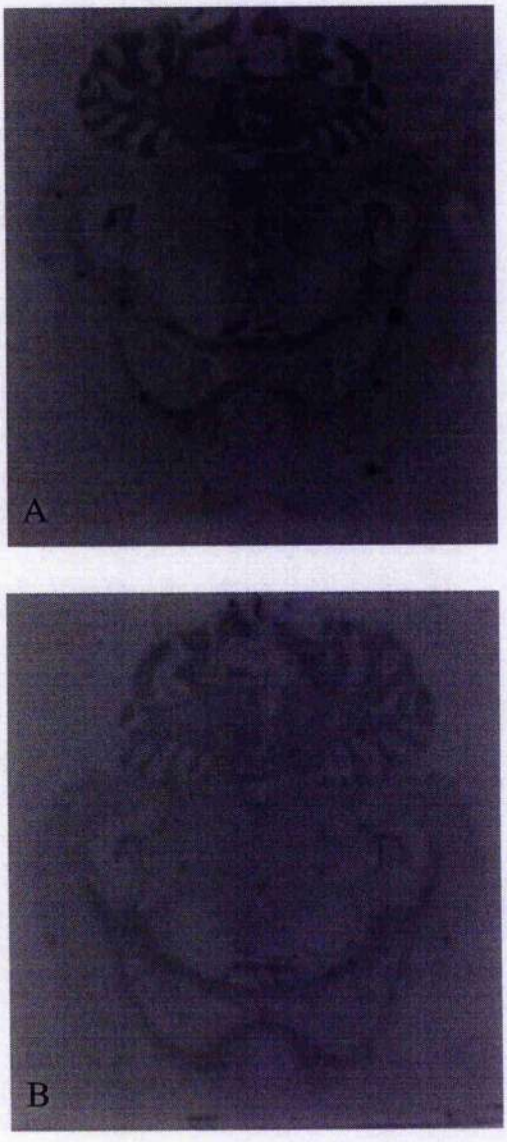


Figure 8.2.3

Image captured from film showing distribution of the oligonucleotide N-FLANK (designed to bind to isoforms of NCAM which do not contain VASE) in adult rat brain (**A**), and how this pattern is altered when a 25-fold excess of the oligonucleotide N-VASE (designed to bind to isoforms of NCAM which contain VASE) (**B**).

Figure 8.2.3



8.3 DISCUSSION

8.3.1 Aims of this study

The lack of knowledge about exactly which exons are responsible for a particular property of a given isoform of NCAM and the large number of different isoforms has made the use of in-situ hybridisation to study the expression of this molecule, and how it can be manipulated, extremely difficult. Prieto *et al.* (1989) studied mRNA distribution of NCAM during development of the chicken, using in-situ hybridisation, but were only able to make the distinction between isoforms based upon the cytoplasmic domain content. Goldowitz *et al.* (1990) studied NCAM gene expression during hippocampal and cerebellar development in the mouse using in-situ hybridisation, but did not distinguish between the different isoforms. There is clearly a dearth in the literature regarding the use of in-situ hybridisation to study NCAM.

One exon which has been studied in more detail than others is VASE, which has been shown to be involved in neurite outgrowth (Doherty *et al.*, 1992a; Walsh *et al.*, 1992; Liu *et al.*, 1993; Saffell *et al.*, 1994) and whose usage is developmentally regulated (Small & Ackeson, 1990). These properties suggest a role for this exon in synaptic plasticity and, with this in mind, we attempted to map the distribution of NCAM isoforms +VASE and -VASE, to see whether their previously reported developmental profiles could be duplicated using in-situ hybridisation.

8.3.2 Distribution and displacement experiments

It was hoped that the distribution of NCAM -VASE mRNA in adult brain might be limited to areas of the brain which are associated with neurogenesis,

supporting the hypothesis that this form was involved with neuronal plasticity, and that NCAM +VASE mRNA would be found in areas of the brain not associated with plasticity.

As previously reported (Small and Ackeson, 1990) we found that levels of NCAM +VASE were higher in the adult than in the embryonic brain and that the opposite was true for NCAM -VASE. The mRNA for both isoforms appeared to be localised in cell bodies and the hippocampus was clearly visible as containing both isoforms. However the displacement studies revealed that both oligonucleotide probes used had a substantial degree of cross reactivity, and N-FLANK appeared to bind in large amounts to the white matter. Further investigation of the regulation of the two isoforms could not be carried out with so much uncertainty over which mRNA species each oligonucleotide was detecting.

It is not clear why the probes did cross react. These sequences were only identical in 2x6 base regions at either end of the N-VASE oligonucleotide and in the middle of the N-FLANK oligonucleotide. The N-VASE oligonucleotide would have to adopt a hairpin shape to bind to NCAM -VASE mRNA and the N-FLANK oligonucleotide would have to bind to only 21/42 nucleotides to cross react with NCAM +VASE isoforms. The high stringency conditions of the hybridisation reaction should ensure that this degree of cross reactivity does not occur. Previous experiments (Morris, unpublished observations) have shown that one or two mismatches within the 45 base oligonucleotide sequence are sufficient to prevent hybridisation at this stringency.

Sequence analysis of the two oligonucleotides did not reveal any homology with any other rat mRNA found in Genbank, although this does not exclude the

possibility that the oligonucleotides are binding to an unsequenced mRNA species.

Assuming that the oligonucleotide probes are specific for NCAM, but not necessarily for the two isoforms they were designed to detect, it can be stated that NCAM mRNA is found in most structures in the brain, and appears mainly in cell body layers.

8.3.3 Conclusions

Although PCR has been used to study the 2 isoforms of NCAM developmentally quite successfully (Small & Ackeson, 1990; Chapter 7 this volume) the method has its limitations in terms of producing quantitative data and information about localisation of mRNA species.

In-situ hybridisation has the advantage of producing semi-quantitative data and giving detailed visual information about mRNA distribution. It is unfortunate that in this case the method proved to be unsuitable, and the pattern of distribution for these two isoforms of NCAM, distinct or otherwise remains unknown.

CHAPTER 9

GENERAL DISCUSSION

This work was undertaken to investigate changes in gene expression associated with synaptic plasticity in the mammalian hippocampus. In particular we have focused on the phenomenon of LTP, which is widely believed to be the mechanism which underlies synaptic plasticity in this region. In addition, we looked at the contribution of glutamate receptors and protein kinase A to changes in gene expression in this region.

To investigate the potential link between the induction of LTP and modulation of cognitive processes we looked at levels of zif/268 and c-fos mRNA following administration of several compounds which affect cognition. Although we found that, on the whole, levels were unaffected by these compounds, this probably implies that their mechanisms of action involve the enhancement or suppression of ongoing processes associated with learning and memory, and that their actions are not sufficient to initiate cognition-related changes. These observations do not necessarily indicate that separate processes are involved in the expression of LTP and learning & memory. It is possible, however that the importance of immediate early genes in plasticity has been overemphasised, especially as zif/268 appears to be induced following the induction of LTP and LTD in the hippocampus. We did see increases in zif/268, but not c-fos, expression in the CA1 region of the hippocampus following the induction of LTP in Schaffer collateral/CA1 synapses, consistent with observations made by Mackler *et al.* (1992). This demonstrates that LTP in this region may involve similar mechanisms to perforant path/granule cell LTP. Levels of αCAMKII mRNA were also found to be increased following the induction of LTP in Schaffer collateral/CA1 synapses, again consistent with previous observations (Mackler *et al.*, 1992) and supporting the theory of common mechanisms for the expression of LTP in these two regions of the hippocampus. In addition, this rise in αCAMKII mRNA levels correlates well with transgenic studies which have implicated this kinase as playing an

important role in the expression of LTP and cognitive function (Silva *et al.*, 1992a & b). We also found that αCAMKII expression is regulated by activation of mGluRs which are thought to play an important role in the generation of LTP and the triggering of second messenger cascades associated with its maintenance. This regulation of αCAMKII expression is likely to be via activation of PLC rather than inhibition of adenylate cyclase as we also found that activation of protein kinase A increased levels of αCAMKII mRNA.

Interestingly, dendritic mRNA levels of αCAMKII and MAP2 were found to be increased following the induction of LTP in the dentate gyrus. The existence of a large number of isoforms of MAP2, which might be differentially regulated according to their particular functions, may account for our failure to detect overall increases in MAP2 mRNA in the cell bodies (our oligonucleotide probe would not distinguish between these different splice variants). The dendritic increases in both these mRNA species may reflect stabilisation of dendritic mRNA brought about following the induction of LTP in perforant path/granule cell synapses. It is possible that all constitutively expressed dendritic mRNA species might be affected and that we have revealed a mechanism which allows enhancement of protein synthesis which can be targeted to specific synapses.

Regulation of structural proteins such as MAP2 may account for morphological changes associated with the induction of LTP (Desmond & Levy, 1986a & b; Geinisman *et al.*, 1991; Hosokawa *et al.*, 1995). We also found evidence for "domino plasticity" (Morris & Johnston, 1995) in the hippocampus following the observation that the induction of LTP in perforant path/granule cell synapses produced increases in the number of cells expressing proenkephalin mRNA. Peptides derived from this mRNA would presumably be transported from these cells to the mossy fibre nerve terminals and act to enhance

transmission in this region. The induction of LTP in mossy fibre/CA3 synapses is NMDA receptor independent and thought to have an opioid component.

Consistent with the theory that enhanced release of glutamate may contribute to the induction of LTP we also found mRNA levels of two isoforms of SNAP (A & B) to be increased following the induction of LTP. These proteins are involved in vesicle fusion and neurotransmitter release and are also thought to be associated with axonal growth, synaptogenesis (SNAP A) and necessary for the functioning of mature synapses (SNAP B) (Boschert *et al.*, 1996). The increased expression of both isoforms indicates that the properties displayed by each are important for the induction and maintenance of LTP.

Although much work has been carried out to uncover the mechanisms which govern the induction and maintenance of LTP it has never been proved unequivocally that this phenomenon underlies synaptic plasticity in mammals. The most informative work has come from studies which have shown that saturation of transmission in the hippocampus prevents spatial learning (Barnes *et al.*, 1994), the demonstration that blockade of LTP in vivo also blocks spatial learning (Morris *et al.*, 1986), the discovery that potentiation of synaptic transmission accompanies exploratory learning in the rat (Moser *et al.*, 1993) and observations made about structural changes following the induction of LTP and certain forms of learning (Desmond & Levy, 1986a & b; Geinisman *et al.*, 1991; Moser *et al.*, 1994; Hosokawa *et al.*, 1995; Vozeh & Myslivacek, 1996). However, it has recently been demonstrated that NMDA receptor-dependent LTP may only be important for certain aspects of learning and memory (Bannerman *et al.*, 1995; Saucier & Cain, 1995).

Several features regarding plasticity and LTP appear to be consistent through many different species. The increase in transmitter release which is associated

with the induction of LTP in the hippocampus (Bliss *et al.*, 1987; Errington *et al.*, 1987) and seen in response to sensitisation in *Aplysia* (Dale *et al.*, 1988). The involvement of cAMP dependent pathways and protein kinase A in the development of long-term facilitation in *Aplysia*, learning and memory in *Drosophila* and the maintenance of LTP in the hippocampus (Frank & Greenberg, 1994) indicates that enzymes and substrates involved in this pathway might be highly conserved in all these species.

The difficulty in relating changes in gene expression observed following the induction of LTP to physiological processes and plasticity stems from the lack of knowledge about how (and possibly whether or not) LTP is linked to learning and memory. Relating changes in gene expression to physiological processes involved in plasticity, learning and memory is further compounded by this lack of knowledge. Designing an experiment to look for changes in gene expression associated with learning is hampered by the lack of information available to act as a guide to determine the optimum time for finding differences in gene expression. Also if the hippocampus is only important for initial storage of memory before its consolidation elsewhere in the brain (Squire & Zola Morgan, 1991) then the physiological significance of LTP which lasts several weeks and the expression of genes associated with this form of LTP (e.g. c-fos) is brought into question. Important information about the relationship between certain genes, LTP, learning and memory (and indeed the link between learning and LTP) has been obtained from transgenic studies and has identified a number of genes as being involved in aspects of LTP and cognitive function. The interpretation of some of these results is not without its problems, and it has recently been suggested that behavioural observations made about some transgenic mice may be invalid as the incorrect control animals have been used for comparison (Gerlai, 1996).

Despite the problems associated with the interpretation and correlation of behavioural and biochemical observations with respect to LTP, I believe that some interesting discoveries have been made about plasticity-related gene expression in the hippocampus in this volume. We have supported some previous observations in the literature and also added more genes to the ever increasing number which are reported to be involved in the expression of LTP. In particular, we have found possible role for two isoforms of SNAP (A & B) in plasticity and found evidence to support a role for enkephalins in hippocampal plasticity. We have also raised the possibility that one of the mechanisms involved in the expression of LTP results in the stabilisation of dendritic mRNA species and allows targeting of proteins to specific synapses. Levels of αCAMKII mRNA are also regulated by mGluR and PKA activation underlining the importance of these receptors and this kinase in hippocampal function. These studies could have been improved, given more time, by carrying out studies with appropriate antagonists to verify that the changes we have reported are specific to the receptor/kinase which we have assumed them to be. Comparison of two molecular biological techniques has revealed that in-situ hybridisation is the optimum method for detection of small, localised changes in gene expression. Although there are many difficulties associated with quantitative RT-PCR, PCR is, nevertheless, a useful technique for detecting extremely low abundance mRNA species. In some cases there would be advantages to running both methods in parallel or carrying out in-situ PCR to localise low abundance transcripts.

Plasticity-related transmission in the hippocampus is associated with the activation of several second messenger systems due to the variety of receptors found in this region. Some of these second messengers are responsible for regulation of the expression of a number of genes. We have found evidence which demonstrates that, following the activation of some of these second

messengers, expression of several different classes of gene is altered. This suggests that many complex transcriptional changes can be achieved by alterations in neurotransmission in this structure, indicating the elaborate nature of synaptic plasticity. The changes in levels of protein kinases, immediate early genes, opioid peptides and structural proteins all have implications for synaptic plasticity in this structure, although the significance of these changes with respect to learning and memory has yet to be fully explained. It seems likely that in the future more information regarding the importance of these and other observations will become available and that the relationship between behavioural, electrophysiological, biochemical, molecular biological and pharmacological observations will be clarified further.

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ADDITIONAL NOTES WITH REFERENCE TO POINTS RAISED DURING THE ORAL EXAMINATION

- *Oligonucleotide sequences*

Arc

5'-GCA TCT CAG CTC GGC ACT TAC CAA TCT GCA GGA TCA CAT TGG-3'

zif/268

5'-CCG TTG CTC AGC AGC ATC ATC TCC TCC AGT TTG GGG TAG TTG TCC-3'

c-fos

5'-GCA GCG GGA GGA TGA CGC CTC GTA GTC CGC GTT GAA ACC CGA GAA-3'

TIMP

5'-TAT GAC CAG GTC CGA GTT GCA GAA GGC TGT CTG TGG GTG GGG TGG GGC ACA GCT-3'

MAP2

5'-TGT CCA GTG TGG TGC CTT TCC TTC GTC TTT CCT CTC GTC AGC CAT-3'

αCAMKII

5'-GCA GCA CCC TTG AGC TTC GAA GCC AGC AAC AGA TTC TCA GGC TTC-3'

cpg

5'-TAA CAG TTC CTT TCC ACA AAC GTG GCT TGG GGT TTG GTT TAA TGA-3'

N-FLANK

5'-CAC CAT GTG CCC ATC TAG AGT CTT TTC TTC ACT GCT GAT GTT-3'

N-VASE

5'-TAG AGT CTC TTG CTT CTC TGG TCG AGT CCA CGA TGC CTT TTC-3'

prodynorphin

5'-GTC CCA CTT AAG CTT GGG GCG AAT GCG CCG CAG GAA GCC CCC ATA-3'

proenkephalin

5'-ATC TGC ATC CTT CTT CAT GAA ACC GCC ATA CCT CTT-3'

- *Quantification*

There are without a doubt countless methods employed in different laboratories for quantifying mRNA levels following in-situ hybridisation all of which will have their own advantages and disadvantages. Before finally deciding on my chosen methods of quantification I experimented with several different approaches including the counting of silver grains, dark field illumination vs. bright field illumination, film vs. emulsion coated slides and variations in the magnification under which the samples were quantified. I do not believe there is necessarily one good method for all types of experiment, but I do feel that the methods which I used in this volume produced results which best represented what had actually occurred during the course of each experiment. Care was always taken to ensure that any changes seen were genuine, and not due to an artefact of the hybridisation reaction or emulsion coating process, by looking at hybridisation signals in regions which should remain unaffected by the experimental procedure (e.g. cortex). With particular reference to the counting of silver grains, I found manual counting of silver grain number per cell produced extremely variable results which I did not feel were not representative of the changes which had visibly occurred. In some cases (e.g. proenkephalin mRNA) the counting of silver grains may not have revealed any changes as it was the number of cells expressing a particular mRNA species which had increased rather than the amount of mRNA being produced per cell.

Some laboratories use radioactive standards so that the optical density of the film exposed to the experimental standard can be expressed as a known amount of radioactivity and also to ensure that the exposure of the experimental sample to the film or emulsion is such that it corresponds to the linear range of the standard curve (as film/emulsion exposure is saturable). This is a good method to use to control for over-exposure, but I do feel that experience with the procedure of in-situ hybridisation means that it becomes obvious when a film or emulsion coated slide has been over-exposed. If, however, a sample has been exposed for so long that it has almost reached saturation it will only mean that any differences between control and experimental samples recorded are reduced in magnitude but not completely abolished. However, I feel that as the units used in quantifying mRNA levels are so arbitrary that it makes no difference whether the are Bequerels, grain number or optical density as it is the differences which the quantification demonstrates which are important. No method will ever give an indication of the actual number of molecules of mRNA present in the tissue.

After careful consideration of these comments I still believe the methods which I used to be valid and have produced data which genuinely represent what has happened to mRNA levels in the tissue. I also believe that flexibility in ones approach to quantification when using in-situ hybridisation is extremely important.

- *Statistics*

To avoid confusion, as this may not have been completely clear in some of the legends, all graphs show mean ± standard error of the mean. However, in the case of the Mann-Witney test (a non-parametric test) the statistics were carried out on the medians obtained from the raw data. The Wilcoxon test assesses whether the hybridisation signal in treated/stimulated tissue is significantly different from control tissue when control levels are normalised to 100%. Analysis of variance is used when there are a large number of samples and it is likely that the more commonly used tests (e.g. Mann-Witney or t-test) will produce a significant result by chance. The post-hoc Fishers test is similar to a Wilcoxon in that it compares the results of the treated samples to values obtained from saline controls.

- *In vivo stereotaxic injections (chapter 6)*

Consistently accurate placement of the needle tip is very important for this technique. Too dorsal and the compound which is being injected will not be effective and too ventral could lead to dendritic or cell body damage. A large number of animals were used during the period of this study which is not reflected in the *n* values presented, but represents the difficulty in achieving correct needle placement consistently using this technique (probably only 30-40% success rate). Had more time been (years rather than months) it would have been more satisfactory to see whether the effects observed were dose-related, but unfortunately it was only possible to examine the effects of one dose of each compound chosen mainly from work published which had examined the effects these compounds have had on LTP or electrophysiological events in the hippocampus (Bashir *et al.*, 1993; Borlotto & Collingridge, 1993; Frey *et al.*, 1993). Experiments which would have clarified the specificity of the effects observed more clearly by using appropriate antagonists would also have been carried out had time been less of a limiting factor. The issue of compound specificity was raised with particular reference to the ACPD results. My conclusions were based purely on what I believed to be the most likely explanation for my observations and I reiterate the fact that it is not possible to reach any definite conclusions without using the appropriate antagonist controls (see comments on the methodology page 146). With particular reference to metabotropic

receptors all three classes of these receptors are expressed in the granule cell layer of the hippocampus (see pages 125-126 for references) meaning that application of ACPD will both decrease cAMP levels and increase production of IP3 and DAG leading to activation of PKC. Although it can not be stated with full certainty the exact mechanisms which underlie the changes in mRNA levels which we observed 24 hours after the application of ACPD, because the aforementioned studies were not performed, I believe that the suggestions made in this volume are not unreasonable (see pages 149-150) given the evidence available and knowledge of the second messenger systems likely to be activated/suppressed by stimulation of these receptors and activation of PKA. I would like to re-emphasise that although the conclusions drawn from this work were done so in the absence of appropriate pharmacological dissection of the pathways and receptors involved, full use of the information available in the literature and logical reasoning were used in arriving at them.

- *CREB*

Since the submission of this volume several important papers have been published concerning the role of CREB in synaptic plasticity. To briefly recap, previous work on *Aplysia* demonstrated that sensitisation of the gill withdrawal reflex is due to changes in synaptic efficacy due to increased presynaptic transmitter release, post translational modification of existing proteins and the synthesis of new proteins. In addition it has also been shown that activation of PKA and phosphorylation of CREB occurs in this model (pages 2 & 3). The role of the cAMP second messenger system in learning and memory in *Drosophila* is also well documented in the literature (pages 3 & 4).

Using the *Drosophila* neuromuscular junction, a synapse which undergoes activity-dependent increases in synaptic growth, the interaction between synaptic activity, cell adhesion molecules (CAMs) and phosphorylation of CREB have been investigated and this has led to the dissociation of structural and functional mechanisms of plasticity. The work showed that synaptic activity or an increase in cAMP triggers the internalisation of CAMs and leads to synaptic sprouting (structural plasticity). However as the pre-existing components of the release machinery are re-distributed rather than increased in number, there is no functional increase in synaptic strength. Similarly the phosphorylation of CREB alone does not produce any increase in synaptic strength because although it leads to synthesis of components of the release machinery (functional plasticity) it does not trigger the internalisation of CAMs and the formation of new synaptic contacts. In order to achieve cAMP dependent enhancement of synaptic strength it appears that both these pathways must be activated in parallel for

stabilisation of the new synapses generated to occur. Alternatively the internalisation of CAMs produced by synaptic activity could in some way "mark" the synapses to which the products of CREB related gene transcription are targeted.

Diesseroth *et al.* (1996) carried out a series of experiments to investigate the mechanisms underlying phosphorylation of CREB in the rat hippocampus using a variety of electrophysiological manipulations. Interestingly they found that two protein kinase A inhibitors failed to affect CREB phosphorylation produced in response to LTP- or LTD- inducing synaptic stimuli suggesting that for these forms of synaptic plasticity the cAMP/PKA pathway does not induce phosphorylation of CREB. Instead it was demonstrated that calcium influx and activation of calcium dependent protein kinases were necessary for the phosphorylation of CREB observed following the induction of LTP or LTD. However, other work by Impey *et al.* (1996) which used a transgenic approach to study CRE-mediated gene expression, demonstrated that this expression was PKA dependent and triggered by the induction of LTP (but not STP). In addition it was also demonstrated that blockade of voltage gated calcium channels (thought to be important for NMDA receptor independent LTP) reduced the duration of LTP and associated increases in CRE-mediated gene expression. This illustrates the important role which calcium must play in CRE-mediated gene expression and also potential roles for calcium dependent proteins such as CAMKII. It was also demonstrated that although the induction of STP and LTP both led to phosphorylation of CREB, this did not trigger CRE-mediated gene expression except in the case of LTP.

These observations imply that even if you do have phosphorylation of CREB and synaptic activity/increases in cAMP levels (as described in the *Drosophila* model) that something additional which may be specific to LTP is still required for CRE-mediated gene expression and the synthesis of new proteins (and hence strengthening of synapses). Activation of CAMKII and/or protein kinase A following the induction of LTP may be the proteins which link CREB phosphorylation to CRE-mediated gene expression and alterations in both functional and structural plasticity.

- *Dendritic targeting*

It has recently been suggested that the induction of LTP leads to the creation of a temporary "synaptic tag" at the potentiated synapse which sequesters certain proteins in that region in order to establish the late phase of LTP (Frey & Morris, 1997). Tagging of synapses in this way does not appear to involve local dendritic protein synthesis and candidates which have been suggested to play the role of the tag are protein kinases.

Conversely, Kang & Schuman (1996) demonstrated than neurotrophin-induced hippocampal synaptic enhancement required immediate protein synthesis for its occurrence which was shown to be independent of the cell body. It was suggested that the site of protein synthesis might be localised to axons or dendrites of pyramidal cells. The observations made in this volume concerning increased dendritic levels of mRNA following the induction of LTP are in agreement with the suggestions made by Kang & Schuman (1996). It is possible that increases in protein synthesis can be localised to particular synapses by targeted increases in mRNA levels of that species, perhaps by stabilising them in some way. It would be necessary to examine dendritic protein levels following the induction of LTP to determine whether the localised increases in mRNA are indicative of increased translation occurring in those synapses. If this hypothesis were proven then it would indicate a mechanism whereby synapses can potentially modify their biochemical properties independently of new transcription occurring in the nucleus.

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