Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

A study of the role of neurotrophins in the mechanisms underlying the expression of long-term potentiation in the dentate gyrus.

by

Marina Casey B.A. (Mod)

Thesis submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College

Submitted September 2001

Department of Physiology
Trinity College
Dublin 2

Table of Contents

Chapter 2 Materials & Methods

Chapter 4 Investigating intracellular signalling mechanisms underlying the actions of NT-3

II Acknowledgements

I wish to thank my supervisor. Prof. Marina Lynch for the constant advice and ever-present encouragement and interest during the past three years.

I wish to express thanks to Prof. Bell for allowing me the opportunity to carry out research in the Department of Physiology.

I acknowledge the Health Research Board for financial support of this study.

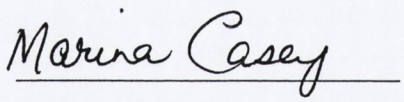
I am grateful to all the members of the technical staff for their much-appreciated assistance: Kieran, Siobhan, Quentin, David, Leslie, Aidan, Alice, Ann, Leslie and Doreen. I would also like to thank Ann, Cormac and the staff of the Bioresources Unit.

A big thanks to all the members, past and present, of our lab: Aine, Eamonn, Conor, Emily, Yvonne, Aileen, Darren, Frank, Veronica and especially my 'twin', Martina.

Special thanks to Paul, Olivia, Caroline and Patrick for their friendship and constant encouragement.

Most importantly, I wish to dedicate this thesis with love to Mam and Dad. This would not have been possible without you.

I declare that this thesis is entirely my own work with the following exceptions: the Western immunoblot examining the effect of low-frequency stimulation on ERK activity was completed by Martina Gooney and the glutamate release experiment to assess the effects of low-frequency stimulation was conducted by Dr. Emily Vereker. This work has not been previously submitted as an exercise for a degree to this or any other university. I give permission to the library to lend or copy this thesis.



Marina Casey B.A. (Mod)

IV Summary

Long-term potentiation (LTP) is a well-described form of synaptic plasticity. This study set out to investigate the role that tyrosine kinase plays in the expression of LTP at the perforant path-granule cell synapses of the rat *in vivo* and to determine the impact of neurotrophin-induced tyrosine kinase cascades.

Increased glutamate release is an established component of the expression of LTP in the dentate gyrus. The focus of this study was, therefore, to examine intracellular events mediated by tyrosine kinase, which impact on glutamate release from presynaptic terminals. The data show that inhibition of tyrosine kinase activity by genistein blocked LTP and the LTP-associated process of glutamate release. Analysis of signalling events following the inhibition of LTP by genistein demonstrated that LTP-induced increases in extra-cellular regulated kinase (ERK) activation and phosphorylation of voltage-operated calcium channel (VOCC) subunit, α_1, in synaptosomes were abolished by pretreatment with genistein. This indicates that phosphorylation of several intracellular tyrosine kinase substrates may mediate glutamate release; these include synaptic vesicle proteins and VOCC subunits and it is possible therefore that blocking tyrosine phosphorylation inhibits LTP because of its effects on glutamate release. In addition, phosphorylation of a transcription factor, cAMP-response element binding protein (CREB) and protein synthesis were investigated as possible mechanisms to mediate long-lasting changes associated with LTP at the perforant path-granule cell synapses. Parallel LTP-related increases in CREB activation and protein synthesis were demonstrated but these effects were inhibited by pretreatment with genistein, indicating that tyrosine phosphorylation is necessary for persistent changes associated with LTP in the dentate gyrus.

Because neurotrophins stimulate a cascade of events initiated by activation of tyrosine kinase and because evidence has accumulated in recent years suggesting a role for neurotrophins in the expression of LTP in the hippocampus, the effect of LTP on Trk-mediated cascades was investigated using the Trk inhibitor, tyrphostin AG879. Inhibition of Trk resulted in failure to sustain LTP at the perforant path-granule cell synapses and abolished LTP-related increases in ERK and CREB activity in the presynaptic terminals and in the postsynaptic region, indicating that events on both sides of the synapse

regulate changes in synaptic function following tetanic stimulation. The possible role for neurotrophin-3 (NT-3) in LTP was assessed. The data showed that NT-3 release was not altered by the expression of LTP but NT-3 release was blocked by pretreatment with tyrphostin AG879.

In conclusion, the evidence suggests that tyrosine kinase participates in signalling cascades mediating LTP at the perforant path-granule cell synapses, by modulating short-term LTP-associated process of glutamate release, and by initiating long-lasting effects through activation of transcription factors and stimulation of protein synthesis. In addition, tyrosine kinase signalling, mediated by Trk receptors, impacts on LTP and its associated biochemical events in presynaptic and postsynaptic regions, indicating that changes occur on both sides of the synapse to support the expression of LTP at the perforant path-granule cell synapses.

V Abbreviations

AA	Arachidonic acid
ACPD	1-amino-cyclopentane-1,3-dicarboxylate
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-proprionate
ATP	Adenosine 5'-triphosphate
BAPTA	1, 2 bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CRE	cAMP-response element
CREB	cAMP-response element binding protein
CO	Carbon monoxide
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine-tetra acetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol bis (β-aminoethylether) *N,N* 'tetraacetic acid
EPSP	Excitatory postsynaptic potential
ERK	Extracellular signal regulated kinase
FGF	Fibroblast growth factor

G-protein	GTP-binding protein
GTP	Guanosine 5'-diphosphate
HFS	High frequency stimulation
HRP	Horseradish peroxidase
IEG	Intermediate early gene
IP_3	Inositol 1,4,5-trisphosphate
KCl	Potassium chloride
LFS	Low frequency stimulation
LTP	Long-term potentiation
MAP Kinase	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
mRNA	Messenger RNA
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NT-3	Neurotrophin-3
NT-4,5	Neurotrophin-4,5
NT-6	Neurotrophin-6
NT-7	Neurotrophin-7
p75 receptor	p75 low affinity neurotrophin
pAB	Polyclonal antibody
PAF	Platelet-activating factor
PIP	Phosphatidylinositol 4-phosphate
PIP_3	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A

PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulphonylfluoride
PSD-95	Postsynaptic density protein 95
SDS	Sodium dodecyl sulphate
TMB	3,3',5,5'-Tetramethyl-benzidine
TCA	Trichloroacetic acid

VI List of Figures

Chapter 1

General introduction

1.1 Neurotrophins

1.1.1 Background

To date the neurotrophin family is composed of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) in mammals. Novel neurotrophins, NT-6 and NT-7 have been identified in fish (Götz *et al.*, 1994; Lai *et al.*, 1998). Neurotrophins are a group of structurally related proteins that promote differentiation and survival of specific neuronal populations. NGF was the first neurotrophin identified and was originally observed as an agent released from mouse sarcoma that increased the number and size of sensory and sympathetic neurons, and their proliferation, in the developing chick (Levi-Montalchini, 1987; Levi-Montalchini and Hamburger, 1951). NGF was subsequently purified from the male mouse submaxillary gland (Varon *et al.*, 1967a). Characterisation of NGF and the finding that it only supported a limited set of neuronal populations stimulated further study, leading to the identification of other family members. BDNF was initially investigated in porcine dorsal root ganglia (Barde *et al.*, 1982). Subsequently, oligonucleotide primers for PCR were used to identify NT-3 (Maisonpierre *et al.*, 1990), followed by isolation of NT-4 (Ip *et al.*, 1992).

The effects of neurotrophins are evident in the peripheral and central nervous systems. Neurotrophins have been shown to regulate neuronal survival. Deletion of genes for either NGF or its receptor, TrkA, results in significant abnormalities in sympathetic and sensory ganglia in the peripheral nervous system, manifest as a 70-80% reduction in cell numbers in the dorsal root ganglion and almost total reduction of cells in the sympathetic superior cervical ganglion (Crowley *et al.*, 1994). Studies using anti-NGF antibodies to bind endogenous NGF demonstrate the role of NGF, in conjunction with TrkA, in regulating the survival of nociceptive sensory neurons during development and adulthood (Hill *et al.*, 1988; Johnson *et al.*, 1980). Similarly, BDNF and TrkB knockouts have decreased numbers of sensory neurons in dorsal root ganglia (Jones *et al.*, 1994). In contrast, differences between the phenotypes of NT-3 and TrkC knockouts have been observed. In both knockout animals, proprioceptive dorsal root ganglion neurons do not

develop (Ernfors *et al.*, 1994; Klein *et al.*, 1994) but the total loss of cells is greater in the NT-3 knockout. This may be a result of the ability of NT-3 to regulate TrkA- and TrkB-mediated cell survival during development (Huang *et al.*, 1999).

Differences in expression of neurotrophins and Trk receptors occur during development. It has been reported that NGF and TrkA are expressed relatively late in development (Williams *et al.*, 1995), whereas NT-3 and TrkC are expressed earlier (Tessarollo *et al.*, 1993; Elkabes *et al.*, 1994). In contrast, BDNF is expressed at high levels during development and adulthood (Ip et al., 2001; Maisonpierre et al., 1990; Friedman et al., 1991; Ringstedt et al., 1993). Expression of neurotrophins may be regulated by activity, which increases levels of neurotrophin mRNA (Gall and Isackson, 1989; Zafra *et al.*, 1990; Ernfors *et al.*, 1991; Lu *et al.*, 1991).

In the central nervous system, the effects of neurotrophins are reported in the visual cortex (Castrén *et al.*, 1992; Galuske *et al.*, 1999; Berardi *et al.*, 1999) and the hippocampus (Crowley *et al.*, 1994; Chen *et al.*, 1999a; Messaoudi *et al.*, 1998). Evidence for one role of neurotrophins in the hippocampus is largely derived from studies of synaptic plasticity in that region. Several reports indicate that neurotrophins serve as neuromodulators of synaptic transmission (Section 1.3.5) and synaptic plasticity, including LTP (Section 1.5.11). Several groups have reported that BDNF, NT-3 and NT-4 can influence the outgrowth of dendrites and axons (Lambelle and Leclerc, 2000; Ip *et al.*, 1993). It has also been reported that neurotrophins regulate gene expression, ion channel gating, protein synthesis and cell growth (Thoenen, 1995). In addition, evidence has recently accumulated in favour of a role for neurotrophins in synaptic plasticity in the central nervous system, which will be addressed later in Section 1.5.11.

1.1.2 Localisation of neurotrophins in the brain

A diverse and partially overlapping distribution of neurotrophins and their cognate receptors, Trk receptors, is reported in several areas of the brain, including the hippocampus, and the subcortex (Yan *et al.*, 1997; Katoh-Semba *et al.*, 1997; Anderson *et al.*, 1995). The hippocampus has received most attention in studies to localise neurotrophins and their Trk receptors in the brain. NGF, BDNF, NT-3, in addition to TrkB and TrkC are expressed at high levels in the hippocampus (Klein *et al.*, 1989; Hofer

et al., 1990, Lambelle *et al.*, 1991; Maisonpierre *et al.*, 1990). NT-3 and its cognate receptor, TrkC are highly expressed in the hippocampus, relative to other brain regions, and, in particular, in the granule cells of the dentate gyrus (Zhou and Rush, 1994; Tokuyama *et al.*, 1999; Merlio *et al*, 1992).

1.1.3 Structure

The structure of NGF was first described by Varon and coworkers (1967b). It was reported that NGF is secreted as a high molecular weight complex of sedimentation coefficient 7S. Further analysis revealed that the complex was composed of one β-subunit (β-NGF), which in turn is composed of two non-covalently associated identical amino acid chains (Varon *et al.*, 1967b), two γ subunits (Mason *et al.*, 1983) and two catalytically inactive α-subunits (Isaackson *et al.*, 1984). The high moecular weight form, 7S NGF, is biologically inactive but dissociation of the α and γ subunits from the complex releases β-NGF, which is active (Varon *et al.*, 1967b). Another form of NGF, 2.5S NGF is a mixture of proteolytically cleaved β-subunits and has different properties from the 7S form (Shao *et al.*, 1993). The β-subunit is entirely responsible for the biological activity of NGF (Young *et al.*, 1988).

The mature or processed part of all neurotrophins displays a high degree of sequence homology and approximately 55% of all sequences are shared by all neurotrophins. This indicates that functional differences between these proteins may be determined by sequences lying outside the conserved region (Maisonpierre *et al.*, 1990). The replacement of 7 amino acids in NT-3 leads to the formation of a neurotrophin with the full biological activity of NGF, BDNF and NT-3, suggesting that NT-3 may have been the neurotrophin ancestor gene (Urfer *et al.*, 1994).

1.1.4 Synthesis and processing

The cDNA coding the precursors or proneurotrophins have been cloned following the identification of members of the nerve growth factor family (Scott *et al.*, 1983; Leibrock *et al.*, 1989; Maisonpierre *et al.*, 1990; Berkemeier *et al.*, 1991; Götz *et al.*, 1994). Sequence data predict that all neurotrophins are generated from 31-35kDa precursors that contain hydrophobic signal peptides adjacent to proregions containing

sequences of basic amino acids. Intracellular cleavage of proneurotrophin to produce active neurotrophins is mediated by a structural motif (Seidah *et al.*, 1994). A family of processing enzymes known as convertases is responsible for processing of precursor proteins (Zhou *et al.*, 1999). Colocalisation and coregulation of convertases and their cognate substrates within the trans-Golgi network of secretory granules (Seidah *et al.*, 1994) led to the finding that many convertases are colocalised with neurotrophin mRNA. One convertase in particular, furin, was found to be most efficient in processing NGF precursor (Seidah *et al.*, 1996a, 1996b) and prohormone convertase 1 (PC1) was reported to convert proBDNF to the bioactive form (Seidah *et al.*, 1996a, 1996b).

More recent studies have focused on intracellular sorting of neurotrophins in hippocampal neurons (Mowla *et al.*, 1999; Farhadi *et al.*, 2000). Pulse chase experiments and immunocytochemistry analysis reveals differential intracellular processing of NGF, BDNF and NT-3. Processing of pro-NGF occurs in the trans-Golgi network and is performed by furin whereas intact pro-BDNF is shunted into secretory vesicles where is cleaved by PC1 (Mowla *et al.*, 1999). Sorting of NGF into the constitutive pathway may allow continuous availability of NGF for dependent neurons, providing a basis for neuroprotection (Mowla *et al.*, 1999). Findings from another study by Blöchl and Thoenen (1995) suggest that NGF may be released in both a constitutive and regulated manner. In contrast, analysis of intracellular sorting of NT-3 indicates that it is primarily processed in the constitutive pathway but the formation of NT-3/BDNF heterodimers (Jungbluth *et al.*, 1994, Robinson *et al.*, 1995) shunts NT-3 into BDNF-containing secretory vesicles (Farhadi *et al.*, 2000).

1.1.5 Neurotrophin release

In the central nervous system, NGF release was first characterised in hippocampal slices and primary cultures of hippocampal neurons (Blöchl and Thoenen, 1995). In this study, it was reported that NGF release was stimulated in response to KCl-induced depolarisation. In addition to this, regulated NGF release was rapid and occurred in response to stimulation of non-NMDA receptors by glutamate or voltage-operated sodium channels by veratridine. The underlying mechanism was reported to depend on extracellular sodium and mobilisation of calcium from intracellular stores. Subsequent to

the work of Blöchl and Thoenen, activity-dependent release of BDNF from hippocampal (Androutsellis-Theotokis *et al.*, 1996; Goodman 1996) and cortical synaptosomes (Androutsellis-Theotokis *et al.*, 1996) and from primary sensory neurons (Balkowiec and Katz, 2000) was observed. Mechanistic differences between NGF release and BDNF release were suggested by Goodman and coworkers (1996), who reported that BDNF release was dependent on extracellular calcium. Their findings were disputed by further work by Griesbeck and coworkers (1999), which suggested that depolarisation-induced release of both NGF and BDNF was dependent on intact intracellular calcium stores. The causal relationship between activity-mediated sodium influx and neurotrophin release was disputed by observations that release of BDNF was independent of sodium influx (Hoener, 2000). Evidence documenting release of NT-3 is sparse and mostly comes from studies of activity-dependent release at peripheral synapses (Liou and Fu, 1997) or PC12 cell lines (Krüttgen *et al*, 1998). Recently, depolarisation-induced release of NT-6 from PC12 and adenovirally transduced hippocampal neurons has been reported (Gartner *et al.*, 2000).

It has been demonstrated that neurotrophin secretion is also regulated by Trk receptor activation, representing a potential feedback mechanism (Canossa *et al.*, 1997; Krüttgen *et al.*, 1998). Krüttgen and coworkers proposed that neurotrophin-stimulated neurotrophin release was mediated by both Trk receptors and p75 receptors but further evidence presented by Canossa and coworkers (2001) suggested that the effects are mediated by Trk receptors alone and further suggest that the differences in findings are explained by the properties of different cells used. In addition to Trk receptor activation, metabotropic glutamate receptors (mGluRI) participate in the modulation of neurotrophin-modulated release of neurotrophins. It is proposed that phospholipase Cγ (PLCγ) may serve as a coincidence detector of simultaneous activation of Trk and mGluRI and may mobilise calcium from intracellular stores via the formation of the ubiquitous second messenger, IP_3.

1.2 Neurotrophin Receptors

1.2.1 Classification

Neurotrophin receptors are divided into two classes; tyrosine receptor kinases or Trks and p75 receptors. The Trk family of receptor tyrosine kinases is a family of high-affinity neurotrophin receptors and comprises TrkA, TrkB and TrkC. The p75 receptor is a low-affinity neurotrophin receptor. NGF, BDNF, NT-3 and NT-4 interact with p75 (Roderiguez-Tebar *et al.*, 1990). Although p75 and Trk do not bind to each other directly there is evidence to suggest that complexes form between the two receptors. As a result of this interaction, increased specificity may be conferred on Trk by p75. BDNF, NT-3 and NT-4 bind to Trk B but only BDNF provides a functional response in the presence of p75 (Bibel *et al.*, 1999). Similarly, NGF and NT-3 bind TrkA but, in the presence of p75, TrkA discriminates in favour of NGF (Benedetti *et al.*, 1993).

1.2.2. Trk

Three *trk* genes have been identified in mammals. The product of the TrkA protooncogene was identified in as the cognate receptor of NGF (Kaplan *et al.*, 1991; Klein *et al.*, 1991). Other work led to the isolation of TrkB (Klein *et al.*, 1991) and TrkC (Lambelle *et al.*, 1991; Condon-Cardo *et al.*, 1991). NGF is the preferred ligand of TrkA and similarly BDNF and NT-4 are preferred ligands for TrkB and NT-3 is preferred ligand for TrkC. These specificities are not absolute. It has been shown that NT-3 is a ligand for both TrkA and TrkB, in addition to TrkC (Barbacid, 1994). Furthermore, each *trk* gene codes more than one transcript. Examples of this are truncated forms of TrkB and TrkC, which lack a cytosolic domain and therefore, cannot propagate a signal (Barbacid, 1994). The purpose of such receptors may be to regulate the levels of neurotrophins in the extracellular medium (Biffo *et al.*, 1995). The tyrosine kinase domains are highly related, displaying 80% amino acid identity. In contrast, the extracellular domains are more divergent, with only 30% homology. Binding of neurotrophins to Trk receptors leads to receptor tyrosine phosphorylation. Ligand-induced dimerisation (Jing *et al.*, 1992) results in the phosphorylation of specific tyrosine

residues, located in the action loop of the tyrosine kinase domain (Cunningham and Greene, 1998). Autophosphorylation controls the signalling and catalytic properties of Trk receptors (Obermeier *et al.*, 1994; Stephens *et al.*, 1994). Phosphorylation of these residues leads to an open conformation of the receptor resulting in *trans*-phosphorylation (Canossa *et al.*, 1996) and allowing substrates to access the kinase domain. Tyrosine residues located within the kinase domain implicated in the kinase activity are Y674 and Y675. Phosphorylation of these residues correlates with activation of the intrinsic tyrosine kinase activity of the Trk receptor (Segal *et al.*, 1996). Another tyrosine residue, Y1162, controls the conformation of the activation loop (Segal *et al.*, 1996). Two specific phosphotyrosine residues, located in the juxtamembrane region, Y490 and Y785, act as docking sites for adaptor molecules, which serve to propagate the neurotrophin-induced signal (Stephens *et al.*, 1994; Vetter *et al.*, 1991). These residues mediate effects through their interaction with Shc and PLCγ respectively (Dikic *et al.*, 1995; Middlemas *et al.*, 1991;Vetter *et al.*, 1991).

1.2.3 p75 receptor

In addition to Trk, neurotrophins bind to p75, which was originally described as the NGF receptor (Johnson *et al.*, 1986; Radeke *et al.*, 1987). The first indication for a signalling function of p75 was the finding that p75 mediates sphingomyelin hydrolysis and production of ceramide following neurotrophin binding (Dobrowsky *et al.*, 1994). Activation of NF-κB was observed following exposure to NGF (Carter *et al.*, 1996) indicating that p75 may mediate cell survival. Other reports demonstrate that p75 activation may cause programmed cell death or apoptosis (Barker, 1998; Friedman, 2000). The activity of p75 has also been implicated in other cellular processes such as stress responses. Despite the molecular characterisation of the p75 receptor, the signal transduction cascades underlying its actions remain poorly defined although inhibition of Jun kinase (JNK) abolishes the p75-mediated signal, indicating that JNK is a component of the signal (Yoon *et al.*, 1998).

1.2.4 Receptor internalisation

Because of the elongated structure of neurons with long processes emanating from the cell body, neurons must rely on transport of proteins within the axon. Retrograde transport of proteins from the nerve terminal to the cell body enables the neuron to respond to its environment, promoting survival or triggering apoptosis, strengthening synaptic connections with target cells or induction of gene expression. It was originally shown that NGF is retrogradely transported within neurons. Injection of radiolabelled NGF permitted the monitoring of axonal transport of NGF in the sympathetic and sensory neurons (Hendry *et al.*, 1974; Johnson *et al.*, 1976). Subsequently, retrograde transport of endogenous NGF was demonstrated (Korsching *et al.*, 1983). Similarly, transport of radiolabelled NT-3 and BDNF to dorsal root ganglion neurons and within motoneurons was shown (DiStefano *et al.*, 1992).

Upon binding with the Trk receptor at the cell surface, the neurotrophins undergo receptor-mediated endocytosis. In PC12 cells, NGF induced rapid internalisation of TrkA receptors into small vesicle-like structures located close to the cell membrane (Grimes *et al.*, 1996) and disruption of internalisation abolishes the cellular response to NGF in PC12. This process is mediated by clathrin (Grimes *et al.*, 1996). The NGF-Trk complex remains intact and NGF-induced signalling may continue, activating PLCγ (Grimes *et al.*, 1996). Once internalised the neurotrophin-Trk complex is propelled along the axon towards the soma (Ehlers *et al.*, 1995) but regulation of this process is not well defined. It has been suggested that PI-3 kinase plays a role in retrograde transport of NGF because PI-3 kinase inhibitor, wortmannin, inhibits retrograde transport of NGF in sympathetic and sensory neurons (Bartlett *et al.*, 1999). In addition, it has been proposed that this process may depend on cytoskeleton structure because retrograde transport is impeded by colchicine, an inhibitor of microtubule polymerisation (Hendry *et al.*, 1974).

1.2.5 Intracellular signalling cascades activated by Trk

The most comprehensively studied route by which neurotrophins mediate intracellular events is the Ras/Raf/ERK pathway, depicted in Figure 1.1. Characterisation of the transduction mechanisms underlying cellular responses to stimulation of receptor

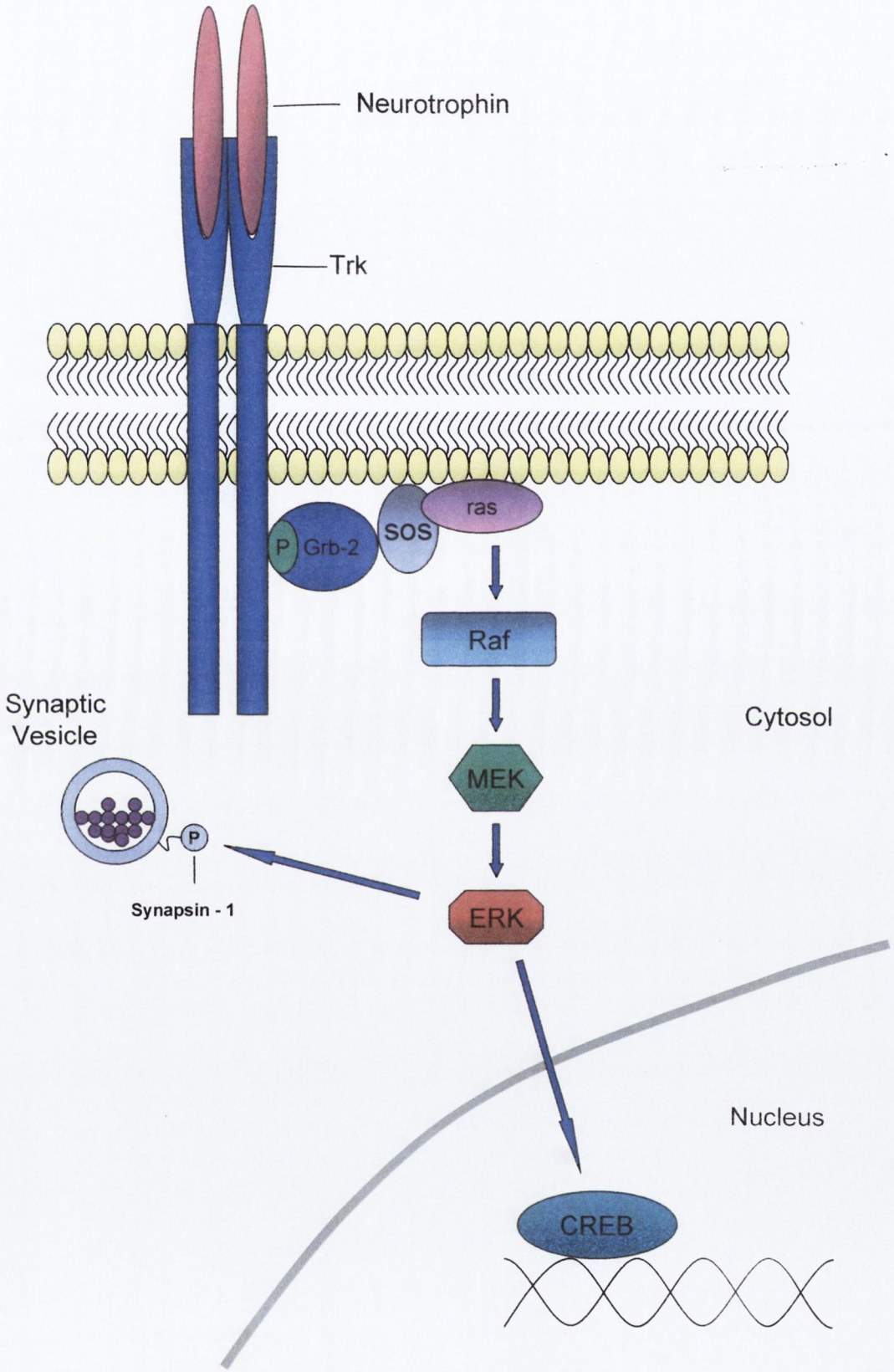
activity by neurotrophins has pinpointed the central importance of tyrosine kinases in propagating the signal in the intracellular domain. The initial event in the cascade is the interaction between the neurotrophin and its cognate receptor, thus stimulating the intrinsic tyrosine kinase activity of the Trk receptor. This leads to dimerisation of Trk receptors followed by autocatalysis of phosphorylation of tyrosine residues in the cytosolic domain of the adjacent Trk receptor (Segal *et al.*, 1996). The resultant phosphotyrosine residues and their neighbouring residues serve as a framework for signal propagation by acting as specific recognition sites for several intracellular kinases and adaptor proteins, such as PLCγ, PI3 kinase and Shc. The interaction between the phosphotyrosine residues of the activated Trk receptor and the effector proteins is governed by a structural motif found on the effector proteins. This motif is known as Src homology 2 (SH2) domain due to its striking similarity to a region of the pp60src tyrosine kinase (Schlessinger *et al.*, 1994; Cohen *et al.*, 1995). Interactions of the Trk receptor with PLCγ, PI3 kinase and Shc allows neurotrophin-mediated signalling to engage two intracellular cascades; Ras-dependent and Ras-independent pathways.

1.2.6 Ras-dependent transduction cascades

Initiation of the Ras-dependent pathway culminates in the sequential activation of a hierarchy of kinases. Shc possesses no catalytic activity but rather serves as an adaptor protein to mediate the interactions to other proteins with the cytosolic domain of Trk receptors. Interaction between Shc and Trk at Y490 allows Shc to become a substrate for the Trk receptor. Tyrosine phosphorylated Shc is recognised by the SH2 domain of Grb-2 (Rozakis-Adcock *et al.*, 1992). Association of Shc and Grb-2 promotes the translocation of Grb-2 to the juxtamembrane region. In addition to a SH2 domain, Grb-2 also possesses a second structural motif, SH3, which acts as a recognition site for proline-rich proteins, such as a small G-protein, SOS. In turn, SOS is responsible for the activation of Ras, a G-protein, by stimulating the exchange of GDP for GTP (McCormick *et al.*, 1994). Raf-1 and B-Raf, 70 – 75kDa serine/threonine kinases, are activated on recruitment to the membrane by GTP-bound Ras. Activation of B-Raf stimulates its ability to dually phosphorylate and thus activate ERK kinase, MEK (Alessi *et al.*, 1994). As no other substrates of MEK other than ERK have been identified (Crews *et al.*, 1992), it is

Figure 1.1 The signalling cascade initiated by the interaction of neurotrophins with Trk

The ras/Raf/ERK cascade is the principal intracellular pathway triggered by neurotrophins and ultimately leads to gene expression. Interaction of Trk with NGF, BDNF or NT-3 results in dimerisation and autophosphorylation of the Trk molecule. The phosphotyrosine residues act as a scaffold for adaptor molecules like Grb-2, which interacts with SOS. The Grb-2-SOS complex activates ras, a small membrane-associated G-protein. Activated ras triggers several sequential phosphorylation events, leading to the activation of ERK. ERK influences events in the nucleus, by activating transcription factor, CREB, and in the cytosol, by phosphorylating synapsin-1, a synaptic vesicle protein.


Neurotrophin
Trk
ras
SOS
P
Grb-2
Raf
Synaptic
Vesicle
Cytosol
MEK
P
Synapsin - 1
ERK
Nucleus
CREB

possible that their marked specificity contributes to the selective activation of their downstream target, ERK. ERK is a member of the mitogen-activated protein kinase (MAP kinase) superfamily (Seger and Krebs, 1995), which includes p38 and JNK.

1.2.7 Ras-independent transduction cascades

In addition to the transduction cascades involving ras, Raf and ERK, Trk activation stimulates other signalling molecules such as PLCγ and PI-3 kinase. Association of PLCγ with Trk occurs via phosphotyrosine residues in the cytosolic domain of Trk (Middlemas *et al.*, 1994; Obermeier *et al.*, 1993; Stephens *et al.*, 1994). This event results in tyrosine phosphorylation and thus activation of PLCγ (Vetter *et al.*, 1991). Activated PLCγ cleaves phosphatidylinositol 4,5-bisphosphate to generate two important lipid-derived signalling molecules, inositol trisphophate (IP_3) and diacylglycerol (DAG). IP_3 stimulates calcium mobilisation from intracellular stores, thereby activating many pathways controlled by calcium while DAG stimulates protein kinase C (PKC) activation (Berridge *et al.*, 1993). It has been shown that activation of PKC is important in mediating ERK activation in response to NGF to achieve neurite outgrowth (Corbit *et al.*, 1999).

Initial studies linking neurotrophin signalling with PI-3 kinase centred on NGF activation of PI-3 mediated by TrkA (Ohmichi *et al.*, 1992). PI-3 kinase is identified as a major mediator of neurotrophin-mediated survival. Activation of PI-3 kinase may be achieved by three adaptor molecules, Shc, Grb-2 and Gab-1. Phosphotyrosine residues in the cytosolic domain of the Trk receptor associates with Shc, which in turn recruits Grb-2 (Rozakis-Adcock *et al.*, 1992). Phosphorylated Grb-2 provides a docking site for Gab-1, which interacts with PI-3 kinase. In PC12 cells, a direct interaction between ras and PI-3 kinase has been identified (Rodiguez-Viciana *et al.*, 1994). Major substrates of PI-3 kinase are the regulatory subunit of PI-3 kinase (Dhand *et al.*, 1993) and Akt, which promotes cell survival (Ashcroft *et al.*, 1999).

1.2.8 Activation and regulation of ERK

A dual phosphorylation event on tyrosine (Tyr-185) and threonine (Thr-183) residues is necessary for the activation of ERK by MEK (Payne *et al.*, 1991; Her *et al.*, 1993). Further analysis revealed that phosphorylation of tyrosine precedes phosphorylation of threonine (Robbins and Cobb, 1992) although phosphorylation of either residue can occur in the absence of the other (Posanda and Cooper, 1992). Because phosphorylation on both residues is required for activation of ERK, phosphatases that remove phosphate from both or either sites can abolish activity.

1.2.9 Consequences of ERK activation

Numerous substrates of ERK have been identified. Because of the broad nature of its substrate recognition, it has been proposed that activation of ERK may have ramifications in the cytosolic (Jonanovic *et al.*, 1994; Matsubara *et al.*, 1996) and nuclear domains (Xing *et al.*, 1996). It seems plausible that ERK mediates both immediate effects of Trk activation by modulating synaptic vesicle proteins or long-term consequences by mediating activation of transcription factors such as Elk-1 and cAMP-binding element protein (CREB). It has been reported that synapsin I, a protein resident in the synaptic vesicle membrane, is an ERK substrate (Jonanovic *et al.*, 1994; Matsubara *et al.*, 1996) and that ERK-induced phosphorylation frees it from the cytoskeletal meshwork, thus potentially increasing the number of available synaptic vesicles in the nerve terminal (Greengard *et al.*, 1993). Alternatively, ERK may modulate patterns of gene expression by activation of transcription factors. ERK has been reported to translocate to the nucleus following phosphorylation and dimerisation (Chen *et al.*, 1992) where it may directly phosphorylate the transcription factor, Elk-1 (Hipskind *et al.*, 1994). Another transcription factor, CREB may be phosphorylated in an ERK-dependent manner by CREB kinase, ribosomal SG kinase, RSK, which is an ERK-substrate (Impey *et al.*, 1998; Xing *et al.*, 1996).

1.2.10 CREB

CREB is a member of a constitutively expressed transcription factor family and was first isolated in studies to determine the signalling mechanisms underlying peptide

relies on a conserved structural motif formed by a heptad of leucine residues, referred to as a leucine zipper, while DNA binding is mediated by a basic domain. The family of transcription factors of which CREB is a member is known as the basic leucine zipper or bZIP superfamily because of these structural motifs. Following the characterisation of CREB, two highly related gene products were isolated: activating transcription factor 1 (ATF-1; Lee *et al.*, 1987) and cAMP response element modulator (CREM; Foulkes *et al.*, 1991). Together these proteins constitute a subfamily of the bZIP superfamily. CREB may homodimerise or may heterodimerise with ATF-1 or CREM before binding DNA (Foulkes *et al.*, 1991; Hurst *et al.*, 1990), CREB homodimers having the longest half-life of between 10 and 20 minutes. The nature of the dimer bound to the target promoter may govern the duration of the CREB-dependent transcription event (Kobayashi *et al.*, 1995).

1.2.11 Activation of CREB

The central event in the activation of CREB is phosphorylation on Ser 133, which confers specificity of neurotrophin-mediated transcription (Bonni *et al.*, 1995). The importance of this residue is underpinned by studies reporting that mutation of this serine residue to alanine abolishes the transcriptional response (Gonzalez *et al.*, 1989) and CREB mutants lacking Ser 133 are unable to induce expression of target genes such as *c-fos,* yet the functional consequences of phosphorylation are subject to debate. Ser 133 is located within a 60-residue stretch of CREB known as the kinase-inducible domain or KID, which is both necessary and sufficient for activation of CREB (Gonzalez *et al.*, 1991).

1.2.12 CREB-mediated transcription

Following its phosphorylation, CREB associates with another nuclear protein, CREB-binding protein (CBP), through a region of the CBP known as the KID interaction domain (Chrivia *et al.*, 1993). It has been suggested that CBP acts as a molecular bridge, allowing upstream transcription factors, such as CREB, to recruit and stabilise RNA polymerase II (Nakajima *et al.*, 1997). In addition, CBP possesses intrinsic histone acetyltransferase (HAT) activity, which makes the DNA template more accessible to transcriptional machinery (Strühl *et al.*, 1998). Transcription of genes by RNA

polymerase II requires assembly of general transcription factors and coactivators around the transcription start site in the gene's promoter (Orphanides *et al.*, 1996). The general transcription factors are necessary for accurate and optimal positioning of RNA polymerase II at the transcriptional start site, facilitating the synthesis of an mRNA transcript (Orphanides *et al.*, 1996). One particular immediate early gene, *c-fos*, is induced by NGF stimulation through a CREB-mediated mechanism (Ginty *et al.*, 1994). Halegoua and coworkers (1996) have shown that certain immediate early genes (IEGs) stimulated by neurotrophins regulate the expression of late response genes (LRG), which encode proteins such as voltage-operated ion channels, neurotransmitter receptors and certain enzymes necessary for the synthesis of neurotransmitters (Dichter *et al.*, 1977; Garber *et al.*, 1989; Fanger *et al.*, 1995; Toledo-Aral *et al.*, 1995).

1.3 Glutamate

1.3.1 Glutamate as a transmitter

Glutamate is the dominant excitatory neurotransmitter at central synapses. To be classified as a neurotransmitter, a substance must not normally be present in the synaptic cleft but released upon depolarisation. It must be released from a presynaptic site in response to a stimulus above a threshold level. Enzymes necessary for its synthesis must be present in the presynaptic terminal. Following its release, the transmitter must be cleared from the synaptic cleft following release by enzymatic degradation or reuptake. It must diffuse across the synaptic cleft and exert an action at the postsynaptic membrane by binding with specific receptors. Glutamate fulfils all these criteria and is therefore classified as a neurotransmitter. The role of glutamate as a neurotransmitter was first described in response to stimulation at spinal synapses. Glutamate is present in high concentrations in the brain (Fonnum *et al.*, 1984) and the hippocampus is one of the areas of highest concentration (Storm-Mathisen and Ottersen, 1987). Two separate compartments of glutamate are observed in neurons; namely a cytosolic pool and a readily releasable pool. The readily releasable pool is stored in vesicles (Maycox *et al.*, 1988) and release of transmitter from these vesicles is reported to be calcium-dependent

(Sandoval *et al.*, 1978). Uptake of glutamate into vesicles relies on a transporter relies on the activity of a proton pump (Maycox *et al.*, 1988). Glutamate uptake carriers, embedded in the cell membrane, are responsible for maintaining extracellular glutamate levels at approximately 1μM. Uptake of glutamate from the synaptic cleft, mediated by sodium-glutamate co-transport, plays as vital role in preventing excitotoxicity by removing excess glutamate from the synaptic cleft (McBean and Roberts, 1985).

Several observations led to the conclusion that glutamate is the dominant excitatory neurotransmitter in the hippocampus. Application of exogenous glutamate stimulated excitatory responses recorded from the hippocampus (Dudar *et al.*, 1974) and further study identified glutamate release from hippocampal neurons as a stimulus-dependent and calcium-dependent process (Nadler *et al.*, 1976). Identification of a presynaptic site of release was reported from experiments investigating glutamate release from hippocampal synaptosomes (Sandoval *et al.*, 1978). In addition, Sandoval and coworkers (1978) also identified uptake systems to clear glutamate from the synaptic cleft following release. Furthermore, lesions to the perforant path resulted in a decrease in glutamate release in the dentate gyrus (Nadler and Smith, 1981). The role of glutamate and glutamate receptors in the expression of LTP is discussed in Section 1.5.5.

1.3.2 Glutamate receptors

Glutamate receptors are broadly categorised as either ionotropic or metabotropic receptors. Glutamate elicits rapid synaptic responses by binding to ionotropic receptors and slower effects through metabotropic receptors. Ionotropic receptors, all of which have a hetero-oligomeric structure, are subdivided into *N*-methyl-*D*-aspartate (NMDA) receptors, kainate and γ-amino-3-hydroxy-5-methyl-5-isoxazole propionic acid (AMPA) receptors. The best studied glutamate receptor subtype is the NMDA receptor. The NMDA receptor consists of one NR1 subunit and four NR2 subunits, designated A, B, C and D, which contain the glutamate-binding domain (Hollmann and Heinemann, 1994). NMDA receptors are selectively antagonised by *D*-2-amino-5-phosphonopentanoic acid (APV; Mayer and Westbrook, 1987). In addition to ligand binding, the NMDA receptor is subject to a unique voltage-dependent blockade by Mg^{2+}. In this manner, the NMDA receptor can function as a 'coincident detector', activated by glutamate binding

simultaneously with membrane depolarisation (Hollmann and Heinemann, 1994). Influx of both Na^+ and Ca^{2+} is permitted by the NMDA receptor-associated channel. The major consequence of NMDA receptor activation is calcium influx (Collingridge and Lester, 1989), which leads to increased cytosolic calcium levels and thence activates several calcium-sensitive enzymes such as CaM kinase II (Omkumar *et al.*, 1996), protein kinase A (Raman *et al.*, 1996), protein kinase C (Chen and Huang, 1992; Tingliey *et al.*, 1993) and src (Yu *et al.*, 1997). It has been reported that NMDA receptor-regulated calcium influx stimulates activation of the Ras/Raf/ERK pathway (Bading and Greenberg; 1991; Kurino et al., 1995). This relies on the activation of PSD-95, a large protein that resides in the postsynaptic density as a macromolecular complex with NMDA receptors (Husi and Grant, 2001). In turn, PSD-95 associates with Syn-GAP, which activates ras (Kim *et al.*, 1998; Chen *et al.*, 1998). CaM kinase inhibits Syn-GAP by phosphorylation (Chen *et al.*, 1998). Src and fyn, non-receptor tyrosine kinases are associated with the cytosolic domain of the receptor and it has been reported that src- and fyn-mediated tyrosine phosphorylation of the 2B subunit potentiates channel opening (Wang and Salter, 1994; Kohr and Seeburg, 1996). In addition, it has been shown that NMDA receptor activity is modulated by BDNF, through a tyrosine kinase mediated pathway (Levine *et al.*, 1998). NMDA receptor activity is a prominent feature of long-term potentiation in the hippocampus and is discussed in detail in Section 1.5.5.

AMPA receptors are composed of a combination of four subunits, Glu1, 2, 3 and 4 (Hollman and Heinemann, 1994). The cation channels associated with AMPA receptors selectively permit Na^+, but not Ca^{2+} influx and therefore, contributes to membrane depolarisation. One notable feature of AMPA receptors is that they undergo rapid clathrin-dependent endocytosis (O'Brien *et al.*, 1998; Carroll *et al.*, 1999). Internalised AMPA receptors may then be reinserted into the membrane (Ehlers *et al.*, 2000). It has been proposed that this mechanism of recycling and reinsertion of AMPA receptors may regulate distribution of AMPA receptors and may play a significant role in synaptic plasticity by functioning at 'silent synapses' as discussed in Section 1.5.9. Kainate receptors have the lowest affinity for glutamate of ionotropic glutamate receptors (Hollman and Heinemann, 1994) and desensitize strongly in the presence of glutamate or

kainate (Huettner, 1990). Calcium permeability of kainate receptors is similar to that of AMPA receptors (Kohler *et al.*, 1993).

Metabotropic glutamate receptors (mGluR) exert their effects by stimulation of receptor-coupled GTP-binding proteins (G-proteins). To date, eight mGluR subtypes, designated mGluR 1 to mGluR 8 (Pin and Duvosin, 1995), have been isolated in the mammalian brain. One subgroup of mGluRs, group I mGluRs, which are primarily located postsynaptically (Shigemoto *et al.*, 1997), are involved in reciprocal interactions with NMDA receptors. Low concentrations of NMDA can enhance group I mGluR-mediated responses to NMDA receptor activation in hippocampal neurons, through a positive feedback mechanism (Fitzjohn *et al.*, 1996). In addition, group I mGluRs can potentiate NMDA-induced excitotoxicity (Bruno *et al.*, 1995) or neurodegeneration (Schoepp *et al.*, 1995). The principal downstream messenger of group I mGluRs is IP_3, generated by phosphoinositol hydrolysis, which gives rise to increases in cytosolic calcium (Nakanishi, 1992).

1.3.3 Proteins involved in glutamate release

Glutamate is stored in synaptic vesicles in the presynaptic terminal and is rapidly released by exocytosis in response to depolarisation of the presynaptic membrane. This process relies on a complex series of intracellular events involving several proteins embedded in the synaptic vesicle membrane. The complex nature of this process confers specificity on depolarisation-induced glutamate release, by ensuring that only adequate stimuli evoke the appropriate response. Exocytosis of synaptic vesicles is mediated by a set of proteins known as soluble *N*-ethylmaleimide-sensitive factor protein (SNAP) receptors, referred to as SNAREs (Söllner *et al.*, 1993). Evidence to support a role for these proteins in exocytosis is provided by work using neurotoxins produced by Clostridia. These neurotoxins block exocytosis and disrupt SNAREs by selectively proteolyzing the individual SNARE proteins (Niemann *et al.*, 1994). SNAREs are classified into two groups; vesicle membrane-associated proteins, v-SNAREs, which include synaptobrevin or (vesicle-associated membrane protein, VAMP), synaptogamin, synpatophysin, synapsin I and rab3A, localised in the synaptic vesicle membrane, and target membrane-associated t-SNARE, such as syntaxin I and SNAP-25, localised on the

cytosolic face of the plasma membrane. It has been reported that the SNARE complex is responsible for tight binding of the vesicle to the plasma membrane, facilitating the large energy demand required for fusion (Hanson *et al.*, 1997). SNARE complexes are formed by coiled-coil interactions of the α-helices of syntaxin, SNAP-25 and synaptobrevin immediately before fusion (Chapman *et al.*, 1995). Synaptotagmin has been proposed to function as a candidate calcium detector (Chen *et al.*, 1999b), which is responsible for rapid calcium-triggered changes in synaptic vesicle conformation, facilitating exocytosis.

A tyrosine kinase substrate, synaptophysin, bind to synaptobrevin and is thought to mediate pore formation (Thomas and Betz, 1990). Synapsin I is a protein peripherally associated with the synaptic vesicle (Greengard *et al.*, 1993) and phosphorylation of synapsin I by ERK (Jonanovic *et al.*, 1996) or CaM Kinase II (Greengard *et al.*, 1993) is thought to allow mobilisation of synaptic vesicles from the actin meshwork to the active site. Mobilisation of vesicles is followed by vesicle docking, which is controlled, at least in part, by rab3A, an abundant G-protein. It has been suggested that rab3A is a negative regulator of neurotransmitter release because it must in the GTP-bound form to permit exocytosis (Geppart *et al.*, 1994; 1997). Syntaxin interacts with SNAP-25, synaptobrevin, MUNC 18 and voltage-operated calcium channels (Schiavo *et al.*, 1994) but its tight binding to SNAP-25 forms a high affinity binding site for synaptobrevin. Cytoplasmic proteins, *N*-ethyl-maleimide-sensitive fusion protein or NSF and Munc 18 interacts with SNARE proteins. NSF, an ATPase, induces dissociation of the SNARE complex, possibly as an initial step in mediating other protein-protein interactions, involving the SNARE complex interactions, preceding membrane fusion (Matveeva *et al.*, 2001), while Munc 18 binds tightly to syntaxin, thereby impeding SNARE complex formation (Pevsner *et al.*, 1994).

1.3.4 Role of calcium

A prominent feature of neurotransmitter release is its dependence on calcium. Several lines of evidence have led to this observation. Firstly, injection of fast-acting calcium chelators, such as BAPTA, into the presynaptic terminal blocks release (Adler *et al.*, 1991). Secondly, measurements of intracellular calcium concentrations indicates very

high calcium concentration during release (Llinas *et al.*, 1992) and action potentials, the stimulus for glutamate release, cause rapid elevations in calcium levels in presynaptic terminals (Mulkey and Zucker, 1991; Yazejian *et al.*, 2000). Thirdly, the close apposition of voltage-operated calcium channels and synaptic vesicles indicates that depolarisation-induced calcium influx via voltage-operated calcium channels impacts on glutamate release (Rettig *et al.*, 1996). There is no consensus on the identity of the calcium-sensitive protein responsible for triggering glutamate release but a likely candidate is synaptotagmin, which can bind several Ca^{2+} ions (Shao *et al.*, 1998; Sutton *et al.*, 1999). It is thought that calcium-bound synaptotagmin participates in membrane changes that underlie exocytosis (Davis *et al.*, 1999). In addition, calcium binding to synaptotagmin may alter its interaction with SNARE proteins, particularly SNAP-25 (Schiaro *et al.*, 1997; Gerona *et al.*, 2000) but the functional outcome of this interaction is not known.

1.3.5 Acute effects of neurotrophins on neurotransmission

The idea of fast-acting effects of neurotrophins is in contrast to the traditional view of characteristically slow actions and long-lasting effects. Initial evidence suggesting that neurotrophins may act neuromodulators was based on observations of synaptic transmission at the neuromuscular junction (Lohof *et al.*, 1993). Exposure to NT-3 and BDNF, but not NGF, resulted in rapid enhancement of synaptic efficacy mediated by a presynaptic mechanism involving Trk receptor activation. Additional research uncovered two major effects of neurotrophins at the neuromuscular synapses; acute modulation of synaptic transmission (Stoop and Poo, 1996; Wang and Poo, 1997, Xie *et al.*, 1997) and long-term regulation of synapse maturation (Liou and Fu, 1997).

Manipulation of calcium influx in response to stimulation by neurotrophins in cultured forebrain neurons (Levine *et al.*, 1995), trk-transfected 3T3 cells (Jiang *et al.*, 1999) and hippocampal synaptosomes (Maguire *et al.*, 1999) points to a putative role in modulating glutamate release. Knipper and coworkers (1994) were the first to suggest acute effects of neurotrophins at central synapses. In their study, acute application of NGF was shown to stimulate glutamatergic transmission in hippocampal neurons. It was proposed that the enhancement was mediated by Trk receptor activation, culminating in the formation of IP_3 subsequent to activation of PLCγ. This early report also found a

direct effect of NGF on the synaptic vesicle protein, synapsin I, providing a putative mechanism by which neurotrophins could alter synaptic vesicle dynamics. Further analysis investigated the effects of BDNF and NT-4 on glutamatergic transmission in the hippocampus (Leßman *et al.*, 1994). The mechanism of modulation by BDNF and NT-4 appeared to be presynaptic in origin, correlating with the findings of Knipper and coworkers (1994).

The presynaptic nature of the modulation of neurotrophin-induced enhancement of glutamate release is further supported by recent studies (Teyler and Pozzo-Miller, 2001; Pozzo-Miller *et al.*, 1999). Electromicroscopy studies (Pozzo-Miller *et al.*, 1999) show a significant reduction in docked vesicles in the active zone of presynaptic terminals of BDNF knockout mice. A decrease in expression of synaptophysin and synaptobrevin (VAMP-2) was detected in these mutants and this may correlate with aberrant fusion and docking of vesicles. Delivery of exogenous BDNF reversed these effects by facilitated mobilisation and docking of vesicles. This indicates that the absence of BDNF causes dysfunction in the exocytotic process and is not a developmental consequence of BDNF gene knockout. Additional analysis supports a role for BDNF in ERK-induced phosphorylation of synapsin I (Jonanovic *et al.*, 1996). Postsynaptic mechanisms of enhancement of excitatory synaptic transmission have also been proposed. Levine and coworkers (1995) suggested a postsynaptic locus of neurotrophin-induced potentiation and reported BDNF-induced enhancement of synaptic transmission in hippocampal neurons and proposed that the effect was governed by postsynaptically-located tyrosine kinases. The effect was not replicated by exposure to NGF or NT-3. Further work by the same group demonstrated increased NMDA receptor activity in response to BDNF treatment (1998), thereby augmenting the responsiveness of the postsynaptic cell. This is most likely mediated by BDNF-induced phosphorylation of NMDA receptor subunits (Suen *et al.*, 1997). In addition, Li and coworkers (1998) used a dominant negative truncated TrkB receptor expressed exclusively in either pre- or post-synaptic membranes to demonstrate that transient potentiation induced by BDNF at developing synapses results from a presynaptic action. Furthermore, Schinder and coworkers (2001) demonstrated that neurotrophins derived from postsynaptic targets acted locally at glutamatergic synapses in the hippocampus to stimulate presynaptic

glutamate release. Neurotrophins acting in this manner as retrograde messengers can selectively reinforce presynaptic changes.

While a wealth of evidence exists to support a role for BDNF in the modulation of synaptic transmission, indications of a role for NT-3 are less well established and are derived from studies investigating the effects of NT-3 on synaptic transmission at peripheral synapses (Yang *et al.*, 2000). The first report of a modulatory function of NT-3 at central synapses indicated that NT-3 potentiates excitatory synaptic responses in cortical neurons (Kim *et al.*, 1994). Kang and Schuman (1995, 1996) have repeatedly reported NT-3-mediated enhancement of excitatory transmission at CA1 synapses in the hippocampus. Two groups of researchers (Kokaia *et al.*, 1998; Asztely *et al.*, 2000) have reported impaired synaptic transmission at the perforant path granule cell synapses of the dentate gyrus using electrophysiological techniques but to date, there is little evidence of a direct effect of NT-3 on the mechanism responsible for glutamate release.

1.4 Calcium

Calcium is cited as the most important and ubiquitous second messenger. Its levels must be carefully regulated as large increases in cytosolic calcium concentrations can trigger activation of proteases and precipitate cell death. Calcium influx into the cytosol is regulated by three mechanisms; the NMDA receptor channels and voltage operated calcium channels in the cell membrane and IP_3-gated receptors in the endoplasmic reticulum membrane. Calcium-induced neurodegeneration is thought to be involved in the aetiology of Alzheimer's disease (Annaert and De Stooper, 1999) and in neuronal ageing (Verhratsky and Toescu, 1998). On the other hand, calcium is involved in the mechanisms supporting morphogenesis (Gomez and Spitzer, 1999) and synaptic plasticity such as LTP. The role of calcium in glutamate release is discussed in Section 1.3.4, and in the induction of LTP is described in Section 1.5.6.

1.4.1 Calcium as a second messenger

Targets of calcium are calcium-activated protein kinases, Ca^{2+}/calmodulin dependent protein kinase II and protein kinase C. The consequences of activation of both

kinases are described in Section 1.5.7. In addition to stimulation of kinases in the cytosol, calcium is a very important stimulus for transcription. Calcium influx, mediated by NMDA receptor activation, culminates in transcription of the IEG, *c-fos* through an ERK-dependent cascade (Xia *et al.*, 1996). In addition, calcium regulates CREB activation indirectly through activation of calcineurin, a phosphatase, which in turn activates nuclear protein phosphatase I (Bito *et al.*, 1996). Through these mechanisms calcium can alter protein synthesis.

1.4.2 Voltage-operated calcium channels

Three types of high-voltage operated calcium channels (VOCC) have been isolated in neurons. N-type, P-type and Q-type mediate calcium influx with different rates of voltage-dependent inactivation, determined by their subunit composition. VOCCs are distinguished on the basis of pharmacological properties. N-type VOCCs are selectively inhibited by ω-conotoxin GVIA, P-type VOCCs are specifically blocked by ω-agaotoxin IVA and Q-type VOCCs are inhibited by ω-conotoxin MVIIC (Catterall, 1991). VOCCs are heterodimeric complexes and consist of a membrane anchored α_1-subunit, a largely extracellular α_2-δ subunit and a cytosolic β-subunit (Walker and DeWaard, 1998).

Activity of VOCC can be regulated by serine/threonine kinases such as PKC and PKA (Hell *et al.*, 1995), G-proteins (Dolphin and Scott, 1989a) and cyclic nucleotides (Dolphin and Scott, 1989b). Recent evidence suggests a modulatory role for tyrosine kinase in calcium channel regulation in smooth muscle (Wijetunge *et al.*, 2000) and dorsal root ganglion neurons (Fitzgerald and Dolphin, 1997). The most probable substrate for tyrosine phosphorylation is the α_1 subunit of VOCCs, which determines the properties of the calcium channel (Hofmann *et al.*, 1994, McCleskey *et al.*, 1994, Walker and DeWaard, 1998) and forms the pore though which calcium ions enter the cell (Catterall, 1991, Walker and DeWaard, 1998). It is therefore likely that phosphorylation would alter channel kinetics and thereby affect calcium influx into the presynaptic nerve terminal and influence glutamate release. Tyrosine kinase inhibitors, genistein and tyrphostin B42 have been shown to block high-voltage activated calcium currents in hippocampal neurons (Potier and Rovira, 1999) and this may provide a tyrosine kinase-regulated mechanism of calcium influx, which has an impact on LTP. Recently, it has been

reported that neurotrophins selectively upregulated the synthesis of VOCCs in hippocampal neurons (Baldelli *et al.*, 2000) and it has previously been reported that NGF enhances calcium influx (Jiang *et al.*, 1999), indicating possible mechanisms by which neurotrophins regulate VOCC activity. Entry of calcium into the presynaptic terminal through VOCCs is the primary stimulus for neurotransmitter release (Augustine *et al.*, 1987). An interaction between VOCCs and SNARE proteins has been suggested (Rettig *et al.*, 1996), demonstrating a major role for calcium and VOCC activity in glutamate release as described in Section 1.3.4.

1.5 Long-term potentiation

1.5.1 Background

Long-term potentiation (LTP), the most studied form of synaptic plasticity, was first characterised by Bliss and Lømo (1973). Delivery of brief high-frequency trains of activation of the perforant pathway, originating in the entorhinal cortex, to the dentate gyrus of rabbits produced a long-lasting selective increase in the synaptic efficacy, indicated by an increase in the slope of the rising phase of the excitatory postsynaptic potential (epsp), depicted in Figure 1.2. Later work led to the characterisation of LTP at the Schaffer collateral-commissural pathway synapses in the CA1 region and in the CA3 region of the hippocampus (Bliss and Collingridge, 1993). The most prominent difference between the signalling mechanisms underlying the induction of LTP in the hippocampal regions is the involvement of the NMDA receptor. Activation of the NMDA receptors is crucial for the successful induction of LTP in response to tetanic stimulation in the dentate gyrus and the CA1 region, whereas LTP in the CA3 region is NMDA-independent (Harris and Cotman, 1986).

1.5.2 Properties

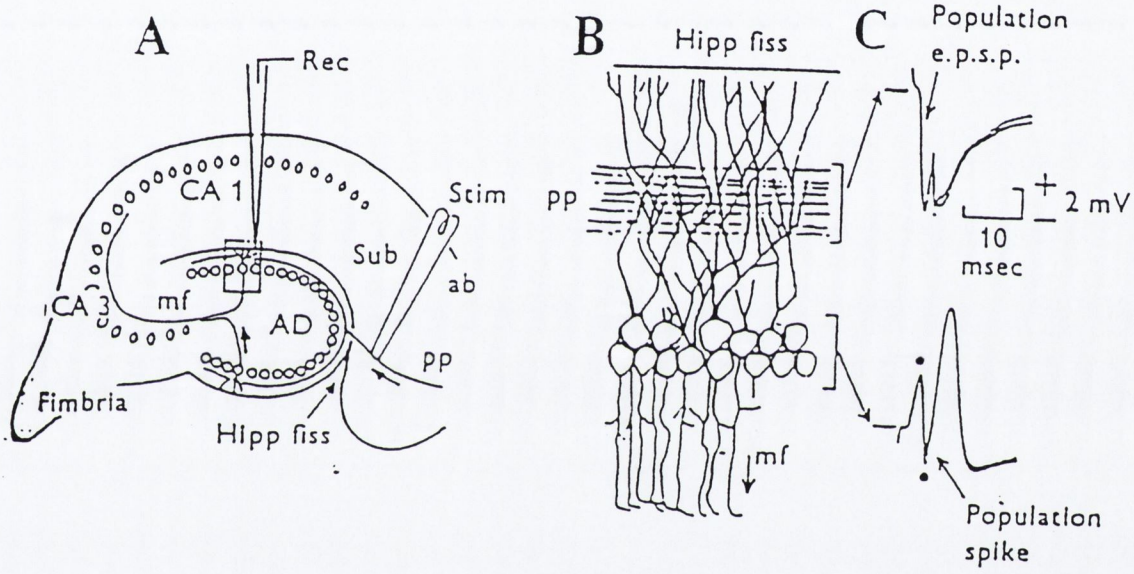
Hippocampal LTP displays three fundamental properties, namely cooperativity, associativity and input-specificity. Cooperativity describes the existence of a threshold for induction. This means that weak tetani stimulating few afferent fibres do not induce

Figure 1.2 A diagrammatic parasagittal section through the hippocampal formation

A. This diagrammatic section through the hippocampus shows a stimulating electrode placed beneath the angular bundle (ab) to activate the perforant path fibres (pp) and a recording electrode in the molecular layer of the dentate area (AD).

B. The region enclosed in the rectangle in (A) is enlarged to show the apical dendritic field of the granule cells, with the perforant path confined to the central one third of the field.

C. The population responses evoked by a strong perforant path volley in the synaptic layer (upper trace) and in the cell body layer (lower trace) are displayed. The spots (upper trace) mark the peaks between which the amplitude of the population spike are measured (From Bliss and Lømo, 1973)


A
Rec
CA 1
Stim
Sub
ab
CA 3
mf
AD
pp
Fimbria
Hipp fiss
B
Hipp fiss
pp
mf
C
Population
e.p.s.p.
+
−
2 mV
10
msec
Population
spike

LTP. This threshold relates to the intensity and the pattern of tetanic stimulation delivery to the afferent input and therefore may rely on cooperativity of several inputs converging on the postsynaptic site (McNaughton *et al.*, 1978). The property of cooperativity stems mainly from the voltage-dependent blockade of the NMDA receptor. Weak stimuli fail to expel the Mg^{2+} from the NMDA receptor cation channel. Associativity refers to the finding that weak inputs, which do not meet the threshold intensity, may lead to potentiation of the synaptic response if spatially and temporally paired with a separate, stronger stimulus, converging on the same postsynaptic site (Levy and Steward, 1979; McNaughton *et al.*, 1978). Finally, LTP is input-specific because other inputs inactive at the time of tetanic stimulation are not potentiated (Andersen *et al.*, 1977; Lynch *et al.*, 1977). The properties of LTP are explained on the assumption that a synapse will only be potentiated if it is active when the dendrite on which it terminates is sufficiently depolarised.

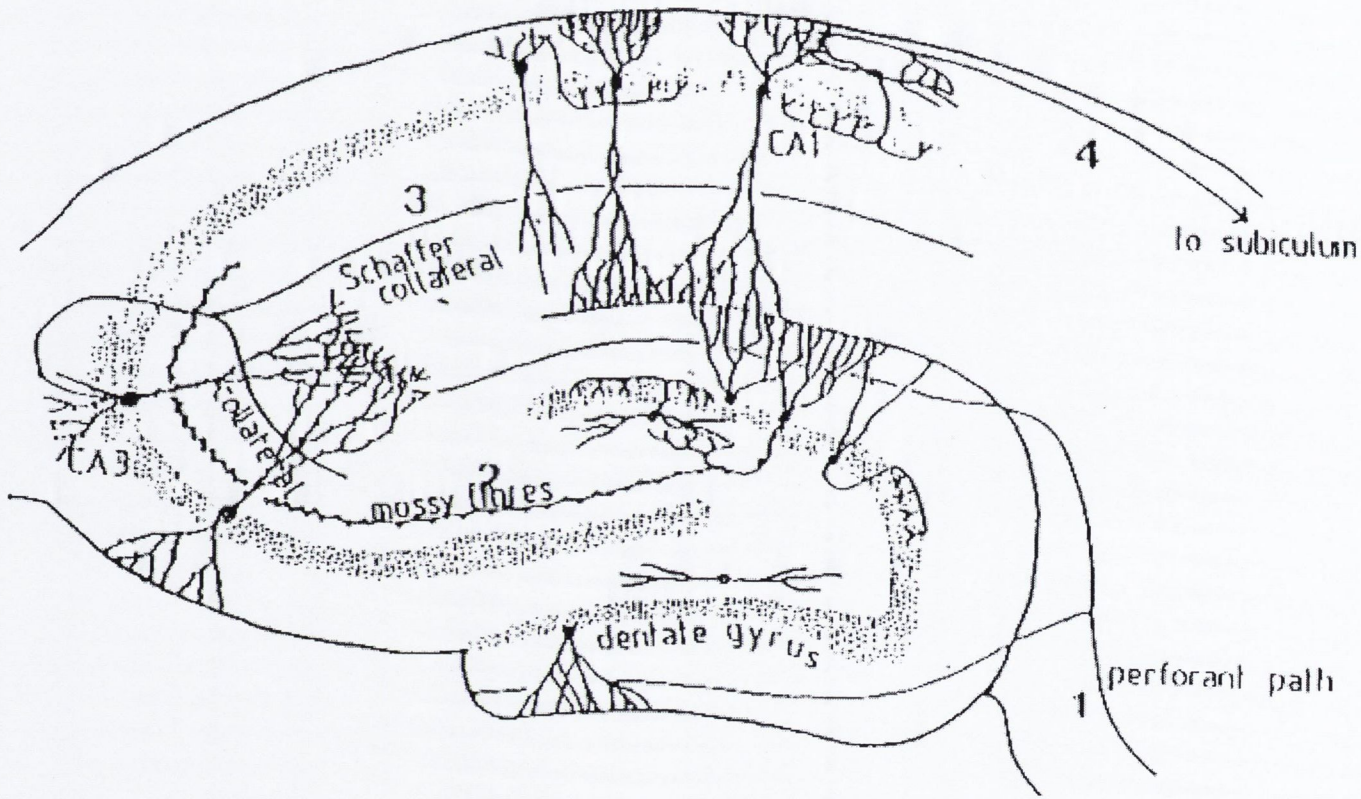
1.5.3 Hippocampus

The hippocampus, a region of the archicortex, consists of two C-shaped structures. Within the hippocampus, four areas have been identified, namely the hippocampus proper, consisting of CA1, CA2 and CA3 regions, the subicular complex, the dentate gyrus and the entorhinal cortex. The dentate gyrus acts as a gate for the passage of neuronal activity from the entorhinal cortex into the hippocampus. Three major excitatory pathways have been identified in the hippocampus, namely the perforant path, the mossy fibre pathway and the Schaffer collaterals, and together, these pathways form a 'trisynaptic-loop' (Anderson, 1987), shown in Figure 1.3. The perforant path projects from layer II stellate cells of the entorhinal cortex (Steward and Scoville, 1976) and terminates on the granule cell dendrites (Hjorth-Simonsen and Jeune, 1972).

Research on the nature of memory focuses on the hippocampus, based largely on the finding that patients with hippocampal lesions show impaired learning and memory. The first indication of hippocampal involvement in such processes was impaired memory formation observed in a patient known as H.M. following bilateral removal of the hippocampus as a treatment for epilepsy. Further study demonstrated that disruption of activity of the hippocampus or some surrounding cortical structures that provide input to

Figure 1.3 The anatomy of the hippocampus

A transverse section through the hippocampus of the rat, indicating inputs from the entorhinal cortex through the perforant path (1). The perforant path synapses with the granule cells of the dentate gyrus and with the apical dendrites of the CA3 pyramidal cells. The granule cells of the dentate gyrus project to the CA3 pyramidal cells via the mossy fibres (2). The CA3 pyramidal cells project via the Schaffer collaterals to the CA1 area (3). CA1 pyramidal cells form an input to the subiculum (4).


CA1
4
3
to subiculum
Schaffer collateral
CA3
collateral
mossy fibres
2
dentate gyrus
perforant path
1

the hippocampus, resulted in a profound inability to form new memories while other cognitative skills and previously formed memories remained intact (Squire and Zola-Morgan, 1991).

1.5.4 Induction of LTP

The most commonly used protocol for the induction of LTP in the hippocampus is by the delivery of high-frequency stimulation to the afferent input to the area of interest. In this study, three trains of tetanic stimuli at 250Hz for 200ms at an intertrain interval of 30s were used. Other methods include electrical and pharmacological induction of LTP. Induction of LTP may also occur following 'theta burst stimulation' or 'primed burst stimulation'. Theta burst stimulation consists of four shocks at 100Hz delivered at an interburst interval of 200ms (Larson *et al.*, 1986), while primed burst stimulation uses a single priming stimulus followed at 200ms by a single burst of four shocks at 100Hz (Rose and Dunwiddie, 1986). The primary pharmalogical agent used to induce LTP is tetraethyl ammonium (TEA), a K^+ channel blocker (Anikszejn and Ben-Ari, 1991; Huang and Malenka, 1993).

1.5.5 Glutamate receptors and the induction of LTP

The first evidence of the involvement of ionotropic glutamate receptors in the induction of LTP was reported by Collingridge and coworkers (1983). In their study, the application of the NMDA receptor antagonist, D-amino phosphonovalerate (APV) blocked the induction of LTP in the CA1 region, but when applied following the induction of LTP, APV had no effect. Subsequent research led to the observation that NMDA receptor activation is necessary for the induction of LTP at the perforant path-granule cell synapses (Errington *et al.*, 1987; Morris *et al.*, 1986), but not for the induction of LTP at the mossy fibre-CA3 synapses, which is NMDA-independent (Harris and Cotman, 1986). The key property of the NMDA receptor in relation to its involvement in LTP is its voltage-dependent ability to regulate calcium influx into the postsynaptic cell (MacDermott *et al.*, 1986). Calcium influx, resulting from NMDA receptor activation by simultaneous glutamate binding and depolarisation, may provide the crucial trigger for intracellular cascades to propagate transient synaptic events by

stimulating protein kinases such as CaM kinase II and protein kinase C, as discussed in Section 1.5.7.

In addition to NMDA receptors, it is reported that mGluRs participate in the induction of LTP in the hippocampus. This was prompted by the finding that mGluR antagonists, 2-amino-3-phosphonopropionate (AP3) and 2-amino-4-phosphono-butanoate (AP4), led to a reduction of the duration of LTP (Izumi *et al.*, 1991; Reymann and Matthies, 1989). Furthermore, application of the selective mGluR agonist, ACPD, augments tetanus-induced LTP (McGuinness *et al.*, 1991) and enables low-frequency stimuli to induce LTP (Radpour *et al.*, 1992). Other evidence to support a role for mGluRs in the induction of LTP is the finding that (R, S)-alpha-methyl-4-carboxyphenylglycine (MCPG) inhibits the induction of LTP in both the CA1 (Bortolotto *et al.*, 1994) and the dentate gyrus (Riedel and Reymann, 1993).

1.5.6 Calcium and the induction of LTP

Elevation of calcium concentrations postsynaptically is essential for the induction of LTP, possibly due to a requirement for the activation of calcium-dependent protein kinases such as protein kinase C and Ca^{2+}/calmodulin protein kinase II, which subserve the expression of LTP, as outlined in Section 1.5.7. The first evidence that calcium was important in the induction of LTP was the finding that application of calcium is sufficient to potentiate synaptic transmission in the CA1 region (Turner *et al.*, 1982). Subsequently, Lynch and coworkers demonstrated the pivotal importance of calcium in the induction phase of LTP (1983). In their early study, injection of the calcium chelator, ethylene glycol bis (β-aminoethylether) N,N' tetraacetic acid (EGTA), into the postsynaptic cell suppressed the induction of LTP. Other studies investigated temporal aspects of the postsynaptic calcium transient. Using photo-activated calcium chelators, it was demonstrated that LTP is induced if the calcium signal is restricted to 3s (Malenka *et al.*, 1992). The major route of calcium influx into the postsynaptic cell during the induction of LTP is the NMDA receptor channel, resulting in rapid and substantial increases in cytosolic calcium levels however, it is possible that calcium from other sources may augment the NMDA receptor-mediated calcium influx. Evidence exists that mobilisation of calcium from intracellular stores participates in the biochemical events underlying the

induction of LTP because application of thapsigargin, which depletes intracellular calcium stores, or dantrolene, which inhibits ryanodine receptors, inhibit LTP (Alford *et al.*, 1992; Bortolotto *et al.*, 1993; Obenaus *et al.*, 19989; Harvey and Collingridge, 1992). In addition, voltage-operated calcium channels participate in the induction of LTP because inhibitors of VOCCs block LTP induction (Grover and Teyler, 1990) but the magnitude of the contribution of VOCCs to LTP is unknown. In addition, Çavus and Teyler (1996) proposed that VOCCs and NMDA receptors underlie two forms of LTP in the hippocampus. These forms of LTP are induced by different stimulation paradigms and did not facilitate or reduce each other's expression. VOCC-mediated LTP was sensitive to tyrosine kinase inhibition but was NMDA antagonist, APV-resistant. Further study confirmed the existence of VOCC-mediated LTP (Morgan and Teyler, 1999).

1.5.7 Protein phosphorylation and the maintenance of LTP

Several protein kinases appear to play a role in the expression of LTP. These include serine/threonine kinases, such as CaM kinase II, protein kinase C, protein kinase A, tyrosine kinases and ERK. Activation of protein kinases may serve to convert the initial synaptic events into long-lasting changes (Recasens, 1995).

1.5.7.1 Calcium/calmodulin-dependent protein kinase II

Calcium/calmodulin-dependent protein kinase II (CaM kinase II) holoenzyme is an oligomer comprising two catalytic subunits, an α-subunit and a β-subunit (Bennett *et al.*, 1983). It is expressed at particularly high levels in the hippocampus where it constitutes 2% of the total protein (Erondu and Kennedy, 1985). Within the hippocampus, it is mainly localised at postsynaptic densities of glutamatergic synapses (Kennedy *et al.*, 1998). CaM kinase II becomes autophosphorylated following activation by Ca^{2+}/calmodulin and the autophosphorylated form remains active in the absence of calcium until it is dephosphorylated by phosphatases (Miller and Kennedy, 1986; Miller *et al.*, 1988). Studies of targeted gene knockouts revealed that LTP is impaired in the absence of CaMK II (Silva *et al.*, 1992); similarly, deficits in spatial learning have also been observed in these animals (Silva *et al.*, 1992). Pharmacological inhibitors of CaM

kinase II were reported to block the induction of LTP in the hippocampus (Malenka *et al.*, 1989; Malinow *et al.*, 1989) while increased activity of calcium-independent CaM kinase II was demonstrated following the delivery of tetanus (Fukunaga *et al.*, 1993, 1995). In addition, application of AP5, an NMDA-receptor agonist abolished tetanus-induced increases in CaM kinase II (Fukunaga *et al.*, 1992). Possible substrates of CaM kinase II are synaptotagmin, rab-3 and synapsin I (Takahashi *et al.*, 1991; Fyske *et al.*, 1994; Fukunaga *et al.*, 1995), constituent proteins of the synaptic vesicle membrane and postsynaptically located AMPA receptors (Tan *et al.*, 1994). Thus, CaM kinase II may contribute to biochemical events underlying LTP.

1.5.7.2 Protein Kinase C

Several lines of evidence point to the involvement of protein kinase C (PKC) in LTP. Delivery of high-frequency stimulation activates PKC by promoting its translocation to the active membrane bound form (Akers *et al.*, 1986; Angenstein *et al.*, 1994). In addition, tetanus increases PKC activation (Klann *et al.*, 1991, 1993) and PKC inhibitors blocked the induction of LTP in the hippocampus (Lovinger *et al*, 1987). The mechanism of its contribution to the signalling cascades underlying LTP was further characterised by a report documenting synaptic potentiation following intracellular injection of the catalytic subunit of PKC (Hu *et al.*, 1987). This effect was replicated by administration of phorbol esters, which stimulate PKC (Malenka *et al.*, 1986). PKC exhibits different patterns of activation in the pre- and postsynaptic regions. Huang and coworkers (1992a) reported transient activation of the postsynaptic kinase while activation of the presynaptic kinase is prolonged. A major substrate of PKC is GAP-43, a synaptic vesicle protein, which shows increased phosphorylation following the expression of LTP (Linden *et al.*, 1988; Gianotti *et al.*, 1992). It has recently been suggested that PKC can override inhibition of LTP by AP5, bypassing the activity of NMDA receptors and indicating that PKC activation is a downstream consequence of NMDA receptor activation (Kleshevnikov and Routtenberg, 2001).

1.5.7.3 Phospholipase C

It has been suggested that phospholipase C (PLC) is involved in the signalling mechanisms underlying the expression of LTP in the dentate gyrus. Consequences of PLC activation include increased hydrolysis of phosphoinositol-bisphosphate, leading to the generation of two important second messengers, inositol trisphosphate, IP_3 and diacylglycerol, DAG. IP_3 stimulates release of calcium from intracellular stores while DAG activates PKC. Phosphorylation and thus, activation of PLCγ is promoted by delivery of LTP-inducing stimuli to the perforant path (Clements *et al.*, 1991; McGahon and Lynch, 1998) but this effect is blocked by tyrosine kinase inhibitor, genistein (McGahon and Lynch, 1998). In addition, it is has been shown that arachidonic acid or NGF applied in combination with the metabotropic glutamate receptor agonist, ACPD, results in increased glutamate release from synaptosomes prepared from the dentate gyrus (McGahon and Lynch, 1998; Kelly *et al.*, 1998; Kelly and Lynch, 1998). Both arachidonic acid and NGF have been demonstrated to stimulate PLCγ activity (McGahon and Lynch, 1998; Vetter *et al.*, 1991). This suggests a functional coupling between PLC activation and enhanced glutamate release and may provide a mechanism by which glutamate release may be augmented during the expression of LTP. It is possible that PLC modulates glutamate release by indirectly increasing intracellular calcium concentrations through the actions of IP_3 and DAG-activated PKC.

1.5.7.4 Protein Kinase A

Evidence supporting a role for protein kinase A or cAMP-dependent kinase (PKA) in LTP is limited. Initial work demonstrated a role for second messenger, cAMP in late phase LTP (Frey *et al.*, 1993), pointing to a role for PKA in the stabilisation of LTP by stimulating protein synthesis. Transient activation of PKA occurs following elevation of cAMP levels in a NMDA-receptor dependent manner (Chetkovich *et al.*, 1991). Conflicting results have been obtained from inhibitor studies. Inhibition of PKA blocks late phase LTP (Frey *et al.*, 1993; Matthies and Reymann, 1993), but the effects of PKA inhibitors on the early phase are disputed. Matthies and coworkers (1993) reported that PKA inhibitors failed to attenuate initial events in the expression of LTP but this is in

conflict with the findings of other groups (Frey *et al.*, 1993; Huang *et al.*, 1994). Injection of PKA inhibitors into the postsynaptic cell completely blocks both early and late phases of LTP (Blitzer *et al.*, 1995). Roberson and Sweatt (1996) characterised the mechanism of PKA activation during LTP as an NMDA-dependent event and reported transient activation of PKA following tetanus. A downstream target of PKA is CREB. It has recently been suggested that crosstalk between PKA and ERK modulates CREB activation (Impey *et al.*, 1998), providing an integrated signalling pathway underlying the expression of LTP. It has also been proposed that PKA may modulate the activity of voltage-operated calcium channels in hippocampal neurons (Hell *et al.*, 1995). PKA-dependent alteration of calcium influx may impact on glutamate release and thus, on LTP. More recently, Nguyen and Kandel (1996) reported the involvement of PKA in LTP in the dentate gyrus and suggested that activation of PKA stimulates protein synthesis.

1.5.7.5 Tyrosine kinases

Tyrosine kinases are broadly classified into two categories; non-receptor tyrosine kinases and receptor tyrosine kinases (Hunter and Cooper, 1985). As high levels of tyrosine kinase have been detected in the hippocampus (Pang *et al.*, 1988), it was speculated that tyrosine kinase may play a regulatory role in synaptic changes such as synaptogenesis (Lai *et al.*, 1994) and long-term potentiation (O'Dell *et al.*, 1991a; Abe and Saito, 1993; Maguire *et al.*, 1999). Early work indicated that the administration of tyrosine kinase inhibitors, genistein and lavendustin A, blocked the induction of LTP in the CA1 region (O'Dell *et al.*, 1991a) while application of herbimycin A and lavedustin A inhibit LTP at the perforant path - granule cell synapses (Abe and Saito, 1993).

In knockouts of c-fyn, a src-related tyrosine kinase, LTP is abolished (Grant *et al.*, 1992) but this deficit is reversed by postnatal expression of fyn transgene and therefore is not a development effect (Kojima *et al.*, 1997). Similarly, the expression of LTP is hampered if the activity of c-src, a non-receptor tyrosine kinase, is inhibited (Lu *et al.*, 1998). Mice expressing constitutively active fyn display enhanced LTP in response to high-frequency stimulation (Lu *et al.*, 1999a). In addition, learning tasks are impaired in fyn mutant mice (Grant *et al.*, 1992) and c-src mutant mice (Zhao *et al.*, 2000). The

functional outcome of activation of fyn and c-src following high frequency stimulation is association with focal adhesion kinase (FAK) and proline-rich calcium sensitive tyrosine kinase 2, (PYK2) in the CA1 region of the hippocampus (Lauri *et al.*, 2000). Increased phosphorylation of PYK2 was detected following high-frequency stimulation. This suggests that association with c-src activates PYK2, which in turn may activate ERK (Lev *et al.*, 1995; Dikic *et al.*, 1996) and contribute to the mechanisms underlying the expression of LTP.

Initial reports of the involvement of receptor tyrosine kinases in the generation of LTP came from studies of the involvement of epidermal growth factor (EGF) and fibroblast growth factor (FGF) in the hippocampus (Abe *et al.*, 1991, 1992; Ishiyama *et al.*, 1991). Subsequent studies reported participation of receptor tyrosine kinase in signalling mechanisms underlying LTP. Inhibition of the tyrosine receptor kinase, Trk receptors, by injection of tyrphostin AG879, completely blocks the expression of LTP in the dentate gyrus (Maguire *et al*, 1999), demonstrating the involvement of Trk-mediated signalling in the expression of LTP at these synapses. Implication of the activity of Trk receptors in LTP is further supported by accumulating evidence of the involvement of neurotrophins in the expression of LTP as discussed in Section 1.5.10.

The consequences of tyrosine phosphorylation may impact on processes in either the pre- or post- synaptic regions, indicated by the distribution of tyrosine kinase substrates on both sides of the synapse. In the presynaptic terminal, high levels of tyrosine kinase substrates are found in the synaptic vesicle membrane (Pang *et al.*, 1988). One synaptic vesicle protein in particular, synaptophysin, has been identified as a tyrosine kinase target (Pang *et al.*, 1988) and phosphorylation of synaptophysin is enhanced by LTP (Mullany and Lynch, 1998). Alternatively, Huang and Hsu (1999) propose a postsynaptic locus of tyrosine kinase activity. In their study, injection of tyrosine kinase inhibitors, genistein and lavendustin A into the postsynaptic cell blocked the induction of LTP. One possible target for tyrosine kinase involved in the expression of LTP is the 2B subunit of the NMDA receptor (Rosenblum *et al.*, 1995; Rostas *et al.*, 1996). Moreover, phosphophorylation of this subunit is rapid and sustained following the delivery of LTP-inducing tetani to the perforant path (Lau and Huangir, 1995). It is likely that tyrosine phosphorylation of the 2B subunit is mediated by src (Yu *et al.*, 1997).

Another candidate for tyrosine phosphorylation is ERK and as discussed in Section 1.5.7.6, ERK is necessary for the expression of LTP in the CA1 region and the dentate gyrus and its activation is a downstream consequence of Trk.

1.5.7.6 ERK and LTP

In addition to ERK phosphorylation in response to depolarisation (Baron *et al.*, 1996) and seizure (Kang *et al.*, 1994), activation of ERK has been demonstrated following the induction of LTP in the CA1 region of the hippocampus (English and Sweatt, 1996) and in the dentate gyrus (McGahon *et al.*, 1998; Maguire *et al.*, 1999). In addition, the MEK inhibitor PD98059 blocked the expression of LTP at CA1 region (Roberson and Sweatt, 1996) and at the perforant path – granule cell synapses in the dentate gyrus (McGahon *et al.*, 1998). LTP-associated increases in ERK activation were occluded in both studies, indicating a coupling of ERK activity and the mechanisms underlying the expression of LTP. Findings from behavioural studies support results from electrophysiological experiments. Enhancement of ERK activity in the hippocampus was observed following learning tasks, known to rely on hippocampal function. Contextual fear conditioning is inhibited by administration of MEK inhibitor, SL327 to the hippocampus (Selcher *et al.*, 1999). Moreover, this form of learning is associated with a rapid and transient increase in ERK phosphorylation following training, which is restored to baseline levels after 2 hours, indicating a possible role of ERK in transducing synaptic activity in to long-lasting effects (Selcher *et al.*, 1999). Likewise, ability to execute the Morris water maze test, a task known to rely on the formation of spatial memory, is impaired by intrahippocampal injection of MEK inhibitors, PD98059 and SL327 (Blum *et al.*, 1999; Selcher *et al.*, 1999). Results of these behavioural studies support the hypothesis that ERK activation is necessary for formation of memory and concurs with data gathered from electrophysiological experiments demonstrating enhancements of ERK activation in LTP, a putative molecular correlate of memory.

Coincidental evidence of ERK activity is presented by the functional coupling of the intracellular signal generated by NMDA activity and downstream ERK activation. These parallel observations are a likely consequence of PKC and PKA activation,

identified as components of LTP-generated signalling mechanisms (Malenka et al., 1986; Nguyen and Kandel; 1996). Given that concerted activities of NMDA receptors, PKC and PKA, coupled with increased glutamate release, are required for the induction of LTP, it is plausible that ERK is activated as a consequence of LTP-inducing stimuli. Similarly, accumulating reports of a role for neurotrophins in synaptic plasticity points to ERK involvement, as a downstream component of Trk activation. Taken together with mounting evidence of the contribution of phospho-CREB in LTP, described in Section 1.5.6, and that inhibition of both Trk (Maguire *et al*, 1999) and ras (Brambilla *et al.*, 1996) abolish LTP-induced increases in ERK activity, it seems extremely likely that ERK signalling is a crucial convergence point for integrating LTP-associated biochemical events.

The functional outcome of ERK activity may support changes in synaptic transmission by facilitating exocytosis of glutamate-containing synaptic vesicles (Jonanovic *et al.*, 1996) or by modifying membrane properties (Adams *et al.*, 2000) and thereby, increasing excitability. Alternatively or simultaneously, ERK activity may induce long-term changes in synaptic architecture by mediating the induction of IEGs (Treisman *et al.*, 1996). Evidence of ERK-associated synaptic remodelling is provided by findings that ERK phosphorylates a neural adhesion molecule homologue, ApCAM, in invertebrates in response to synaptic activity (Martin *et al.*, 1997). In this manner, ERK may initiate synaptic remodelling and thus facilitate formation of new synaptic contacts. A parallel scenario in mammalian models has yet to be investigated.

1.5.6.7 CREB and LTP

Several lines of evidence, both direct and indirect, point to a role for CREB in the expression of LTP. Recruitment of PKA, a CREB activator, occurs following LTP-inducing tetanus (Stanton and Sarvey, 1984) and another CREB stimulator, cAMP, which also stimulates PKA, is involved in late-phase LTP (Frey *et al.* 1993). Secondly, protein synthesis inhibitors block LTP (Krug *et al.*, 1984, Otani *et al.*, 1989), implying activation of one or several transcription factors. In addition, the major neurotransmitter involved in LTP, glutamate, has been shown to induce CREB phosphorylation via the ERK pathway (Vanhoutte *et al.*, 1999). Activation of CREB results in an increase in dendritic spine

density (Murphy and Segal, 1997) and induction of immediate early genes (Ahn *et al.*, 1998), putative targets of LTP (Richardson *et al.*, 1992). Direct evidence of a role for CREB in LTP is convincing. Bourtchuladze and coworkers (1994) showed that targeted mutation in CREB impairs water maze performance and that LTP in the CA1 of these mutants was not maintained. Other studies reported increased CREB phosphorylation following LTP in the CA1 (Matthies *et al.*, 1997) and dentate gyrus (Schulz *et al.*, 1999, Davis *et al.*, 2000). CREB may also provide a means of transducing the transient potentiation of neurotransmitter release induced by exposure to neurotrophins (Finkbeiner, 2000) and membrane depolarisation (Sheng *et al.*, 1990) into a long-lasting form of enhancement of synaptic transmission by initiating protein synthesis.

1.5.7 Locus of expression of LTP

Although there is general consensus for the postsynaptic locus of induction of LTP, whether the long-term cellular changes accompanying increases in synaptic efficacy reside in the pre- or postsynaptic cell has been subject to debate (Bliss and Collingridge, 1993). The notion of a presynaptic locus is supported by several findings indicating elevated glutamate release in response to LTP-inducing stimuli. The first report recording the effect of LTP on glutamate release indicated increased release of newly synthesised glutamate following delivery of tetanic stimulation to the perforant path (Dolphin *et al.*, 1982). Furthermore, when LTP is blocked, the LTP-associated enhancement of glutamate release is similarly inhibited. Quantal analysis of fluctuations of synaptic currents and failure rates preceding and following the induction of LTP indicates the involvement of presynaptic increases in the probability of transmitter release (Bekkers and Stephens, 1990; Malinow and Tsien, 1990). Taken together, these lines of evidence provide convincing support for a presynaptic mechanism in the maintenance phase of LTP in the perforant path-granule cell synapses.

Alternatively, substantial evidence exists suggesting that the sensitivity of the postsynaptic membrane to increased glutamate release is enhanced following the induction of LTP. This may occur by increased numbers of postsynaptically located receptors (Baudry *et al.*, 1980) or by modulation of receptor characteristics (Kauer *et al.*, 1988). In addition, potentiation of the epsp slope, which correlates with an increase in the

probability of the postsynaptic cell firing an action potential, is an essential component of LTP (Bliss and Lomo, 1973). It has also been suggested that AMPA receptors play a prominent role in the maintenance of LTP, which is discussed in Section 1.5.9. Evidence of a causal link between increased glutamate release and postsynaptic NMDA receptor function is suggested by the work of Canevari and coworkers (1994). In their study, pretreatment with NMDA receptor antagonist, MK-801, prevented the LTP-induced increase in glutamate release in the dentate gyrus, indicating a coupling of pre- and postsynaptic mechanisms. In an attempt to reconcile findings that the expression of LTP is dependent on both pre- and postsynaptic process, it was proposed a retrograde signal from the postsynaptic site to the presynaptic terminal was required (Bliss *et al.*, 1986). The criteria set down for classification of retrograde messengers indicate that the molecule must be released from a postsynaptic site, it must be present in the synaptic cleft following the induction of LTP and must exert an action of the presynaptic terminal. Candidate retrograde messengers are described in Section 1.5.10.

1.5.8 The 'Silent Synapse' theory

One theory supporting a postsynaptic locus of the maintenance of LTP has come to the fore in recent years. This theory is largely based on two observations. Firstly, LTP selectively increases postsynaptic currents mediated by AMPA receptors (Muller *et al.*, 1988; Kauer *et al.*, 1988) and secondly, LTP increases the sensitivity exogenous AMPA (Davies *et al.*, 1989). In addition, the technique of analysis of the frequency of failures of synaptic transmission, originally attributed to a presynaptic mechanism, is now viewed as a predominantly postsynaptic phenomenon. This is due to the unique property of the NMDA receptor, which demands activation by glutamate and concurrent depolarisation of the membrane. In the absence of membrane depolarisation, glutamate does not elicit NMDA receptor activity and therefore, failure is not caused by a failure to release glutamate but rather by a failure of the NMDA receptor to respond adequately (Nowak *et al.*, 1984). Postsynaptic responses mediated entirely by activation of NMDA receptors that do not produce a synaptic response at resting membrane potential are known as 'silent synapse'. The model proposed by this theory suggests functional AMPA receptors are delivered to membranes previously devoid of AMPA receptors from a non-synaptic

site (Liao *et al.*, 1995). It is suggested that inserted AMPA receptors may explain increases in quantal size (Kullman *et al.*, 1992; Liao *et al.*, 1992; Stricker *et al.*, 1991) because AMPA receptors are active during low-frequency synaptic transmission. This may also explain decreases in synaptic failures following the induction of LTP because previously 'silent synapses' are now functional at resting membrane potential. 'Silent synapses' have been reported in the CA1 region (Liao *et al.*, 1995; Durand *et al.*, 1996; Isaac *et al.*, 1995) and in the dentate gyrus (Min *et al.*, 1998).

1.5.9 Retrograde messengers

Several candidate membrane-permeant, diffusible molecules have been proposed as retrograde messengers; arachidonic acid (AA), nitric oxide (NO), carbon monoxide (CO) and platelet activating factor (PAF). Evidence to support the identification of AA as a retrograde messenger is derived from findings that inhibition of phospholipase A_2 (PLA_2), which generates AA, blocks the induction of LTP (Lynch *et al.*, 1989) and that AA potentiates synaptic transmission in the dentate gyrus and in the CA1 region (Williams *et al.*, 1989). This is further supported by later work indicating increased levels of AA in perfusate following the induction of LTP (Lynch *et al.*, 1991; Clements *et al.*, 1991). The effect of AA on glutamate release from presynaptic terminals is also relevant to its classification as a retrograde messenger. Lynch and Voss (1991) reported that KCl-stimulated glutamate release from synaptosomes was enhanced in the presence of high concentration of AA. Similarly, co-application of lower concentrations of AA and metabotropic glutamate receptor agonist, *trans*-1-amino-cyclopentyl-1,3-dicarboxylate (ACPD), increases glutamate release from synaptosomes prepared from the dentate gyrus (McGahon *et al.*, 1994), suggesting a synergistic action of both agents. Interestingly, AA and ACPD, applied in combination, stimulated an immediate increase in the epsp slope recorded from the CA1 region (Collins and Davies, 1993). Furthermore, co-application of AA and ACPD stimulates ERK activity (McGahon *et al.*, 1999) and as discussed in Section 1.5.7.6., ERK activation is crucial to the expression of LTP in the CA1 region (English and Sweatt, 1996) and the dentate gyrus (McGahon *et al.*, 1999).

Nitric oxide was also proposed as a retrograde messenger based on several observations. Firstly, nitric oxide synthase (NOS), the enzyme that generates NO is

located in the postsynaptic cell and inhibition of NOS blocks the induction of LTP (O'Dell *et al.*, 1991b, Schuman and Madison, 1991). Secondly, addition of haemoglobin, a NO binding protein, inhibits LTP. Haemoglobin does not penetrate the cell membrane, indicating that NO must diffuse into the extracellular space to exert its effect (O'Dell *et al.*, 1991b, Schuman and Madison, 1991). Thirdly, NMDA receptor activation stimulates NO release (Garthwaite *et al.*, 1988). It is therefore, likely that NMDA-dependent LTP promotes increased NO production. In sharp contrast to this evidence, Sequeira and coworkers (2001) have recently suggested that activation of presynaptic NMDA receptors inhibits glutamate release in the hippocampus through the formation of NO. However, NOS knockout mice demonstrate impaired LTP (Son *et al.*, 1996) and NO signalling contributes to late-phase LTP by stimulating CREB phosphorylation (Lu *et al.*, 1999b), further supporting its status as a retrograde messenger.

Stevens and Wang (1993) proposed carbon monoxide as a retrograde messenger, based on observations that inhibition of haem oxygenase, which generates CO, blocks LTP. In addition, inhibitors of haem oxygenase suppress glutamate release, supporting a presynaptic action of CO. Platelet-activating factor (PAF), another putative retrograde messenger, is a membrane-derived phospholipid produced by PLA_2 and acts as a second messenger. PAF augments synaptic transmission in hippocampal neurons by a presynaptic mechanism (Clark *et al.*, 1992) A role for PAF in the mechanisms underlying LTP is suggested by reports that inhibition of PAF suppresses LTP in the CA1 region (Kato *et al.*, 1994; Wieraszko *et al.*, 1993) and that induction of LTP results in increased release of PAF into the extracellular medium (Kornecki *et al.*, 1994). A direct effect of PAF on glutamate release has not been reported.

Neurotrophins have also been proposed as retrograde messengers because neurotrophin release is activity-dependent (Blöchl and Thoenen, 1995; Goodman *et al.*, 1996) and because they modulate glutamatergic transmission, as outlined in Section 1.3.5 and participate in signalling mechanisms subserving LTP in the hippocampus as discussed in Section 1.5.10.

1.5.10 Role of neurotrophins in LTP

Evidence of a role for neurotrophins in LTP and synaptic plasticity in the hippocampus is derived from accumulating information, firstly, implicating members of the neurotrophin family and their respective receptors in the modulation of synaptic transmission and secondly, from the high expression of Trk receptors in the hippocampus (Ernfors *et al.*, 1992; Muragaki *et al.*, 1995). Furthermore, activity-dependent regulation of neurotrophin expression (Gall and Isackson, 1989; Zafra *et al.*, 1990; Ernfors *et al.*, 1991; Lu *et al.*, 1991) and activity-induced neurotrophin release (Blöchl and Thoenen, 1995, 1996; Goodman *et al*, 1996) also point to a putative role for neurotrophins in LTP. Investigation of interplay between synaptic activity and neurotrophin expression revealed upregulation of neurotrophin expression following epileptiform activity in the hippocampus (Gall and Isackson, 1989; Zafra *et al.*, 1990) and dentate gyrus (Ernfors *et al*, 1991). In addition to seizure-stimulated expression of neurotrophin mRNA, depolarisation was observed to increase mRNA of BDNF and NGF (Lu *et al.*, 1991; Zafra *et al.*, 1990; Lindholm *et al.*, 1994). Subsequent studies uncovered rapid and selective regulation of BDNF mRNA following induction of LTP in the hippocampus (Patterson *et al.*, 1992; Castrén *et al.*, 1993; Dragunow *et al*, 1993). Interestingly, Castrén and coworkers (1993) observed increased BDNF mRNA with a concomitant reduction in NT-3 mRNA following the delivery of LTP-inducing tetanic stimuli.

In addition to upregulation of mRNA of neurotrophins in response to tetanic stimulation, tyrosine kinase inhibitors were shown to inhibit expression of LTP in CA1 region of the hippocampus (O'Dell *et al.*, 1991a, Huang and Hsu, 1999) and in the dentate gyrus (Abe and Saito, 1993; McGahon *et al.*, 1998), suggesting a possible parallel between signalling events stimulated by neurotrophins via Trk receptors and the underlying signalling mechanisms supporting the expression of LTP. Furthermore, the activation of ERK, a downstream messenger of Trk activation, has been reported to be a crucial event in the expression of LTP in the CA1 region (English and Sweatt, 1996) and the dentate gyrus (McGahon and Lynch, 1998, Maguire *et al.*, 1999). Participation of Trk-mediated signalling cascades in the biochemical pathways subserving expression of LTP is suggested by reports that tyrphosphin AG879, a Trk inhibitor, blocks LTP in the perforant path-granule cell synapses (Maguire *et al.*, 1999). Likewise, inhibitors of

several components of neurotrophin signalling mechanisms, such as PI-3 kinase (Kelly and Lynch, 2000) and MEK (McGahon and Lynch, 1998) inhibit LTP in the dentate gyrus. This effect is correlated with increased phosphorylation of Trk in synaptosomes prepared from dentate gyri that sustained LTP (Maguire *et al.*, 1999). In addition, Bramham and coworkers (1996) also proposed that LTP induced increased expression of trk mRNA, further supporting a role for Trk in the expression of LTP.

Additional research showed deficits in LTP at the CA1 synapses of the hippocampus in BDNF knockout mice. Application of exogenous BDNF (Patterson *et al*, 1996) or reintroduction of BDNF by transfection of hippocampal slices with BDNF adenovirus (Korte *et al.*, 1996) resulted in a 'rescue' effect, restoring the ability to sustain LTP. Similarly acute intrahippocampal infusion of BDNF was reported to potentiate synaptic activity, causing a rapid and marked increase in synaptic strength at the perforant path (Messaoudi *et al.*, 1998). Further studies using TrkB-IgG fusion proteins to chelate endogenously released BDNF and thus, impairing TrkB-mediated signalling by BDNF was found to diminish expression of LTP (Figurov *et al.*, 1996; Kang *et al.*, 1997). Chen and coworkers (1999a) reported similar effects on LTP expression using antibodies raised against BDNF. Consistent with observations from LTP studies, contextual learning, a behavioural correlate of hippocampal LTP, was impaired in BDNF knockout mice (Hall *et al.*, 2000).

Apart from an unsubstantiated report that NGF inhibits the induction of LTP (Tancredi *et al*, 1995), involvement of NGF in the expression of LTP is suggested by observations that genetically hypertensive (GH) rats, known to be deficient in NGF (Messina and Bell, 1991), fail to sustain LTP upon delivery of tetanic stimuli to the perforant path (Kelly *et al.*, 1998). This impairment is reversed following intra-cerebroventricular injection of NGF (Kelly *et al.*, 1998). Evidence suggests that NGF may fulfil the criteria of a retrograde messenger at the perforant path-granule cell synapses and thereby, serve as a means of communication between the pre- and postsynaptic neurons (Thoenen, 1995). Firstly, depolarisation induces NGF release from the granule cells of the dentate gyrus (Thoenen, 1995; Kelly *et al.*, 2000). Secondly, the expression of LTP is associated with increases in NGF mRNA (Zafra *et al.*, 1991) and TrkA phosphorylation (Maguire *et al.*, 1999). Finally, NGF has been shown to enhance

glutamatergic transmission (Knipper *et al.*, 1994). It is therefore possible that neurotrophins function as retrograde messengers as they fulfil many of the criteria for classification as a retrograde messenger.

Circumstantial evidence of a role for neurotrophins in LTP is derived from previously discussed reports that BDNF-mediated facilitation of glutamatergic transmission at hippocampal synapses and the expression of LTP is correlated to increased phosphorylation of NMDA receptor subunit 2B (Rostas *et al.*, 1996; Rosenblum *et al.*, 1995). Subsequent analysis implicated BDNF in the phosphorylation of NMDA subunits (Suen *et al.*, 1997; Levine *et al.*, 1998), pointing to a possible mediator of NMDA receptor phosphorylation following the induction of LTP. Furthermore, BDNF has been demonstrated to modulate AMPA receptors (Narisawa-Saito *et al.*, 1999), which are central to the 'silent synapse theory' of LTP. In addition, it has been proposed that BDNF alters synaptic vesicle protein, synaptophysin (Jonanovic *et al.*, 1996) while synaptophysin has been reported to be upregulated by the expression of LTP (Mullany and Lynch, 1998). This may provide a means by which BDNF plays a permissive role in the increase in glutamate release observed following the induction of LTP.

Several lines of evidence underlie a role for neurotrophins in late phase LTP. Firstly, electrophysiological experiments indicate that application of TrkB-IgG proteins, which scavenge endogenous BDNF, following the onset of LTP completely reverses established LTP (Kang *et al.*, 1997; Korte *et al.*, 1998). The requirement for neurotrophins in late phase LTP may reside in the finding that maintenance of LTP requires formation of new synaptic contacts, described in Section 1.5.11. Exposure to NT-3 and BDNF has been found to accelerate the appearance of synapses in the hippocampus (Vicario-Abejon, 1998; Martinez *et al.*, 1998; Murphy and Segal, 1999). Furthermore, neurotrophin-stimulated synaptogenesis may enable the expression of LTP in neonatal hippocampus. The inability of juvenile rats to sustain LTP (Figurov *et al.*, 1996) is correlated with extremely low levels of endogenous BDNF expression reported in the neonatal hippocampus (Maisonpierre *et al.*, 1990; Friedman *et al.*, 1991; Ringstedt *et al.*, 1993). Administration of exogenous BDNF to the developing hippocampus promotes the expression of LTP in response to tetanus (Figurov *et al.*, 1996; Gottschalk

et al., 1998); BDNF may mediate this rescue effect by inducing synaptogenesis required for the establishment of new synaptic contacts in late phase LTP. Moreover, the maintenance of LTP requires protein synthesis (Krug *et al.*, 1984, Otani et al., 1989) and neurotrophin-stimulated protein synthesis has been demonstrated in the hippocampus (Baldelli *et al.*, 2000; Kang and Schuman, 1996). In addition, induction of the IEG, *c-fos* has been observed in late phase LTP (Jeffrey *et al.*, 1990; Richardson *et al.*, 1992) and parallel increases in c-fos induction have been reported in response to stimulation by NGF (Ginty *et al.*, 1994).

The involvement of NT-3 in biochemical events underlying LTP is controversial as studies of NT-3 knockout mice reveal that NT-3 is not required for LTP in the CA1 region (Ma *et al.*, 1999). Work by Patterson and coworkers (1992) suggests that induction of LTP in the CA1 areas of the hippocampus leads to a increase in NT-3 mRNA but this is contradicted by Castrén and coworkers, who reported a decrease in NT-3 mRNA levels following the expression of LTP. Analysis of a possible role for NT-3 in the mechanisms underlying LTP in the perforant path–granule cell synapses, where greater expression of NT-3 and TrkC is reported, has not been investigated.

1.5.11 Long-lasting changes involved in LTP

Synaptic changes that support the persistence of synaptic efficacy induced by LTP are subject to intense investigation. The exact mechanisms underpinning the long-lasting nature of LTP have not been fully elucidated but it seems obvious that both protein synthesis and synaptic remodelling are necessary components of any such mechanism.

1.5.12 Morphological changes

The maintenance of LTP is correlated with synaptic remodelling indicated by the early but persistent increases new dendritic spines (Geinisman *et al.*, 1993; Engert and Bonhoffer, 1999) and changes in spine area (Fifkova and Van Harreveld, 1977). In addition, evidence exists that late-phase LTP occurs in parallel with the formation of new synaptic contacts demonstrated by formation of perforated synapses and multiple synaptic boutons (Buchs and Muller, 1996; Toni *et al.*, 1999). Presynaptic changes include changes in the distribution of synaptic vesicles as more vesicles are located in the

active zone (Applegate *et al.*, 1987) and increases in synaptic vesicle number (Meshul and Hopkins, 1990). Postsynaptic changes include increases in postsynaptic surface area (Desmond and Levy, 1988) by modification of synaptic curvature (Desmond and Levy, 1990). Changes in synaptic structure of this nature may govern the stabilisation of LTP.

1.5.13 Protein synthesis

Morphological changes in synaptic morphology require availability of proteins to support modifications, provided by *de novo* protein synthesis (Bailey and Kandel, 1993). Duffy and coworkers (1981) reported increased release of newly synthesised proteins into the extracellular medium in the CA1 region and the dentate gyrus following the delivery of LTP-inducing stimuli. Subsequent research by Fazeli and coworkers (1993) demonstrated an increase in the concentration of newly synthesized proteins in the extracellular medium accompanying the expression of LTP in the dentate gyrus. Similarly, Otani and coworkers (1992) reported increased extracellular protein concentrations in the CA1 following induction of LTP *in vitro,* which was blocked by the application of protein synthesis inhibitor, cycloheximide. It was postulated that additional proteins present in the extracellular medium following the induction of LTP are proteases, which may promote dendritic spine formation (Otani *et al.*, 1992).

The stimulation of protein synthesis during late phase LTP is well documented. The protein synthesis inhibitor, anisomysin blocks maintenance of LTP in the CA1 region *in vitro* (Otani *et al.* 1989; Stanton and Sarvey, 1984; Mochida *et al*, 2001) and in the dentate gyrus *in vivo* (Krug *et al.*, 1984, Otani *et al.* 1989, Mullany *et al.*, 1997). The increase in the synthesis of new proteins may support enhanced glutamate release in response to the expression of LTP (Dolphin *et al.*, 1982). A feature of LTP is enhanced distribution and greater numbers of presynaptic glutamate-containing vesicles (Applegate *et al.*, 1987; Meshul and Hopkins, 1991) and production of new component proteins of the synaptic vesicle reflected by increased synthesis of synaptophysin (Mullany and Lynch, 1998; Lynch *et al.* 1994), synapsin I, and synaptotagmin (Lynch *et al.* 1994). Protein synthesis may also facilitate morphological changes which accompany maintenance of LTP by providing other structural proteins to permit synaptic remodelling and to increase spine formation (Geinisman *et al.*, 1993) and postsynaptic surface area

(Desmond and Levy, 1990). In recent years, several studies have indicated that neurotrophin mRNA is stimulated by the expression of LTP (Patterson *et al.*, 1992; Castrén *et al.*, 1993; Dragunow *et al*, 1993) and this indicates that tetanic stimulation induces the synthesis of some neurotrophins, which may serve to reinforce cellular events associated with LTP.

1.6 Objectives of this study

The objective of this study was to examine the role of tyrosine kinase on the expression of LTP at the perforant path-granule cell synapses and to assess whether neurotrophins impact on this mechanism by modulating tyrosine kinase activity.

- This study focuses on presynaptic mechanisms underlying the expression of LTP and the role of tyrosine kinases in the modulation of such mechanisms by investigating the effect of tyrosine kinase inhibitors on the LTP-related processes of glutamate release and calcium influx, as well as intracellular signalling events, in synaptosomes prepared from untetanised and tetanised dentate gyrus, which mediate this change.

- The effect of NT-3 on intracellular signalling cascades was examined.

- The involvement of *Trk* on the expression of LTP in the dentate gyrus was analysed by investigating the impact of a *Trk* inhibitor on signalling events in the pre- and postsynaptic areas.

- The impact of NT-3 on the expression of LTP at the perforant path-granule cell synapses was examined.

Chapter 2

Materials and Methods

2.1 Materials

The full names and addresses of the sources listed below are given in Appendix IX.

Materials	Source
t-ACPD	Tocris
Acrylamide	Sigma
Antiphosphotyrosine antisera	Affiniti
Anti-pan α_1 subunit antisera	Alomone
Anti- active ERK	Promega
Anti-phospho CREB	NE Biolabs
Bio-Rad	Bio-Rad Laboratories
ß-mercaptoethanol	Sigma
Bovine serum albumin (BSA)	Sigma
Brain-derived neurotrophic factor (BDNF)	Alomone
Bromophenol blue	Sigma
[^{45}Ca] $CaCl_2$ (Specific activity: 77.7MBq/ml)	Amersham
Cellulose Acetate strips	Sartorius
Cocktail-T	Lennox
Dimethyl sulphoxide (DMSO)	Sigma
Enhanced chemiluminescence (ECL) detection kit	Amersham
ECL supersignal kit	Pierce
Genistein	Calbiochem
Pre-stained molecular weight markers	Sigma
Nerve growth factor (NGF)	Alomone
Neurotrophin-3 (NT-3)	Alomone
Nitrocellulose membranes	Sartorius
Normal donkey serum	Sigma
NT-3 E_{max} ImmunoAssay System kit	Promega

Protein-A sepharose	Sigma
[^{35}S] methionine	
(Specific activity: 37Tbq/mmol)	Amersham
Sodium dodecylsulphate (SDS)	Sigma
Sodium orthovanadate	Sigma
3,3', 5,5'-Tetramethyl-benzidine (TMB)	Sigma
Triton X-100	Sigma
Tyrphostin AG879	Calbiochem
Urethane	Sigma
Whatman filter paper	Whatman
All other basic chemicals	Sigma and Lennox

2.2 Animals

Male Wistar rats (250-350g; 3months) were used in this study; rats were obtained from the BioResources Unit, Trinity College Dublin. They were housed in groups of 6, maintained under veterinary supervision with a 12 hour light schedule and at an ambient temperature of 22-23°C. Food and water were provided *ad libitum.*

2.3 Preparation of tissue

Animals were killed by cervical dislocation and decapitation. The brain was rapidly removed and the hippocampi, dentate gyri and entorhinal cortices were dissected free on ice.

2.3.1 Preparation of slices for freezing

Tissue slices were prepared as described below and frozen according to the method of Haan and Bowen (1981). Freshly dissected tissue was sliced bidirectionally to a thickness of 350μm using a McIlwain Tissue chopper and rinsed in ice-cold Krebs solution (composition: NaCl, 136 mM; KCl, 2.54mM; KH_2PO_4, 1.18mM; $Mg_2SO_4.7H_2O$, 1.18; $NaHCO_3$, 16mM, glucose, 10mM) containing $CaCl_2$ (final concentration: 2mM). Slices were allowed to settle and rinsed twice with ice-cold Krebs solution containing $CaCl_2$ (2mM) and dimethyl sulfoxide (DMSO: final

concentration: 10% Krebs-DMSO). Slices were stored in this solution at -80 °C until required for further analysis. When required, slices were thawed quickly by agitation and rinsed three times in ice-cold Krebs solution containing $CaCl_2$ (2mM). Slices were used for analysis of protein synthesis, CREB phosphorylation and NT-3 release.

2.3.2 Preparation of synaptosomes

Synaptosomes were prepared from either freshly dissected dentate gyri or frozen slices according to the method of Gray and Whittaker (1962). When using fresh tissue, the dentate gyri were homogenised in sucrose (1ml, 0.32M). To prepare the crude synaptosomal pellet, P_2, the homogenate was spun at 5,000 rpm for 5min. The supernatant, which contained synaptosomes, was removed and used to prepared P_2 as described below. The pellet, P_1, was retained in some experiments for analysis. This pellet was a crude preparation containing glial cells and neuronal cells and will hereafter be referred to as a postsynaptic preparation. The supernatant was centrifuged at 15,000 rpm for 15min at 4°C. The resulting pellet was a synaptosomally enriched preparation and was resuspended in oxygenated Krebs solution at 37 °C. When using frozen tissue, slices were quickly thawed and rinsed with ice-cold Krebs solution containing $CaCl_2$ (2mM). Slices were then homogenised and spun as for fresh tissue. Synaptosomes were used for analysis of calcium influx, glutamate release, tyrosine kinase activity, NT-3 release and protein phosphorylation.

2.4 Protein quantitation using the Bradford assay

Protein concentrations were assessed using the method of Bradford (1976). Samples (5µl) were diluted with distilled water (155µl). Standards were prepared from a stock solution of 200µg/ml bovine serum albumin (BSA), ranging in concentration from 2µg/ml to 100µg/ml, to a final volume of 160µl. Biorad dye (40µl; Biorad Laboratories) was added to samples and standards and incubated at room temperature for 5min. Preparations were mixed and aliquots (200µl) were transferred to a 96-well plate. Absorbance readings were recorded at a wavelength of 630nm using a Sigma Diagnostic EIA Multiwell plate reader. Protein concentrations of tissue samples were calculated by linear regression using absorbances of BSA standards.

2.5 Induction of LTP *in vivo*

2.5.1 Recording apparatus

The recording chamber consisted of a stereotaxic frame permanently fixed to a bench and surrounded by a Faraday cage to isolate the signal from environmental interference. All instruments in the cage were grounded to eliminate 50Hz cycle noise.

2.5.2 Preparation of animals

Rats were anaesthetized with urethane (1.5g/kg i.p.). Loss of consciousness was indicated by absence of pedal reflex. Scalp fur was trimmed and rats were placed in the head holder of the stereotaxic frame. A midline incision was made and the periosteum was scraped back to expose the skull plates. This allowed identification of bregma and lambda. In some experiments, rats were randomly assigned to groups of 6 and a intracerebroventricular (i.c.v) injection of saline, genistein (final concentration: 50μM; O'Dell *et al.*, 1991a), tyrphostin AG879 (final concentration: 100μM; Maguire *et al.*, 1999) or NT-3 (final concentration: 20ng/ml; Kang and Schuman, 1995) was administered to the third ventricle (co-ordinates: 0.5mm lateral to, and 2.5 posterior to, bregma) 45min prior to the delivery of tetanic stimulation.

2.5.3 Electrode implantation

A dental drill was used to remove a window of the skull to expose the underlying cortex. The dura mater was carefully peeled back to allow implantation of electrodes. A bipolar stimulating electrode (Clark Electromedical, UK) was placed on the surface of the brain 4mm lateral lamba and initially lowered to a depth of 2mm. A unipolar recording electrode (Clark Electromedical, UK) was placed on the surface of the brain 2.5mm lateral and 3.9mm posterior to bregma and lowered to a depth of 2mm. A 4V pulse of 0.1ms duration and 2ms delay was generated and delivered through the stimulating electrode. The evoked response was detected by the recording electrode and displayed on the monitor screen. The stimulating electrode was lowered incrementally by approximately 2.2mm into the perforant path and the recording

electrode was lowered approximately 3mm. Stimuli were delivered at 10s intervals and the recording electrode lowered to the cell layer of the dentate gyrus.

Final positions of the electrodes were adjusted to give an evoked response of approximately 2mV in amplitude. Positions below the surface of the brain were 2-2.5mm for the stimulating electrode and 3.5-4.5mm for the recording electrode.

2.5.4 epsp recordings

The slope of the population field excitatory postsynaptic potential (field epsp) was used as a measure of excitatory synaptic transmission in the hippocampus. An epsp was achieved by passing a single square wave pulse of current at low frequency (0.033Hz, 0.1s duration, 2ms delay) generated by a constant current isolation unit to the bipolar stimulating electrodes. The evoked response was transmitted via a preamplifier to an analogue-to-digital converter (MacLab/2e, Analog Digital Instruments). This digitized system was linked to an Apple Macintosh computer (Performa 200) which interfaced with the converter via a specialised software application (Scope, Version 3.36), customised to control both the generation of square wave pulses and recordings of the evoked potentials. The field epsps were displayed on-screen and could be analysed at the time of recording or at a later time.

The slope of the field epsp was taken as the main indicator of excitatory transmission and was measured as the middle 1/3 of the rising phase of the epsp. Test shocks were delivered every 30s for a 10min period to establish stable baseline recordings. The average epsp slope was 1.83mV/min (± 0.10, SEM) and varied from 0.80 to 2.40mV/min over the course of the experiments for 24 rats. This was followed by delivery of 3 trains of stimuli (250Hz for 200ms) at 30s intervals. Recording at test shock frequency then resumed for 40min. In the case of low frequency control experiments, test shocks were delivered at a rate of 1/12sec for the 50min recording period, so that rats received the same total number of stimuli but without the high frequency train.

2.6 Analysis of endogenous glutamate release

Synaptosomes (P_2) prepared from hippocampus or dentate gyrus were resuspended in ice-cold Krebs solution containing $CaCl_2$ (2mM) and preincubated in

the presence of Krebs solution, genistein (final concentration: 50μM; O'Dell *et al.*, 1991a), BDNF (final concentration: 200ng/ml; Kang and Schuman, 1995), NT-3 (final concentration: 20ng/ml; Kang and Schuman, 1995) or ACPD (final concentration: 50μM; McGahon and Lynch, 1998) at 37°C under constant oxygenation for 15min. Samples were aliquoted onto Millipore filters (pore size 0.45μM) in a manifold and washed 15 times with oxygenated Krebs solution containing $CaCl_2$ (2mM). Synaptosomes were incubated in oxygenated Krebs solution containing $CaCl_2$ (2mM) in the absence or presence of KCl (final concentration: 50mM) for 3 minutes at 37°C. Filtrate was collected under vacuum and stored at -80 °C for analysis (Section 2.7).

2.7 Analysis of glutamate concentration by immunoassay

Glutamate concentration was assessed as previously described (Ordronneau *et al.*, 1991). Coating buffer (250μl; composition: NaH_2PO_4, 100mM; glutaraldehyde, 0.5% v/v; pH4.5) was added to 96-well plates and plates were blocked for 60min at 37°C before washing twice with NaH_2PO_4 (100mM, pH 8.0). Triplicate samples (50μl) or glutamate standards (50μl; 50nM to 10μM prepared in Na_2HPO_4 buffer, pH 8.0) were added to the coated wells and incubated at 37°C for 2h, before washing twice with Na_2HPO_4 buffer. Ethanolamine (250μl; 0.1M in 100mM Na_2HPO_4 buffer) was added to bind unreacted aldehydes. Plates were incubated for 60min at 37°C and washed with phosphate-buffered saline containing Tween-20 (0.2% Tween; PBS-T). Non-specific binding was blocked by adding normal donkey serum (250μl; 3% v/v in PBS-T) for 1h at 37°C. Anti-glutamate antibody (raised in rabbit; 100μl; 1:5000 in PBS-T; Sigma, UK) was added to each plate. In the case of blank wells, normal donkey serum (100μl; 3% v/v in PBS-T) was added. Plates were incubated at 4°C overnight and then washed thoroughly in PBS-T. Anti-rabbit horseradish peroxidase–linked secondary antibody (100μl; 1:10000 in normal donkey serum, 3% v/v in PBS-T; Amersham, UK) was added. Plates were incubated at room temperature for 60min and washed with PBS-T. 3,3',5,5'-tetramethylbenzidine (TMB; 100μl; Sigma) liquid substrate was added as a chromogen and incubation proceeded for 60min at room temperature. H_2SO_4 (4M; 50μl) was added to stop the reaction and the optical

densities were determined at 450nm. Values were calculated with reference to the standard curve.

2.8 Analysis of $^{45}Ca^{2+}$ influx

Synaptosomes (P_2) prepared from hippocampus were resuspended in ice-cold oxygenated incubation buffer (NaCl, 128mM; KCl, 4.8mM; KH_2PO_4, 1.2mM; $NaHCO_3$, 7.5mM; $CaCl_2$, 1.3mM; glucose, 11mM; ascorbic acid, 0.1mM; HEPES, 15mM; EDTA, 0.3mM) and incubated for 5s at 37°C in oxygenated incubation buffer containing $^{45}Ca^{2+}$ (final concentration 1μCi/ml; specific activity, 2.1mCi/ml; Amersham, UK) in the presence of low (4.8mM) or high concentrations (50mM) of KCl. In some experiments, genistein (final concentration: 50μM) was added during the incubation. Reaction was terminated by addition of 1ml of ice-cold "stop" buffer (NaCl, 118 mM; KCl, 48 mM, $CaCl_2$, 1.3 mM; glucose, 11mM; oubain, 10 mM). Samples were aliquoted onto Millipore filters (pore size 0.45μM) on a filtration manifold and rinsed at least 20 times with ice-cold 'stop' buffer under vacuum. Filters were added to scintillation fluid (Cocktail-T) and radioactivity levels were assessed using a Packard 1500 liquid scintillation counter.

2.9 Analysis of NT-3 release

NT-3 release was analysed from fresh or frozen slices. It was also assessed from synaptosomes prepared from freshly dissected tissue or from synaptosomes prepared from untetanised and tetanised dentate gyri frozen following electrophysiological recording. Slices of dentate gyrus, prepared from freshly dissected tissue or frozen slices quickly thawed, and rinsed three times with ice-cold Krebs solution containing $CaCl_2$ (2mM). Slices were incubated in Krebs solution containing $CaCl_2$ (2mM) at 37°C for 3min under continuous oxygenation. Slices were centrifuged at 4°C for 1min at 5,000, the supernatant discarded and the slices were incubated in Krebs solution (100μl) containing $CaCl_2$ (2mM) for 3min at 37°C under continuous oxygenation. The slices were centrifuged at 5,000rpm for 1min and the supernatant was retained for analysis. The slices were incubated under continuous oxygenation at 37°C for 3min in Krebs solution (100μl) containing $CaCl_2$ (2mM) and

KCl (50mM) to cause depolarisation. Samples were centrifuged for 1min at 5,000rpm. The supernatant was retained for analysis of NT-3 concentration and the pellet was retained for analysis of protein concentration. Unstimulated and KCl-stimulated supernatants were assayed for NT-3 concentration using a two-site immunoassay described (Section 2.11).

2.11 Analysis of NT-3 concentration by two-site immunoassay

Concentrations of NT-3 were assessed by ELISA using NT-3 E_{max} ImmunoAssay System kits (Promega). Immunoplates (Nunc MaxiSorp) were coated with polyclonal anti-human NT-3 antibody (Promega) in coating buffer (composition: $NaHCO_3$, 25mM; $NaCO_3$, 25mM; pH 9.7). The wells were sealed and incubated for 16h at 4°C. Plates were washed vigorously with TBS-Tween and blocked with manufacturer's blocking solution for 60min at room temperature. Plates were washed with TBS-Tween. Samples (25µl) or standard NT-3 samples (25µl; ranging from 4.7pg/ml to 300pg/ml) were added to wells. Plates were sealed, incubated for 6h at room temperature and vigorously washed with TBS-Tween. Monoclonal anti-NT-3 antibody was diluted 1:4,000 with blocking buffer and this solution (100µl) was added to wells. Plates were incubated for 16h at 4°C and washed. HRP-conjugated anti-mouse IgG was diluted 1:2000 with blocking buffer and this solution (100µl) was added to wells. Plates were gently agitated for 2.5h at room temperature and washed 5 times with TBS-T. TMB substrate was prepared 30min prior to colour development by combining equal volumes of TMB solution and peroxidase substrate. This solution (100µl) was added to wells and incubated at room temperature for 15min to allow maximal colour development. Phosphoric acid (1M; 100µl) was added to terminate the reaction. Absorbances were read at a wavelength of 450nm using a 96 well plate reader, with the absorbance of substrate solution (50µl) taken as blank. NT-3 concentrations were calculated with reference to the standard curve, protein concentrations were determined using the Bradford assay and results were expressed as pg/mg of protein.

2.12 Immunoprecipitation

2.12.1 Preparation of Protein-A sepharose

Protein-A sepharose beads (125mg) were incubated in 1ml immunoprecipitation buffer (composition: NaCl, 190mM; Tris-HCl pH 7.4, 60mM; EDTA pH 8.0, 6mM; Triton X-100, 1.25% v/v; aprotonin, 10U/ml) at room temperature for 3-4h to allow swelling of the sepharose beads. The suspension was spun at 15, 000rpm for 1min, the supernatant discarded and the beads resuspended in 750µml immunoprecipitation buffer. The suspension was vortex-mixed and centrifuged at 15,000rpm for 1min before being stored at 4°C until required.

2.12.2 Immunoprecipitation of proteins with anti-phosphotyrosine

Immunocomplexes were prepared by incubating tissue with immunoprecipitation buffer (155µl; Triton-X 100, 1.25%, NaCl, 1.9mM; Tris-HCl, 60mM, pH 7.4; EDTA, 6µM, pH 8; aprotonin, 10U/ml) for 1h at 37°C, in the presence of anti-phosphotyrosine (5µl, Affiniti Laboratories, UK) and sodium orthovanadate (25µM). Protein-A sepharose beads (50µl; 12.5%) were added and incubation proceeded at room temperature for 1h. Samples were centrifuged at 14,000g for 1min. The pellet was resuspended in wash buffer containing SDS (1ml; Triton-X 100, 0.1%; SDS, 0.02%; NaCl, 150mM; Tris-HCl, 50mM, pH 8.5; EDTA, 5mM; aprotonin, 10U/ml) and centrifuged at 14,000g for 1 min. This step was repeated twice. Supernatant was removed while the pellet was resuspended in wash buffer (1ml; NaCl, 12.5mM; Tris-HCl, 12.5mM, pH 8.5; EDTA, 2.5mM; aprotonin, 0.2U/ml) and spun for 14,000g for 1 min. The pellet was resuspended in sample buffer (30µl; Tris-HCl, 0.5mM, pH 6.8; glycerol, 10%; SDS, 10%; ß-mercaptoethanol, 5%; bromophenol blue, 0.05% w/v) and boiled for 10 min. Supernatants were loaded on to gels and separated using gel electrophoresis.

2.13 Preparation of samples for gel electrophoresis

Samples were equalised for protein concentration using the Bradford assay and equal volumes of sample were added to sample buffer (composition: Tris-HCl pH

6.8, 0.5M; SDS, 10% w/v; glycerol, 10% v/v; 2-β-mercaptoethanol, 5% v/v; bromophenol blue, 0.05% w/v). Samples were boiled for 5 min.

2.14 Gel electrophoresis

Gel electrophoresis was used to separate proteins and the gels used in these studies were 7.5%, 10% or 14% SDS-polyacrylamide. Electrophoresis was performed in a vertical slab gel apparatus (Biometra, UK). The dimensions of the gels were 8cm by 10cm. A 14-well comb was placed in the top of the well during polymerisation to form sample wells. Pre-stained molecular weight markers (Sigma, UK) and samples were loaded onto the gel. A constant current of 36mA was applied to the gel for approximately 30min to achieve protein separation. Following electrophoresis, bands were visualised by silver staining or probed for specific proteins by Western immunoblotting.

2.15 Silver staining

In some experiments, protein bands were visualised using silver staining. The gel slab placed in a Pyrex staining tank and was immersed in a fixing solution ([% as v/v unless otherwise stated] methanol, 50%; trichloroacetic acid, 20%; copper chloride, 2% (w/v); formaldehyde, 0.1%) at room temperature with gentle agitation (35rpm) for 25min. The gel was washed (ethanol, 10%; acetic acid, 5%) for 15min and incubated in potassium permanganate (0.01% (w/v)) for 15min at room temperature before washing (ethanol, 10%; acetic acid, 5%) for 15min. The gel was then washed in 10% ethanol for 15min and distilled H_2O for 15min. Protein bands were stained by adding silver nitrate (0.1% (w/v)) for 15min at room temperature, followed by rinsing in distilled H_2O. The gel was then incubated in potassium carbonate solution (10%) for 1min. The stained bands were visualised by incubation in a formaldehyde/potassium carbonate solution (0.02%; 2% (w/v) respectively). The reaction was terminated once the bands appeared by addition of a solution containing ethanol (10%) and acetic acid (5%) before washing 3 times in distilled water. Protein bands were quantitated by densitometric analysis.

2.16 Western Immunoblotting

The gel slab was gently rinsed in transfer buffer (Tris base, 25mM; glycine, 200mM; methanol, 20% v/v; SDS, pH 8.3, 0.5%). Separated proteins were transferred to a nitrocellulose sheet (Sartorius) by electrophoretic transfer. This was achieved by preparing a sandwich of a sheet of prewetted filter paper (Whatman, number 3), nitrocellulose paper, SDS polyacrylamide slab gel and a sheet of prewetted filter paper. Air bubbles were removed and the sandwich was placed on the anode of a Biometra semi-dry blotter. The cathode lid was secured to the anode and screwed in place. A current of 225mA or 250mA was applied to the unit. The transfer time depended on the molecular size of the protein analysed. Nitrocellulose sheets were blocked for 1h at 37°C with a solution of TBS-T containing non-fat dry milk. The blots were washed with TBS-T at room temperature and incubated with primary antibody overnight at 4 °C. Nitrocellulose membranes were washed with TBS-T at room temperature and incubated with secondary antibody, horseradish peroxidase-linked anti-rabbit IgG (Amersham, UK). Immunoblots were washed and protein complexes were visualised using enhanced chemilluminescense (Amersham, UK). Nitrocellulose membranes were exposed to X-ray film (Amersham, UK) and processed using a Fuji X-ray processor. Protein bands were quantitated by densitometric analysis, using the Gelworks ID and Grab-It packages. A molecular weight calibration curve was obtained for a mixture of molecular weight markers by plotting $\log_{10}M_w$, where M_w is molecular weight, against migration distance, R_f. As the background of the densitograms is not linear, it was necessary to apply a manual baseline so that a more reliable value for the peak area was obtained.

2.17 Analysis of protein synthesis

Slices of entorhinal cortex were thawed rapidly by agitation, rinsed 3 times in Krebs solution containing $CaCl_2$ (2mM). Slices were preincubated in Krebs solution containing $CaCl_2$ (2mM) for 10min at 37°C under constant oxygenation. Supernatant was removed from gravity-packed slices and incubation buffer (composition: $CaCl_2$, 2 mM; ATP, 3.5 mM) containing [^{35}S] methionine (specific activity, 37TBq/mmol; 0.2µl/ml) was added. Slices were incubated for 60min at 37°C under continuous oxygenation. Ice-cold trichloroacetic acid (final concentration: 10%) was added to

aliquots and incubated for 5min on ice. TCA-precipitated proteins were homogenised. Aliquots were transferred to a Millipore filter (pore size 0.45μm) and rinsed under vacuum at least 10 times with excess TCA (final concentration: 5% v/v). An aliquot was retained for determination of protein concentration. Filters were added to a scintillant (Cocktail-T) for assessment of radioactivity. Data were expressed as cpm per milligram of protein.

2.18 Liquid scintillation counting

[^{45}Ca] or [^{35}S] isotopes were measured using a Packard 1500 scintillation spectometer. The scintillation fluid used was Cocktail-T (Lennox) and average counting efficiency for the isotope was calculated to be 60-63%, based on quench curves relating counting efficiency to the spectral index of the external standard.

2.19 Statistical analysis

In each experiment, the sample number, n, refers to the number of animals used, usually 6. For the LTP experiments, rats were randomly assigned to different treatment groups. For experiments involving analysis of several conditions, the tissue from one animal was divided into a number of samples according to the number of treatments. An unpaired *t*-test was performed to determine whether there was a statistical difference between conditions. Welch's correction was used to ensure that significant changes were not due to non-uniform variability.

Chapter 3

Establishing a role for tyrosine kinase in LTP

3.1 Introduction

Several kinases are involved in the mechanisms underlying the expression of LTP in the hippocampus. They include PKC (Akers *et al.*, 1986, Lovinger and Routtenberg, 1988), PKA (Roberson and Sweatt, 1999), CAM kinase II (Malenka *et al.*, 1989, Malinow *et al.*, 1988) and tyrosine kinases. Inhibition of tyrosine kinase has been repeatedly found to block LTP (O'Dell *et al.*, 1991a, Huang and Hsu, 1999; Abe and Saito, 1993; Maguire *et al.*, 1999, McGahon *et al.*, 1998) and phosphorylation of some presynaptic tyrosine kinase substrates, such as synaptophysin (Mullany and Lynch, 1998) and PLCγ (McGahon and Lynch, 1998), accompanies LTP. Inhibition of non-receptor tyrosine kinases such as c-src and fyn (Grant *et al.*, 1992; Kojima *et al.*, 1997) has also been demonstrated to abolish LTP, while a role for receptor tyrosine kinases has also been suggested (Maguire *et al.*, 1999).

A role for increased glutamate release in LTP in the perforant path-granule cell synapses is widely reported (Dolphin *et al.*, 1982; Canevari *et al.*, 1994). Because increases in cytosolic calcium concentrations are a necessary prerequisite step for glutamate release, modulation of calcium influx following LTP may be responsible for augmented glutamate release in response to depolarisation. One subunit of the voltage-operated calcium channel, the pore-forming α_1 subunit, is a possible target of tyrosine kinase in non-neuronal tissues (Boixel *et al.*, 2000; Arnoult *et al.*, 1997; Fitzgerald and Dolphin, 1997; Wijetunge *et al.*, 1995) and may contribute to a presynaptic mechanism for modifying depolarisation-induced glutamate release in LTP.

Protein synthesis is essential for the maintenance of LTP and the protein synthesis inhibitor, anisomysin, blocks maintenance of LTP (Otani *et al.* 1989, Stanton and Sarvey, 1984, Krug *et al.*, 1984, Otani *et al.* 1989, Mullany *et al.*, 1997). Protein synthesis may facilitate morphological changes, which accompany maintenance of LTP by providing structural proteins to permit synaptic remodelling (Geinisman *et al.*, 1993, Desmond and Levy, 1990).

The aim of this study is 1) to assess the contribution of tyrosine kinase to presynaptic mechanisms underlying the expression of LTP in the perforant-path granule cell synapses and 2) to identify substrates of tyrosine kinase that may impact on glutamatergic transmission.

3.2 Methods

3.2.1 Analysis of endogenous glutamate release

Analysis of glutamate release was described in detail in Section 2.6. Briefly, synaptosomes were prepared from hippocampus and resuspended in oxygenated Krebs solution containing $CaCl_2$ (final concentration: 2mM). Some samples were preincubated with genistein (final concentration: 50μM) at 37°C for 15min. Following incubation, aliquots were transferred to a Millipore filter in a filtration manifold, washed and incubated in Krebs solution containing $CaCl_2$ (final concentration: 2mM) in the presence or absence of KCl (50mM). Filtrates were collected under vacuum and stored at -80°C for later analysis. Analysis of glutamate concentration in standards (ranging from 25nM to 2μM) and filtrates is described in detail in Section 2.6. Synaptosomal suspensions were used to determine protein concentrations.

3.2.2 Analysis of $^{45}Ca^{2+}$ influx

This method was described in detail in Section 2.8. Synaptosomes were prepared from hippocampus and resuspended in ice-cold incubation buffer. Aliquots were incubated at 37°C for 10s in incubation buffer containing $^{45}CaCl$, and in cases that depolarisation-dependent calcium influx was studied, with KCl (50mM). Samples were incubated in genistein (final concentration: 50μM), in the presence of KCl (50mM). The reaction was terminated with ice-cold stop buffer. Samples were then aliquoted onto a Millipore filter and washed under vacuum with ice-cold stop buffer. Filters were placed in scintillation fluid and samples were counted for radioactivity. Protein concentrations were determined from synaptosomal suspension. Results were expressed as nmol $^{45}Ca^{2+}$/mg protein.

3.2.3 Induction of LTP *in vivo*

Induction of LTP is described in detail in Section 2.5. Rats were anaesthetised with urethane (1.5g/kg i.p) and secured in a stereotaxic frame. In some experiments, pretreatment with genistein (5μl; final concentration: 50μM) or saline (5μl) was delivered

by intracerebroventricular injection 30min prior to recording. Test shocks were delivered to the perforant path every 30s for a 10min control period. This was followed by delivery of 3 trains of high frequency stimulation (250Hz for 200ms) at 30s intervals. Recording at test shock frequency resumed for a 40min period. In low-frequency experiments, test shocks were delivered for the duration of the experiment as described in Section 2.5. At the end of the recording period, rats were killed by cervical dislocation and decapitation. Hippocampi, dentate gyri and entorhinal cortices were dissected free on ice and tissue slices were stored at -80 °C for later analysis.

3.2.4 Measurement of ERK activity *in vivo*

ERK activity was analysed in synaptosomes prepared from tetanised and untetanised dentate gyri of rats pretreated with either genistein or saline. Proteins were separated on 10% SDS-polyacrylamide gels as outlined in Section 2.14 and immunoblotted with anti-active ERK (1.5:1000 in TBS-T containing 2% non-fat dried milk; Promega, USA) as described in Section 2.16.

3.2.5 Measurement of phosphorylation of α_1 subunit of voltage operated calcium channels *in vivo*

Phosphorylation of the α_1 subunit was assessed in synaptosomes prepared from tetanised and untetanised dentate gyri of rats pretreated with either genistein or saline. Proteins were immunoprecipitated (see Section 2.12) with anti-pan α_1 subunit (1:25; Alomone Laboratories, Israel) and then separated on 7.5% SDS-polyacrylamide gels. Separated proteins were immunoblotted with anti-phosphotyrosine (1:160 in TBS-T containing 2% non-fat dried milk; Affiniti, UK) as described in Section 2.16.

3.2.6 Analysis of protein synthesis

The method used was described in Section 2.17. Slices, prepared from entorhinal cortices from either tetanised or untetanised sides of the brain of rats pretreated with either genistein or saline, were incubated under constant oxygenation and at 37°C in Krebs solution containing $CaCl_2$ (2mM), ATP (3.5mM) and [^{35}S]-methionine (0.2µl/ml) for 60min. The reaction was terminated and [^{35}S]-methionine labelling of TCA-

precipitated proteins was assessed. Protein concentrations were determined from incubated slices.

3.2.7 Analysis of CREB phosphorylation

CREB phosphorylation was analysed in homogenate prepared from entorhinal cortices from either tetanised or untetanised dentate gyri of rats pretreated with either genistein or saline or from rats subjected to low frequency stimulation. Proteins were separated on 14% SDS-polyacrylamide gels as described in Section 2.14. Separated proteins were immunoblotted with anti-phospho CREB (1:1000 in 5% BSA in TBS-T) as outlined in Section 2.16.

3.3 Results

3.3.1 Genistein inhibits glutamate release from hippocampal synaptosomes *in vitro*

To investigate the role of tyrosine kinase activity in glutamate release, KCl-stimulated glutamate release from hippocampal synaptosomes was examined in the presence or absence of genistein (50μM), a tyrosine kinase inhibitor. Figure 3.1 shows addition of KCl (50mM) caused a significant increase in glutamate release from hippocampal synaptosomes from 0.159 μmol/mg (± 0.012, SEM; n=6) to 0.237 μmol/mg (± 0.024, SEM; n=6; * $p<0.05$, unpaired *t*-test with Welch's correction). KCl failed to elicit the same response in hippocampal synaptosomes incubated in the presence of genistein (50μM). In this group, unstimulated glutamate concentration was 0.074μmol/mg (± 0.009, SEM; n=6) and KCl-stimulated glutamate concentration was 0.083μmol/mg (± 0.007, SEM; n=6). Incubation in the presence of genistein resulted in a significant decrease in unstimulated release of glutamate (+ $p<0.001$, unpaired *t*-test with Welch's correction).

3.3.2 Depolarisation stimulates calcium influx in hippocampal synaptosomes *in vitro*

Figure 3.2 shows the effect of genistein (50μM) on $^{45}Ca^{2+}$ influx in hippocampal synaptosomes. Calcium influx was assessed in the presence and absence of KCl (50mM). The depolarising pulse of KCl significantly increased $^{45}Ca^{2+}$ influx from 335.538 nmol/mg (± 20.462, SEM; n=6) to 520.719nmol/mg (± 42.029, SEM; n=6; * $p<0.01$, unpaired *t*-test with Welch's correction). This response was reduced but not inhibited by the presence of genistein (50μM; * $p<0.05$, unpaired *t*-test with Welch's correction). Mean values in this group were 424.541 nmol/mg (± 27.013, SEM; n=6).

3.3.3 Expression of LTP in the dentate gyrus is blocked by genistein.

The effect of genistein on LTP in perforant path – granule cell synapses was examined by comparing the effect of intracerebroventricular injection of saline with that of genistein (5μl; 50μM). The mean slope of the population excitatory postsynaptic potential (epsp) is expressed as a percentage of the mean epsp in the two-minute

recording period immediately preceding the three high frequency trains of stimuli delivered to the perforant path. The data presented in Figure 3.3 indicate that the genistein-injected group show a small post-tetanic potentiation, which rapidly decayed. LTP was completely blocked in this group. In contrast, the group pretreated with saline showed an immediate increase in EPSP slope after tetanic stimulation. LTP was sustained without decrement until the end of the 40-minute recording period. The mean percentage increases in the population epsp slope following the delivery of tetanising stimuli to the perforant path compared to the recording period prior to high-frequency stimulation were 143.730 (± 5.760, SEM; n=6) and 115.400 (± 2.740, SEM; n=6) in the saline- and genistein-injected groups respectively. The corresponding values in the last 5 minutes of the experiment were 130.080 (± 1.580, SEM; n=6) and 107.870 (± 2.110, SEM; n=6).

3.3.4 Genistein inhibits LTP-induced increase in ERK activity

Figure 3.4A shows a representative immunoblot indicating an increase in ERK activity in synaptosomes prepared from tetanised dentate gyri (lane 2) compared with synaptosomes prepared from untetanised dentate gyri (lane 1) of saline-treated rats. There was no increase in ERK activity in synaptosomes prepared from tetanised dentate gyri (lane 4) compared with synaptosomes prepared from untetanised dentate gyri (lane 3) of genistein-treated rats. Densitometric data from 6 replicate electrophoresis experiments (Figure 3.4B) show a significant increase in ERK phosphorylation in synaptosomes prepared from tetanised dentate gyri of rats preinjected with saline, compared with synaptosomes prepared from untetanised dentate gyri (* $p < 0.05$; unpaired *t*-test with Welch's correction). In the saline-treated groups, mean values for ERK activity in synaptosomes prepared from untetanised and tetanised dentate gyri were 18.459 (± 8.071, SEM; n=6) and 30.291 (± 4.208, SEM; n=6) respectively. Tetanisation failed to elicit an increase in ERK activity in synaptosomes prepared from genistein-treated animals. Mean values (±SEM; n=6; in arbitrary units) were 11.501 ± 2.921 and 21.038 ± 2.101 in synaptosomes prepared from untetanised and tetanised dentate gyri of genistein-treated rats.

3.3.5 Genistein blocks LTP-associated increase in α_1-subunit phosphorylation

One candidate substrate for tyrosine kinase is the α_1-subunit of the voltage-operated calcium channel. Figure 3.5A shows a Western immunoblot indicating enhanced tyrosine phosphorylation of the α_1-subunit of the voltage-activated calcium in synaptosomes prepared from tetanised, compared with untetanised, dentate gyri of saline-injected rats. A comparable trend was absent from synaptosomes prepared from dentate gyri of genistein-injected rats. Figure 3.5B shows the densitometric analysis of 6 Western immunoblotting experiments. In the group pretreated with saline, a significant increase in tyrosine phosphorylation of the α_1-subunit was found in synaptosomes prepared from tetanised dentate gyri compared with untetanised tissue (* $p < 0.05$; unpaired *t*-test with Welch's correction). Phosphorylation of this subunit on a tyrosine residue was 18.355 (± 1.53, SEM; n=6; in arbitrary units) in synaptosomes prepared from untetanised dentate gyri and 26.736 (± 3.143; SEM; n=6; arbitrary units) in synaptosomes prepared from tetanised dentate gyri. In contrast, the tetanus-associated increase was absent in synaptosomes prepared from the genistein-pretreated group. Mean values (±SEM; n=6; in arbitrary units) in the genistein-pretreated group were 18.736 ± 6.6202 in synaptosomes prepared from untetanised dentate gyri and 26.8422 ± 9.709 in synaptosomes prepared from tetanised dentate gyri.

3.3.6 Genistein inhibits LTP-stimulated protein synthesis

Analysis of protein synthesis (Figure 3.6) in the cell bodies of the perforant path synapses, located in the entorhinal cortex, indicated that [^{35}S]-methionine incorporation into new proteins was significantly enhanced (* $p < 0.01$; unpaired *t*-test with Welch's correction) in homogenate prepared from slices of entorhinal cortex prepared from the side of the brain that received tetanic stimulation (ipsilateral; 476.164 cpm/mg ± 29.41, SEM; n=6) compared with the contralateral side (999.387 cpm/mg ± 71.706, SEM; n=6). There was a significant decrease in protein synthesis in contralateral side from genistein-treated animals compared with saline-treated animals (+ $p<0.001$; unpaired *t*-test with Welch's correction). Pretreatment with genistein also abolished the tetanus-related increase in protein synthesis. Mean values (± SEM; n=6) in this group were 156.858

cpm/mg ± 20.677 in homogenate prepared from the side of the brain contralateral side of the brain and 230.390 cpm/mg ± 31.530 from the ipsilateral side of the brain.

3.3.7 Genistein inhibits LTP-associated CREB phosphorylation

In parallel with the enhanced protein synthesis, serine-phosphorylation and thus activation of the transcription factor, CREB was also significantly increased following induction of LTP. Figure 3.7A shows a sample immunoblot which demonstrates that CREB phosphorylation was enhanced in homogenate prepared from the ipsilateral entorhinal cortex (lane 2) compared to the contralateral side (lane 1) in the saline groups and that this LTP-associated increase was inhibited in cells prepared from ipsilateral (lane 4) and contralateral (lane 3) sides of the entorhinal cortex of genistein-injected animals. Analysis of densitometric data (Figure 3.7B) from Western immunoblotting experiments indicated an LTP-related increase in CREB phosphorylation from 10.852 (± 0.949, SEM; n=6) to 17.579 (± 2.102, SEM; n=6) in saline-treated rats (* $p < 0.01$; unpaired *t*-test with Welch's correction). In the genistein-injected animals, tetanic stimulation failed to elicit the same response. In the genistein-treated group, mean values (± SEM; n=6; in arbitrary units) were 9.390 ± 1.634 and 11.658 ± 0.839 in samples prepared from untetanised and tetanised dentate gyri respectively.

3.3.8 Low frequency stimulation does not affect the epsp slope at the perforant path-granule cell synapses

Figure 3.8 shows that low frequency stimulation delivery to the perforant path every 30s for an 50min experimental period did not cause an increase in the epsp slope of the evoked response measured from the granule cell layer of the dentate gyrus.

3.3.9 Low frequency stimulation does not increase glutamate release

Glutamate release from synaptosomes prepared from dentate gyri subject to low frequency stimulation was assessed. KCl-depolarisation stimulated a significant increase in glutamate release from synaptosomes prepared from the contralateral side (* $p<0.05$, unpaired *t*-test with Welch's correction). Incubation in the presence of KCl failed to induce a further increase in glutamate release from synaptosomes from the ipsilateral

dentate gyrus (* $p<0.05$, unpaired *t*-test with Welch's correction). Mean values of glutamate released from synaptosomes prepared from the contralateral side were 0.460 μmol/mg (± 0.060, SEM; n=6) and 0.610μmol/mg (± 0.070, SEM; n=6) for unstimulated and KCl-stimulated release respectively. Glutamate concentrations from synaptosomes prepared from the ipsilateral dentate gyrus were 0.390 μmol/mg (± 0.050, SEM; n=6) and 0.560 μmol/mg (± 0.060, SEM; n=6).

3.3.10 Low frequency stimulation does not enhance ERK phosphorylation

ERK activation was measured in synaptosomes prepared from dentate gyri subjected to low frequency stimulation and from the contralateral side. Figure 3.10A shows a representative immunoblot indicating no change in ERK activity in synaptosomes prepared from dentate gyri following low frequency stimulation (lane 1) compared with ERK activity in synaptosomes prepared from the contralateral side of the dentate gyrus (lane 2). Analysis of densitometric data presented in Figure 3.10B indicates that ERK activity in synaptosomes prepared from dentate gyrus following low frequency stimulation was not enhanced compared with ERK activity in synaptosomes prepared from the contralateral dentate gyrus ($p>0.05$, unpaired *t*-test with Welch's correction). Mean values (±SEM, n=6; in arbitrary units) were 27.601 ± 3.600 in the ipsilateral side and 27.971 ± 5.290 in the contralateral side.

3.3.11 Low frequency stimulation does not stimulate CREB phosphorylation

CREB phosphorylation was measured in synaptosomes prepared from dentate gyri subjected to low frequency stimulation and from the contralateral side. Figure 3.11A shows a representative immunoblot showing no increase between CREB phosphorylation following low frequency stimulation (lane 1) and in the absence of stimulation (lane 2). Densitometric data from Western immunoblotting experiments shown in figure 3.11B indicated no difference in CREB phosphorylation in synaptosomes prepared from the ipsilateral and contralateral sides of the dentate gyri following low frequency stimulation ($p>0.05$, unpaired *t*-test with Welch's correction). Means values (±SEM, n=6; in arbitrary units) were 10.550 ± 0.580 for the ipsilateral side and 12.056 ± 0.732 for the contralateral side.

Figure 3.1: Genistein inhibits glutamate release from hippocampal synaptosomes *in vitro*

Addition of KCl (50mM) caused a significant increase in glutamate release in synaptosomes prepared from hippocampus (* $p<0.05$; unpaired *t*-test with Welch's correction). KCl-stimulated glutamate release was blocked in hippocampal synaptosomes incubated in the presence of genistein (50μM). Unstimulated glutamate release was significantly reduced in synaptosomes incubated in the presence of genistein compared with synaptosomes incubated in the absence of genistein (+ $p<0.001$; unpaired *t*-test with Welch's correction). The values are presented as the mean (± SEM) of 6 independent observations and are expressed as μmol glutamate/mg protein.

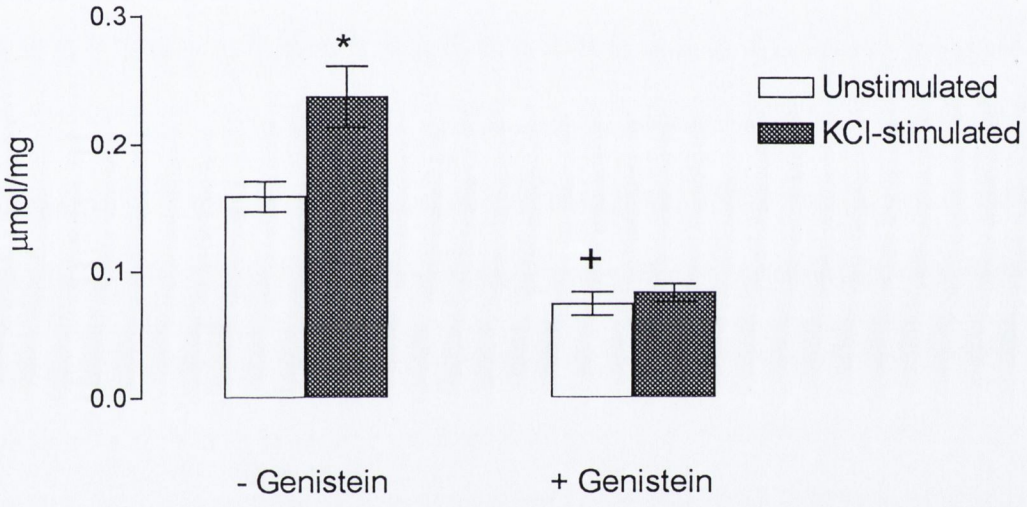

0.3
0.2
0.1
0.0
µmol/mg
*
+
Unstimulated
KCl-stimulated
- Genistein
+ Genistein

Figure 3.2: Depolarisation stimulates calcium influx in hippocampal synaptosomes *in vitro*

Intracellular calcium concentration was significantly increased by addition of KCl (50mM) to synaptosomes prepared from hippocampus (*$p<0.05$; unpaired *t*-test with Welch's correction). The effect of KCl on calcium influx was not inhibited in the presence of genistein (50μM). The values are expressed in nmol Ca^{2+}/mg protein and are means (± SEM) of 6 independent observations.

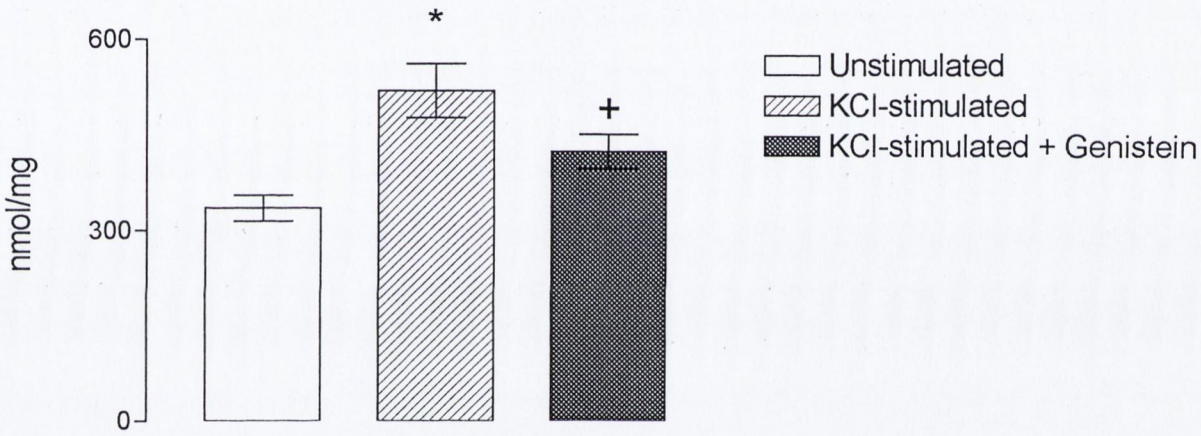

600
300
0
nmol/mg
*
+
Unstimulated
KCl-stimulated
KCl-stimulated + Genistein

Figure 3.3. Expression of LTP in the dentate gyrus *in vivo* is inhibited by genistein. Expression of LTP was inhibited by intracerebroventricular injection of genistein (Final concentration: 50μM). The slope of the epsp recorded from the granule cell layer of the dentate gyrus evoked by delivery of test stimuli to the perforant path at 30s intervals before and after tetanus in saline- and genistein-pretreated rats is shown. An arrow indicates the delivery of tetanus. The results are expressed as mean % changes in EPSP slope with reference to the epsp slope in the 2 minutes immediately preceding tetanisation. Results are expressed as means of 6 observations for each group. SEM values are shown for every 10^{th} response.

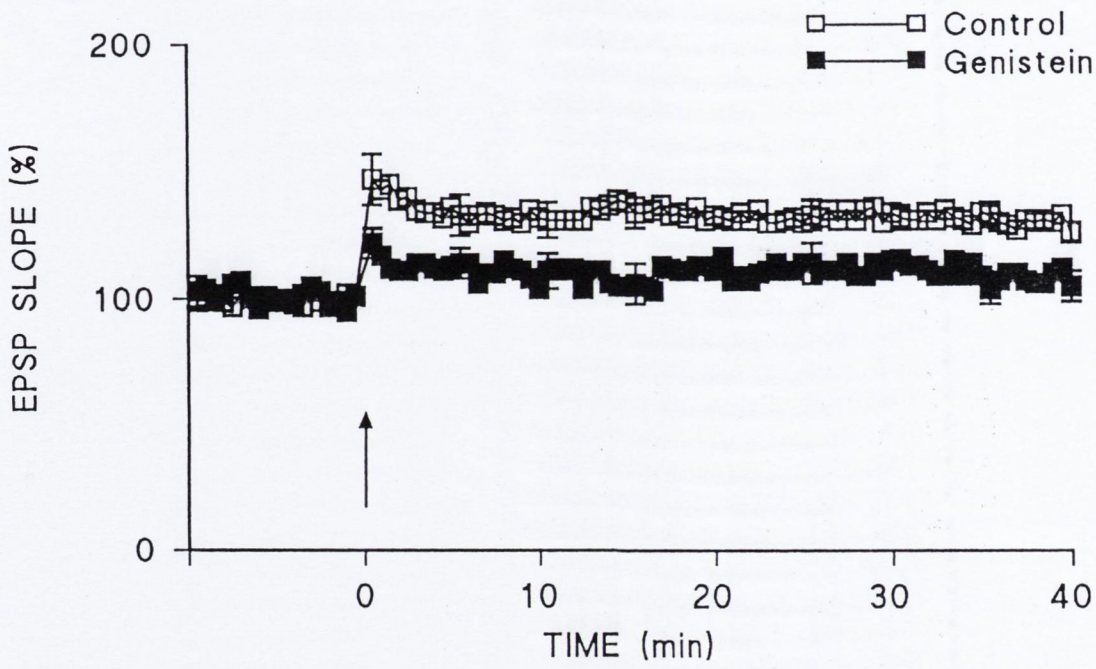
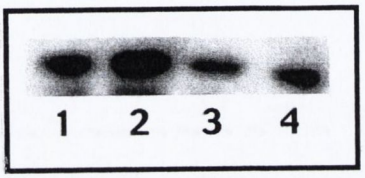

Control
Genistein
200
100
0
EPSP SLOPE (%)
0
10
20
30
40
TIME (min)

Figure 3.4. LTP-associated increase in ERK phosphorylation in dentate gyrus was inhibited by genistein.

A. Following the induction of LTP in rats pretreated with saline, phosphorylation of ERK was greater in synaptosomes prepared from dentate gyrus in tetanised dentate gyri (lane 2) compared with untetanised dentate gyri (lane 1). This tetanus-induced increase in ERK phosphorylation was blocked in synaptosomes prepared from untetanised (lane 3) and tetanised (lane 4) dentate gyri of rats pretreated with genistein.

B. Analysis of densitometric data indicates that ERK phosphorylation was significantly increased following tetanisation in dentate gyrus tissue prepared from rats pretreated with saline (* $p<0.05$; unpaired *t*-test with Welch's correction). The LTP-associated increase was blocked in synaptosomes prepared from the dentate gyri of rats preinjected with genistein. Results are expressed as arbitrary units and are means (± SEM) of 6 observations.

A.


1
2
3
4

B.

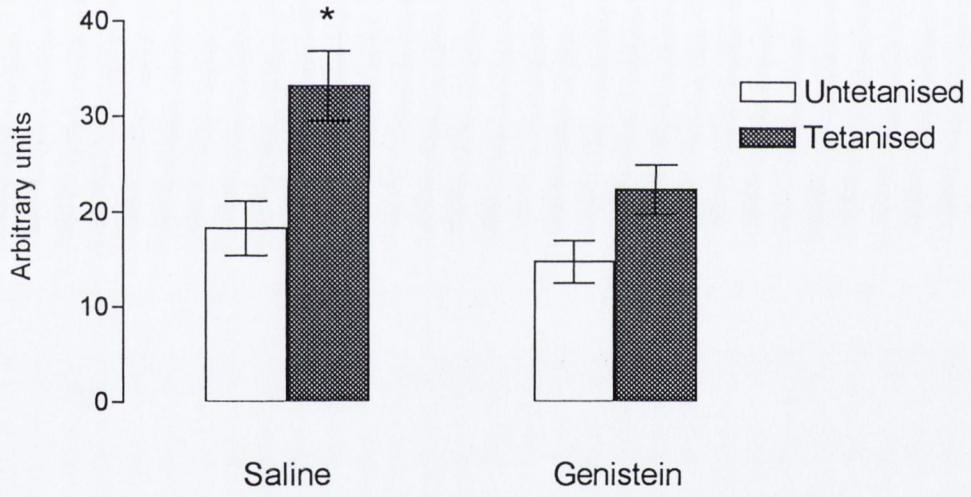
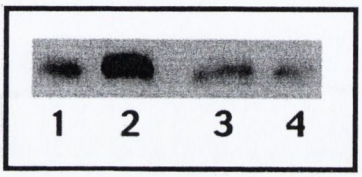

Arbitrary units
40
30
20
10
0
*
Untetanised
Tetanised
Saline
Genistein

Figure 3.5: LTP-induced increase in tyrosine phosphorylation of the calcium channel α_1 subunit in dentate gyrus was blocked by genistein.

A. Tyrosine phosphorylation of the calcium channel α_1 subunit was greater in synaptosomes prepared from tetanised (lane 2) compared with untetanised (lane 1) dentate gyrus of saline-injected rats as shown in this representative immunoblot. In contrast, pretreatment with genistein blocked the tetanus-associated increase in the calcium channel α_1 subunit phosphorylation in synaptosomes prepared from tetanised (lanes 3) compared with untetanised dentate gyri (lane 4).

B. Analysis of densitometric data indicates that in saline-injected rats, mean values of tyrosine phosphorylation of the α_1 subunit of calcium channel were significantly increased in synaptosomes prepared from dentate gyrus following expression of LTP (*$p<0.05$; unpaired *t*-test with Welch's correction). This effect of tetanus was inhibited by pretreatment with genistein. Results are given as arbitrary units and are expressed as means ± SEM of 6 observations.

A.


1
2
3
4

B.

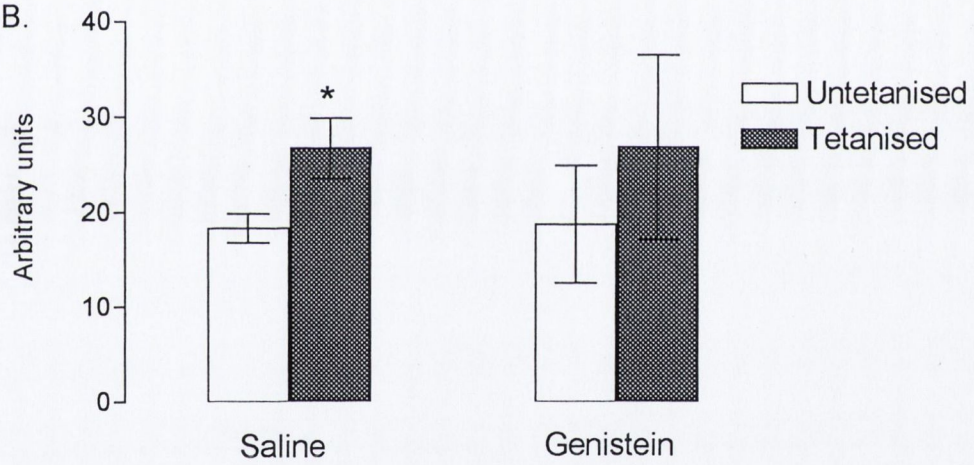

40
30
20
10
0
Arbitrary units
*
Untetanised
Tetanised
Saline
Genistein

Figure 3.6. Increased [^{35}S]-methionine labelling of TCA-precipitated proteins in the entorhinal cortex following tetanus is inhibited by genistein.

Protein synthesis was increased in homogenate prepared from ipsilateral entorhinal cortices compared with homogenate prepared from contralateral entorhinal cortices following tetanus (* $p<0.001$, unpaired *t*-test with Welch's correction). In contrast, there was no change in protein synthesis following tetanus in homogenates prepared from the entorhinal cortices of the genistein-pretreated group. Protein synthesis was significantly decreased in homogenate prepared from ipsilateral entorhinal cortices of the genistein-injected group compared with the saline-injected group (+ $p<0.001$; unpaired *t*-test with Welch's correction). Results are expressed as cpm/mg protein and are means (± SEM) of 6 observations.

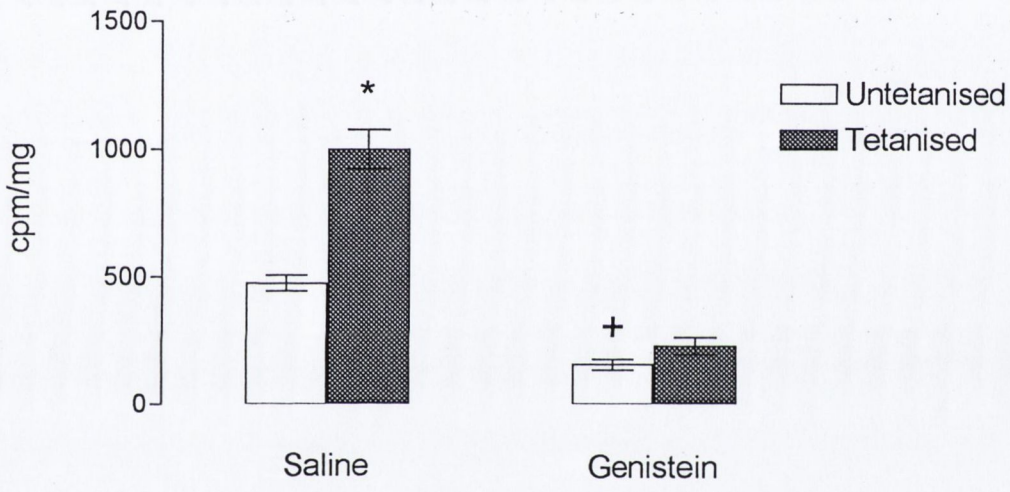
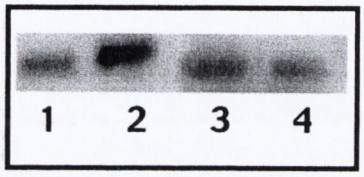

1500
1000
500
0
cpm/mg
*
+
Untetanised
Tetanised
Saline
Genistein

Figure 3.7: LTP-associated phosphorylation of CREB in the entorhinal cortex is blocked by genistein.

A. Phosphorylation of CREB was greater in homogenates prepared from ipsilateral entorhinal cortices (lane 2) compared with the contralateral entorhinal cortices (lane 1) of saline-injected rats following tetanisation as shown in this representative immunoblot. In contrast, pretreatment with genistein blocked the increase in CREB phosphorylation in homogenate prepared from the ipsilateral entorhinal cortex (lane 4) compared with homogenate prepared from the contralateral side (lane 3) following tetanus.

B. Analysis of densitometric data indicates that in saline-injected rats mean values of CREB phosphorylation were significantly increased in homogenates prepared from entorhinal cortex (*$p<0.01$; unpaired *t*-test with Welch's correction). This effect was absent from homogenate prepared from entorhinal cortices of genistein-treated rats. Results are given as arbitrary units and are expressed as means (± SEM) of 6 observations.

A.


1
2
3
4

B.

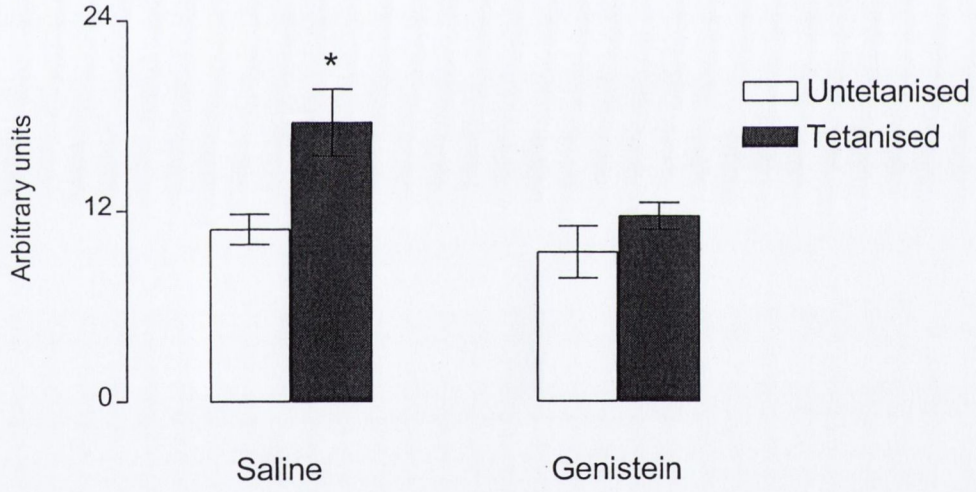

24
12
0
Arbitrary units
*
Untetanised
Tetanised
Saline
Genistein

Figure 3.8. Low frequency stimulation does not affect the epsp slope in the perforant path-granule cell synapses

Low frequency stimulation was delivered to the perforant path and the response was measured from the granule cell layer of the dentate gyrus. Test shocks were applied at 30s intervals for the 50min experimental period.

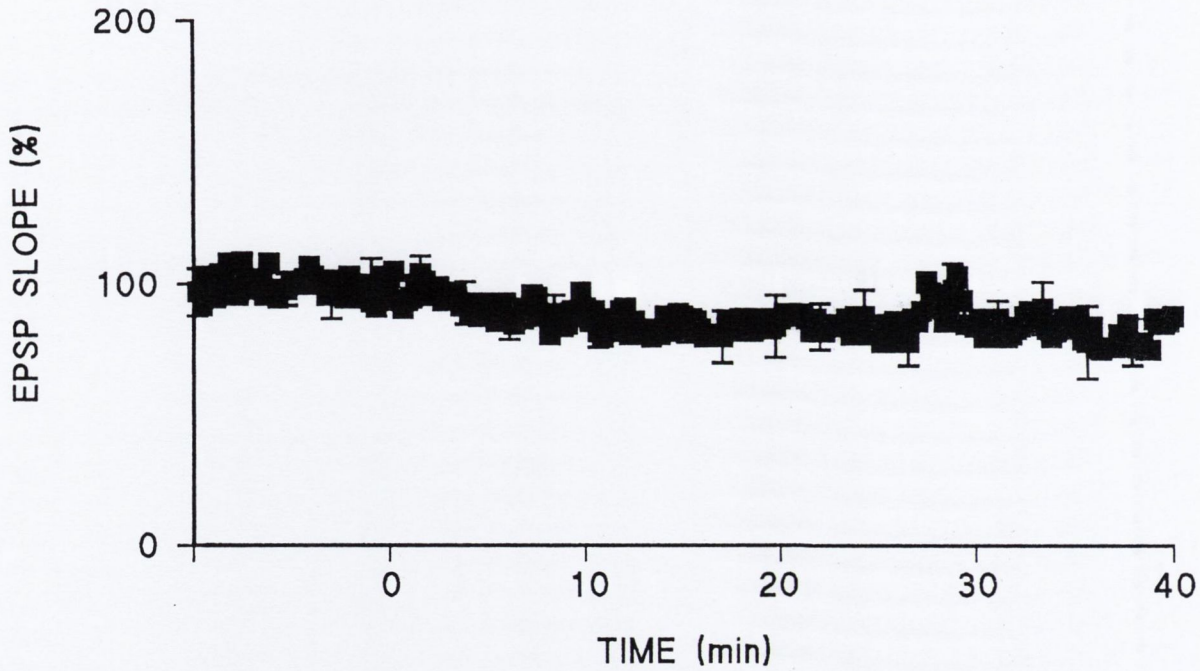

200
100
0
EPSP SLOPE (%)
0
10
20
30
40
TIME (min)

Figure 3.9. Glutamate release is not enhanced by low frequency stimulation

KCl-stimulated glutamate release was not significantly increased in synaptosomes prepared from dentate gyri following low frequency stimulation (**LHS**) compared with glutamate release from synaptosomes prepared from the contralateral dentate gyri (**RHS**). Results are expressed as μmol glutamate/mg protein and are means (± SEM) of 6 observations.

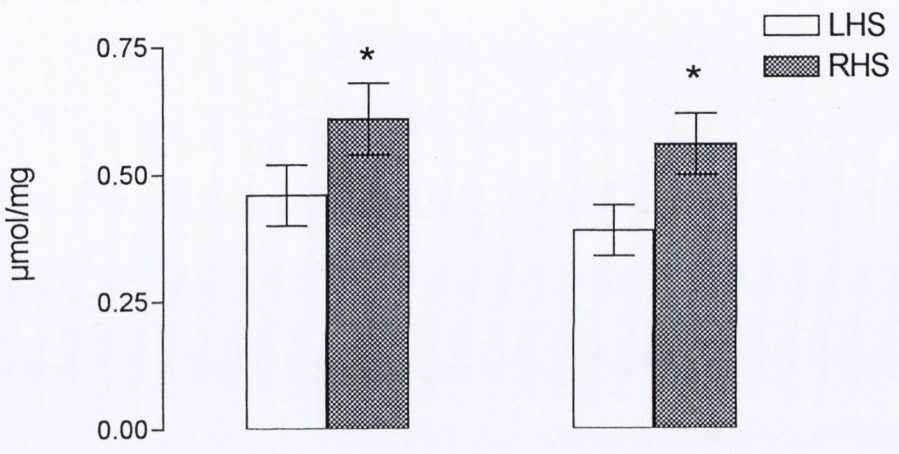
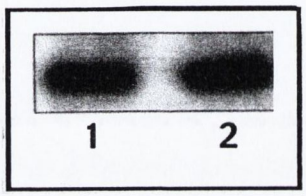

LHS
RHS
0.75
0.50
0.25
0.00
µmol/mg
*
*

Figure 3.10. ERK activity is not increased by low frequency stimulation

A. The representative immunoblot shows activation of ERK was not significantly greater in synaptosomes prepared from dentate gyrus following low frequency stimulation (lane 1) compared with samples prepared from contralateral dentate gyrus (lane 2).

B. Analysis of densitometric data demonstrates that mean values of ERK activity were not significantly increased in synaptosomes prepared from ipsilateral (**LHS**) or contralateral dentate gyri (**RHS**) following low frequency stimulation ($p>0.05$; unpaired *t*-test with Welch's correction). Results are given as arbitrary units and are expressed as means (± SEM) of 6 observations.

A.



B.

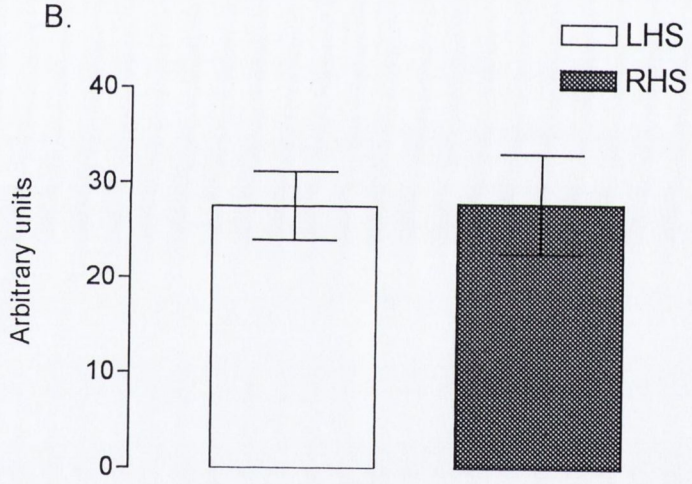
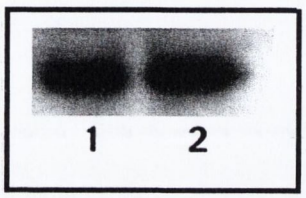


Figure 3.11. CREB phosphorylation is not stimulated by low frequency stimulation

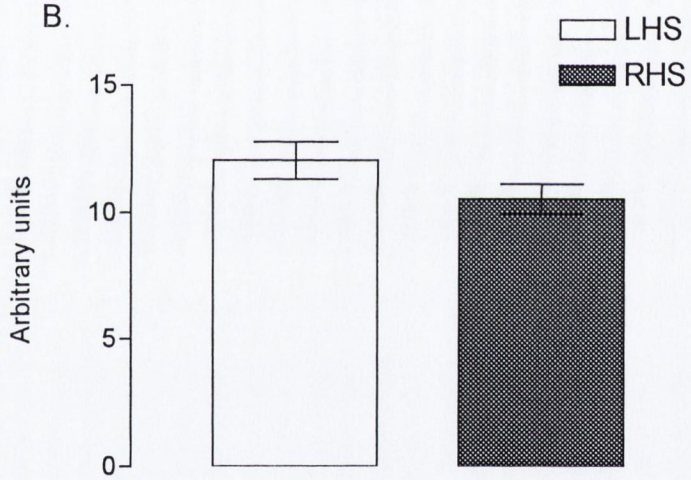
C. The representative immunoblot shows phosphorylation of CREB was not significantly greater in homogenates prepared from entorhinal cortex following low frequency stimulation (lane 1) compared with samples prepared from contralateral entorhinal cortex (lane 2).

D. Analysis of densitometric data demonstrates that mean values of CREB phosphorylation were not significantly increased in homogenates prepared from slices of ipsilateral or contralateral entorhinal cortices following low frequency stimulation ($p>0.05$; unpaired *t*-test with Welch's correction). Results are given as arbitrary units and are expressed as means (± SEM) of 6 observations.

A.



B.



3.4 Discussion

The aim of this study was to investigate the possible role of tyrosine kinase in LTP at perforant path-granule cell synapses. The data show 1) tyrosine kinase activity is involved in the LTP-associated process of glutamate release in hippocampal synaptosomes, 2) genistein blocks the LTP-induced increase in phosphorylation of ERK and voltage-operated calcium channel subunit in the dentate gyrus and 3) genistein inhibits increased protein synthesis and CREB phosphorylation following tetanisation. These findings support a multi-faceted role for tyrosine kinase in the expression of LTP in the dentate gyrus and in the maintenance of LTP resulting from changes in synthesis of new proteins in the entorhinal cortex. Significantly, low-frequency stimulation to the perforant path did not cause changes in glutamate release or ERK activation in the dentate gyrus and CREB phosphorylation in the entorhinal cortex.

Addition of KCl to hippocampal synaptosomes resulted in a significant increase in endogenous glutamate release but this was inhibited by the presence of the tyrosine kinase inhibitor, genistein. This finding is consistent with previous findings in the hippocampus (Mullany *et al.*, 1996) and the cortex (Phillis *et al.*, 1996). This indicates a role for tyrosine kinase in transmitter release, possibly by the inhibition of tyrosine phosphorylation of the synaptic vesicle protein, synaptophysin, thus impeding formation of the fusion pore (Thomas and Betz, 1990) and therefore blocking exocytosis. Other possible means of inhibition are the prevention of ERK phosphorylation, which may impact on synapsin 1 phosphorylation (Jonanovic *et al.*, 1996) and therefore on glutamate release. Genistein also occluded the release of glutamate in the absence of KCl-depolarisation. This may indicate a disturbance of the cytosolic glutamate pool, but the mechanism underlying this needs further investigation. Two possible ways in which this occurs are 1) by alteration of membrane properties or 2) by modulation of the activity of the sodium-glutamate co-transporter located in the synaptosomal membrane. Genistein is an antibiotic and antibiotics are reported to have ionophoric properties (Grigoriev *et al.*, 1997); Formation of an ionophore may result in indiscriminate leakage of glutamate across the synaptosomal membrane. Alternatively, genistein may modulate the activity of Na^+-glutamate co-transporter (Robinson and Dowd, 1997) in an ATP-dependent manner

as genistein is an inhibitor of ATP-binding enzymes (Akiyama *et al.*, 1987, Huang *et al.*, 1992b); this would result in a decrease in unstimulated glutamate release.

Because inhibition of calcium entry into the presynaptic terminal may impact on calcium-dependent glutamate release, the effect of genistein on calcium influx into hippocampal synaptosomes was assessed. Analysis of $^{45}Ca^{2+}$ influx in synaptosomes prepared from rat hippocampus indicates that KCl-induced depolarisation significantly increases influx of the isotope. This finding is consistent with previous reports of depolarisation-induced calcium influx in hippocampal synaptosomes (Kelly and Lynch 1998, Kelly and Lynch, 2000). Genistein attenuated this response but did not abolish it. This finding is in contrast to previous findings that suggest that tyrosine kinase is involved in the regulation of voltage-dependent calcium channels (Fitzgerald and Dolphin, 1997, Potier and Rovira, 1999). The magnitude of the calcium transient in the presynaptic terminal is likely to have direct implications for glutamate release due to its calcium-dependent nature (Mullany *et al.*, 1996). Parallel increases in tyrosine kinase activity and glutamate release induced by a depolarising pulse suggest a possible cascade effect that is abolished with the inhibition of tyrosine kinase activity by genistein. This supports the proposal that activity-dependent changes in the presynaptic terminal may result in increased glutamate release (Maguire *et al.*, 1999; Canevari *et al.*, 1994) and points to tyrosine phosphorylation as a possible component of the upregulating mechanism.

The expression of long-term potentiation in the granule cell–perforant path synapses in the dentate gyrus was inhibited by the intracerebroventricular administration of genistein prior to the delivery of tetanising stimuli. This is corroborated by previous findings which reported that tyrosine kinase inhibitors block LTP in both the CA1 (O'Dell *et al.*, 1991a) and dentate gyrus (Abe and Saito, 1993; Maguire *et al.*, 1999). The locus of inhibition may be presynaptic or postsynaptic due to the localisation of possible substrates. The mechanism of inhibition may be due to additive effects of phosphorylation of several substrates of tyrosine kinase pre- and postsynaptically such as synaptophysin (Pang *et al.*, 1988), synaptogyrin (Baumert *et al.*, 1990), phospholipase Cγ (McGahon *et al.*, 1998), ERK (Maguire *et al.*, 1999) or the 2B subunit of the NMDA receptor (Rostas *et al.*, 1996, Rosenblum *et al.*, 1996). Other studies have reported the involvement of non-receptor tyrosine kinases, *src* and *fyn* in LTP (Grant *et al.*, 1992;

Huang and Hsu, 1999) and the ability of src to phosphorylate the NMDA 2B subunit is well-documented (Yu *et al.*, 1997). Evidence implicating neurotrophins in the expression of LTP (Thoenen, 1995) and pointing to activity-dependent expression of neurotrophins (Zafra *et al.*, 1990, Gall *et al.*, 1989) may provide the identity of the extracellular trigger for tyrosine kinase in LTP, as members of the nerve growth factor family stimulate tyrosine kinase cascades (Segal and Greenberg, 1996).

As glutamate release is blocked by genistein, synaptosomes prepared from the dentate gyri were analysed for possible changes in tyrosine phosphorylation of candidate proteins such as ERK and calcium channel α_1 subunit. Phosphorylation of ERK was elevated in samples prepared from dentate gyri that sustained LTP in saline-injected rats and this trend was less pronounced in the genistein-injected group. Tetanus-associated increases in ERK have been previously reported in the CA1 (English and Sweatt, 1996) and in the dentate gyrus (Maguire *et al.*, 1999). In addition to this, inhibitors of MEK, which stimulates ERK, have been shown to block LTP (Liu *et al.*, 1999, McGahon *et al.*, 1999). Failure of the tetanising stimuli to elicit increased ERK phosphorylation may contribute to the inhibition of LTP by genistein, perhaps by blocking the LTP-associated increase in glutamate release. Abolition of ERK-dependent phosphorylation of synapsin-1, as suggested by Jonanovic and coworkers (1996), may attenuate glutamate release and therefore block LTP. ERK may serve as a convergence point for PKA, PKC and CaM kinase signals (Roberson *et al.*, 1999, Vanhoutte *et al.*, 1999, Impey *et al.*, 1998), suggesting cross-talk between different signalling pathways. Integration of these signals at ERK may allow increased activation of CREB mediated by RSK (Xing et al., 1996). Activation of CREB is a consequence of the expression of LTP in the dentate gyrus (Davis et al., 2000) and may govern the persistence of LTP, by upregulating of immediate early genes (Richardson *et al.*, 1992).

Because glutamate release is a calcium-dependent process, analysis of tyrosine phosphorylation of the α_1 subunit of the voltage activated calcium channel was conducted. The data indicate an increased phosphorylation of the α_1 subunit on tyrosine residues associated with the expression of LTP in synaptosomes prepared from dentate gyrus in saline-treated rats. Phosphorylation of the channel subunit on tyrosine residues may have a functional significance as it is the pore-forming subunit of the channel and has been reported in several tissue types such as atrial myocytes (Boixel *et al.*, 2000),

spermatogenic cells (Arnoult *et al.*, 1997), dorsal root ganglion neurons (Fitzgerald and Dolphin, 1997), smooth muscle cells (Wijetunge *et al.*, 1995) and hippocampal neurons (Potier and Rovira, 1999). Alteration of channel configuration by phosphorylation of the α_1 subunit may alter calcium influx by delaying the closure of the pore in the channel thus having a knock-on effect on increasing the calcium-dependent release of glutamate. Furthermore, the α_1 subunit has been shown to interact with synaptic vesicle proteins (Rettig et al., 1997) and thus, may modify synaptic vesicle activity and ultimately glutamate release. In synaptosomes prepared from the dentate gyri of genistein-injected rats, the tetanus-associated increase in tyrosine phosphorylation of the α_1 subunit was not observed. This is consistent with the blockade of high-voltage calcium currents in hippocampal cells by genistein reported by Potier and Rovira (1999). Furthermore, it has been suggested that the activity of voltage-gated calcium channels may underlie a form of LTP, VOCC-LTP, independent of NMDA receptor activity, in the CA1 region (Morgan and Teyler, 1999). Application of tyrosine kinase inhibitors, genistein and lavendustin A blocked VOCC-LTP while NMDA-mediated LTP remained unaffected (Cavus and Teyler, 1996).

Calcium plays an important role in regulation of gene expression (Xia *et al.*, 1995). Therefore, modulation of calcium influx through voltage-operated calcium channels by this means may alter gene expression in hippocampal neurons or may be an additional mechanism by which neurotrophins can regulate gene expression. Alternatively, activity-dependent increases in cytosolic calcium concentrations can regulate the expression of several IEGs in part through the Ras/Raf/ERK pathway (Impey *et al.*, 1998; Xia *et al.*, 1995). This mechanism of calcium-induced ERK activation and subsequent IEG expressions has been thought to support activity-dependent changes in neuronal function (Finkbeiner *et al.*, 1996) thus modulation of calcium channel activity by tyrosine kinase, perhaps initiated by neurotrophin signalling, may modify calcium-dependent gene expression. Absence of tyrosine kinase activity, manifest as reduced tyrosine phosphorylation of ERK and of the calcium channel α_1 subunit in this group, may have contributed to the failure to sustain LTP. Other candidate components of this cascade and the exact mechanism underlying the inhibition by genistein remain to be fully elucidated.

The site of protein synthesis for relevant plasticity-related proteins is the cell body (Wenzel *et al.*, 1993), although some synthesis may occur in the dendrites (Schuman, 1997). The perforant path, the input to the dentate gyrus, originates in the entorhinal cortex and this area was therefore examined for changes in transcription factor regulation and protein synthesis. In agreement with a previous report (Davis *et al.*, 2000), increased phosphorylation of the transcription factor CREB was found following LTP-inducing tetanus in the saline-injected group, but this increase was not mirrored in the genistein-injected group. Tyrosine kinase involvement in the activation of CREB may rely on ERK activation (Finkbeiner, 2000). The parallel increase of CREB activation and protein synthesis may point to a coupling of these processes to stabilise LTP, by facilitating long-term synaptic changes in morphology. Analysis of synthesis of new proteins was therefore conducted on the entorhinal cortex and revealed that [^{35}S] methionine incorporation into new proteins was significantly greater following the induction of LTP at the perforant path-granule cell synapses. This is consistent with previous findings that protein synthesis inhibitors block the induction of LTP in both the CA1 (Stanton and Sarvey, 1984) and dentate gyrus (Krug *et al.*, 1984). It also agrees with findings suggesting an LTP-associated increase in protein synthesis (Mullany and Lynch, 1997) and CREB-mediated protein synthesis in the perforant path following tetanus (Nguyen and Kandel, 1996). This may support the morphological changes that accompany the expression of LTP such as the increase in spine number and spine area (Fifkova *et al.*, 1977) and also in the increase in synaptic vesicle proteins, including synaptophysin, synapsin and synaptotagmin (Lynch *et al.*, 1994). Increased levels of synthesis of presynaptic proteins may underpin the sustained increase in glutamate release observed following the induction of LTP. Genistein reduces the level of protein synthesis in the absence of KCl stimulation. This finding may be due to the effects of genistein on ATP-dependent activity (Akiyama *et al.*, 1987, Huang *et al.*, 1992).

To investigate the effects of low-frequency stimulation on synaptic activity, glutamate release and activation of ERK were assessed in the dentate gyrus and CREB activity was assessed in the entorhinal cortex. The delivery of low-frequency stimulation to the perforant path failed to elicit enhanced synaptic activity in the perforant path-granule cell synapses. This is consistent with previous reports (Lauri et al., 1999; Fukunaga *et al.*, 1999) that only tetanising stimuli induce LTP. Glutamate release and

ERK activity were not increased in the dentate gyrus following low-frequency stimulation. This is consistent with previous reports that ERK activity was not increased by low-frequency trains in the CA1 region (Fukunaga *et al.*, 1999). In their study, Fukunaga and coworkers demonstrated that phosphorylation of synapsin I, a synaptic vesicle protein and ERK substrate (Jonanovic *et al.*, 1996), was not stimulated by low-frequency trains. This may explain why low-frequency stimulation failed to enhance glutamate release. CREB phosphorylation was not increased following low-frequency stimulation of the perforant path. This is in agreement with previous findings of CREB activation that is only induced by stimuli capable of inducing a persistent enhancement of synaptic strength (Schulz *et al.*, 1999). It seems reasonable therefore to conclude that the changes reported here are related to the induction of LTP and are not due to non-selective effects of stimulation.

Tyrosine phosphorylation of proteins such as ERK and the voltage activated calcium channel α_1 reported in this study, in parallel with tyrosine kinase activity in the LTP-associated feature of glutamate release, points towards a role for presynaptic tyrosine kinase in the transduction mechanism underlying long-term potentiation in the dentate gyrus. The contribution of ERK phosphorylation, as a possible route to modulate synaptic transmission or consolidate LTP by upregulating protein synthesis via activation of CREB, is abolished by tyrosine kinase inhibition. Tyrosine phosphorylation of α_1 subunit may be a necessary prerequisite step in LTP-enhanced glutamate release, which is occluded by genistein. The exact sequence of events leading to ERK stimulation and α_1 subunit modulation has yet to be elucidated and the kinases that result in their phosphorylation identified. Similarly, the consequences of CREB phosphorylation and subsequently increased protein synthesis in the entorhinal cortex following tetanus have to be further investigated to identify possible protein products. The data here, however, argue the important role of tyrosine kinase in synaptic changes in the hippocampus by providing immediate means of modulation of synaptic activity as well as more persistent modifications to support long-term changes in synaptic architecture.

Chapter 4

Investigating intracellular signalling mechanisms underlying the actions of NT-3

4.1 Introduction

Neurotrophins propagate intracellular signals through a tyrosine kinase cascade, initiated by a receptor tyrosine kinase, Trk, and involving several tyrosine kinase substrates such as ERK and PI3-kinase (Segal and Greenberg, 1996). The physiological consequences of such signalling cascades in the nervous system are the subject of much interest. As neurotrophin release is activity-dependent, it is possible that they act as neuromodulators. Possible short-term activity-related effects are increased glutamate release (Lessman *et al.*, 1994, Knipper *et al.*, 1994) or neurotrophin-induced neurotrophin release (Canossa *et al.*, 1997; Kruttgen *et al.*, 1998).

Activity-dependent NGF (Blöchl and Thoenen, 1995) and BDNF release (Goodman *et al.*, 1996; Androutsellis-Theotokis *et al.*, 1996) has been reported at central synapses but neurotrophin release was abolished in the presence of Trk inhibitors (Canossa *et al.*, 1997; Krüttgen *et al.*, 1998), pointing to a possible causal relationship between neurotrophin-mediated signalling and neurotrophin release. Recent work has indicated that metabotropic glutamate receptor activity and Trk receptor activity may combine to regulate neurotrophin release by stimulating PLCγ (Canossa *et al.*, 2001).

Neurotrophins can exert both short-term and long-lasting effects. Knipper and coworkers (1994) were the first group to suggest that neurotrophins can modulate glutamatergic synaptic transmission in the CA1 region of the hippocampus (1994). Subsequent to this, BDNF, NT-4 (Lessmann *et al.*, 1994) and NT-3 (Kokaia *et al.*, 1998) were found to enhance glutamatergic transmission in the hippocampus, providing evidence of a short-term modulatory action of neurotrophins. It is likely that neurotrophin signalling may lead to long-term effects such as modification of synaptic structure (Martinez *et al.*, 1998) or neuronal morphology (Lambelle and Leclerc, 2000), which may necessitate protein synthesis.

The aims of this study were 1) to investigate mechanisms which control NT-3 release and 2) to elucidate the consequences of neurotrophin signalling with specific interests in glutamate release, second messenger activation and protein synthesis.

4.2 Methods

4.2.1 Analysis of endogenous glutamate release

Synaptosomes prepared from dentate gyri were incubated in the presence of 3 different concentrations of BDNF (40ng/ml, 200ng/ml, 1000ng/ml) or NT-3 (4ng/ml, 20ng/ml, 100ng/ml) for 15min at 37°C under continuous oxygenation. In other experiments, synaptosomes were incubated in the presence of NT-3 (final concentration: 20ng/ml) or ACPD (final concentration: 50μM), alone or in combination for 15min at 37°C under continuous oxygenation. Aliquots were then incubated in the presence or absence of KCl (50mM) for 3min. Filtrates were collected under vacuum as described in Section 2.6. Synaptosomal suspensions were retained for analysis of protein concentrations. Filtrates were analysed for glutamate concentrations as described in Section 2.7.

4.2.2 Measurement of ERK activity

Synaptosomes prepared from dentate gyri were incubated with KCl (50mM) or alone or in combination with NT-3 (final concentration: 20ng/ml) and tyrphostin AG879 (final concentration: 100μM) for 15min at 37°C under constant oxygenation. Samples were separated using 10% SDS-polyacrylamide gels and immunoblotted with anti-active ERK (1.5:1000 in TBS-T containing 2% non-fat dried milk; Promega, USA) as described in Section 2.16.

4.2.3 Analysis of protein synthesis

The method used was described in Section 2.17. Slices of entorhinal cortices and hippocampi were incubated with NT-3 (20ng/ml) or NGF (50ng/ml) for 45min at 37°C under constant oxygenation. Supernatant was removed and slices were incubated in Krebs solution containing $CaCl_2$ (2mM), ATP (3.5mM) and [^{35}S]-methionine (0.2μl/ml) for 60min at 37°C under constant oxygenation. The reaction was terminated and [^{35}S]-methionine incorporation into TCA-precipitated proteins was determined by liquid scintillation counting.

4.2.4 Analysis of NT-3 release

To assess the effect of depolarisation on NT-3 release, slices and synaptosomes were prepared from dentate gyri and samples were collected as outlined in Section 2.10. In some experiments, synaptosomes were incubated in the presence of NGF (final concentration: 50ng/ml), BDNF (final concentration: 200ng/ml) or tyrphostin AG879 (final concentration: 100mM) for 15min at 37°C under continuous oxygenation prior to preincubation in Krebs solution containing $CaCl_2$ (2mM). Supernatants were analysed for NT-3 concentrations as outlined in Section 2.11 and protein concentrations were determined from synaptosomal suspensions.

4.3 Results

4.3.1 Tyrphostin AG879 blocks NT-3-induced ERK activity.

Figure 4.1A, a representative immunoblot, indicates greater ERK activity in synaptosomes prepared from dentate gyrus incubated in the presence of NT-3 compared with samples incubated in the absence of NT-3. Co-incubation with NT-3 and tyrphostin AG879 suppressed this NT-3-induced effect. Incubation with tyrphostin alone had no effect on ERK activity. Analysis of densitometric data, shown in Figure 4.1B, demonstrates the significant stimulatory effect of NT-3 on ERK activation in synaptosomes prepared from dentate gyrus (*$p<0.05$, unpaired *t*-test with Welch's correction). Mean values (±SEM; in arbitrary units) for 6 observations were 27.077 ± 2.378 and 43.552 ± 4.599 in unstimulated and NT-3-stimulated samples respectively. In contrast, incubation with NT-3 together with tyrphostin AG879 blocked ERK activation. Tyrphostin AG879 alone had no effect on ERK activation. Mean values (±SEM; n=6; in arbitrary units) in the tyrphostin groups were 28.342 ± 3.353 and 34.342 ± 4.737 in the absence and presence of NT-3 respectively.

4.3.2 Depolarisation induces NT-3 release from synaptosomes prepared from dentate gyrus.

Figure 4.2 demonstrates that NT-3 release in synaptosomes prepared from dentate gyrus was significantly increased in the presence of KCl (50mM) compared with samples incubated in the absence of KCl (* $p<0.01$, unpaired *t*-test with Welch's correction). Mean values (±SEM; n=6) of 6 independent observations were 0.121 pmol NT-3/mg protein ± 0.007 and 0.250 pmol NT-3/mg protein ± 0.020 from unstimulated and KCl-stimulated synaptosomes respectively.

4.3.3 Neither NGF nor BDNF enhance depolarisation-stimulated NT-3 release from slices prepared from dentate gyrus.

Figure 4.3 shows the effect of NGF and BDNF on NT-3 release in slices prepared from the dentate gyrus. NT-3 release was induced in slices incubated in the absence of a neurotrophin by KCl-stimulated depolarisation (+ $p<0.01$, unpaired t-test with Welch's correction). Mean values (±SEM; n=6) were 0.145 pmol NT-3/mg protein ± 0.032 and 0.450 pmol NT-3/mg protein ± 0.063 in unstimulated samples and KCl-stimulated samples respectively. Incubation with NGF did not cause a further increase in depolarisation-induced NT-3 release (* $p<0.05$, unpaired t-test with Welch's correction). NT-3 values (±SEM; n=6) in this case were 0.080 pmol/mg protein ± 0.016 and 0.341pmol/mg protein ± 0.061 in NGF-pretreated synaptosomes incubated in unstimulated and KCl-stimulated samples respectively. In the case of slices preincubated with BDNF, KCl did not significantly increase NT-3 release; mean values (±SEM; n=6) were 0.129pmol NT-3/mg protein ± 0.049 and 0.300 pmol NT-3/mg protein ± 0.070 in synaptosomes incubated in the absence and presence of KCl respectively. Similarly, NT-3 release was not stimulated by depolarisation in slices preincubated with tyrphostin AG879. In this case, NT-3 concentrations (±SEM; n=6) were 0.150 pmol/mg protein ± 0.051 and 0.314 pmol/mg protein ± 0.089 in unstimulated and KCl-stimulated samples respectively.

4.3.4 NGF or BDNF do not enhance depolarisation-stimulated NT-3 release from synaptosomes prepared from dentate gyrus.

The effect of BDNF and NGF on NT-3 release was investigated in synaptosomes prepared from dentate gyrus, as shown in Figure 4.4. KCl-stimulated depolarisation resulted in a significant increase in NT-3 release from synaptosomes prepared from dentate gyrus (* $p<0.05$, unpaired *t*-test with Welch's correction). NT-3 concentrations (±SEM; n=6) were 0.802 pmol/mg protein ± 0.240 and 3.939 pmol/mg protein ± 0.931 in synaptosomes incubated in the absence and presence of added KCl respectively. KCl-stimulated NT-3 release was not further enhanced by addition of NGF or BDNF. In the case of synaptosomes preincubated in NGF, mean values (±SEM; n=6) were 1.269 pmol NT-3/mg protein ± 0.573 and 3.468 pmol NT-3/mg protein ± 0.662 in synaptosomes

incubated in the absence and presence of KCl respectively. Following incubation with BDNF, concentrations of NT-3 (±SEM; n=6) were 0.574pmol/mg protein ± 0.272 and 3.821 pmol/mg protein ± 1.010 from unstimulated and KCl-stimulated samples. KCl significantly increased release in both cases (* $p<0.05$, unpaired t-test with Welch's correction). Preincubation with tyrphostin AG879 blocked depolarisation-stimulated NT-3 release. In this case, mean values (±SEM; n=6) were 1.085 pmol NT-3/mg protein ± 0.181 and 2.889 pmol NT-3/mg protein ± 0.889 in synaptosomes incubated in the absence and presence of KCl respectively.

4.3.5 NGF and NT-3 promote protein synthesis in slices prepared from hippocampus.

The effect of preincubation with NGF or NT-3 on [^{35}S]-methionine incorporation into TCA-precipitated proteins was measured in slices prepared from hippocampus. As shown in Figure 4.6, preincubation with NGF or NT-3 significantly stimulated protein synthesis compared with slices incubated in the absence of a neurotrophin (* $p<0.05$, unpaired *t*-test with Welch's correction). Mean values (±SEM; n=6) were 279.050 cpm/mg protein ± 44.040, 682.566 cpm/mg protein ± 110.469 and 767.566cpm/mg protein ± 162.750 in slices incubated in the absence of NGF or NT-3, in slices incubated with NGF and in slices incubated with NT-3 respectively.

4.3.6 Neither NGF nor NT-3 promotes protein synthesis in synaptosomes prepared from entorhinal cortex.

Figure 4.6 indicated that preincubation with NGF or NT3 did not enhance protein synthesis in slices prepared from entorhinal cortex compared with slices incubated in the absence of NGF ($p>0.05$, unpaired t-test with Welch's correction). Preincubation with NT-3 had no stimulatory effect on protein synthesis. Mean values (±SEM; n=6) were 287.550 cpm/mg ± 37.849, 417.030 cpm/mg ± 42.948 and 317.070 cpm/mg ± 31.373 in slices incubated in the absence of NGF or NT-3, in slices incubated with NGF and in slices incubated with NT-3 respectively.

4.3.7 Changes in NT-3 concentration do not affect KCl-stimulated glutamate release.

The effects of NT-3 on KCl-stimulated glutamate release were assessed in synaptosomes prepared from dentate gyrus. Three different concentrations of NT-3 were used; 4ng/ml, 20ng/ml and 100ng/ml. In the absence of NT-3, KCl (50mM) stimulated a significant increase in glutamate release. Mean values (±SEM; n=6) were 0.436 μmol glutamate/mg protein ± 0.035 and 0.670 μmol glutamate/mg protein ± 0.066 in unstimulated and KCl-stimulated samples respectively (* $p<0.05$, unpaired *t*-test with Welch's correction). Following incubation with NT-3 (4ng/ml), glutamate concentrations (±SEM; n=6) were 0.395μmol glutamate/mg protein ± 0.036 and 0.606μmol glutamate/mg protein ± 0.035 in unstimulated and KCl-stimulated samples respectively (+ $p<0.01$, unpaired *t*-test with Welch's correction). In the case of preincubation with NT-3 (20ng/ml), glutamate concentrations were 0.463 μmol glutamate/mg protein ± 0.050 and 0.717 μmol/mg ± 0.060 in samples in the absence and presence of KCl respectively (+ $p<0.01$, unpaired *t*-test with Welch's correction). Finally in the presence of NT-3 (100ng/ml), glutamate concentrations (±SEM; n=6) were 0.507μmol glutamate/mg protein ± 0.044and 0.760 μmol glutamate/mg protein ± 0.067 in unstimulated and KCl-stimulated samples respectively (* $p<0.05$, unpaired *t*-test with Welch's correction).

4.3.8 Changes in BDNF concentration do not affect KCl-stimulated glutamate release.

Figure 4.8 shows the effects of 3 different concentrations of BDNF on KCl-stimulated glutamate release from synaptosomes prepared from the dentate gyrus. Incubation in the presence of KCl (50mM) significantly stimulated glutamate release (* $p<0.05$, unpaired *t*-test with Welch's correction). Mean values were 0.353μmol glutamate/mg protein ± 0.023 and 0.460 μmol glutamate/mg protein ± 0.027 (± SEM; n=6) in unstimulated and KCl-stimulated samples respectively. Incubation in the presence of BDNF (40ng/ml), prior to addition of KCl, did not enhance depolarisation-stimulated glutamate release; mean values were 0.361μmol glutamate/mg protein ± 0.024 and 0.481μmol glutamate/mg protein ± 0.031 (± SEM; n=6) in absence and presence of KCl respectively (* $p<0.05$, unpaired *t*-test with Welch's correction). Addition of BDNF (200ng/ml) to the incubation medium failed to enhance KCl-induced glutamate release (*

$p<0.05$, unpaired *t*-test with Welch's correction). In this case, glutamate concentrations were 0.345μmol glutamate/mg protein ± 0.022 and 0.504μmol glutamate/mg protein ± 0.0369 (± SEM; n=6) in unstimulated and KCl-stimulated samples respectively. Furthermore, addition of BDNF (1000ng/ml) to the medium did not enhance depolarisation-stimulated glutamate release (*$p<0.05$, unpaired *t*-test with Welch's correction); mean values were 0.394μmol glutamate/mg protein ± 0.030 and 0.545 μmol glutamate/mg protein ± 0.034 (± SEM; n=6) in unstimulated and KCl-stimulated samples respectively.

4.3.9 NT-3 and ACPD do not increase KCl-stimulated glutamate release.

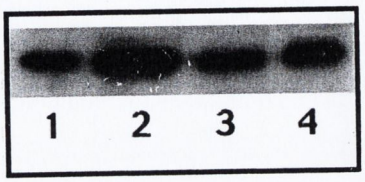
The effect of NT-3, alone and, in combination with ACPD on KCl-stimulated release from synaptosomes prepared from dentate gyrus was assessed; the data are shown in Figure 4.10. Addition of KCl (50mM) to the incubation medium resulted in significantly increased glutamate release from synaptosomes prepared from the dentate gyrus (* $p<0.05$, unpaired *t*-test with Welch's correction). Mean values were 0.278 μmol glutamate/mg protein ± 0.025 and 0.428 μmol glutamate/mg protein ± 0.040 (± SEM; n=6) in unstimulated and KCl-stimulated samples respectively. Addition of NT-3 (20ng.ml) to the incubation medium did not result in enhancement of KCl-stimulated glutamate release (* $p<0.05$, unpaired *t*-test with Welch's correction); mean values were 0.243μmol glutamate/mg protein ± 0.029 and 0.404μmol glutamate/mg protein ± 0.061 (± SEM; n=6) in unstimulated and KCl-stimulated samples respectively. Incubation with NT-3 in combination with ACPD (50μM) had no effect on KCl-stimulated glutamate release (+ $p<0.01$, unpaired *t*-test with Welch's correction). In this case, glutamate concentrations were 0.408 μmol glutamate/mg protein ± 0.038 and 0.760 μmol glutamate/mg protein ± 0.100 (± SEM; n=6) in unstimulated and KCl-stimulated samples respectively. Incubation with APCD alone did not enhance KCl-induced glutamate release (* $p<0.05$, unpaired *t*-test with Welch's correction); mean values (± SEM; n=6) were 0.288μmol glutamate/mg protein ± 0.035 and 0.534 μmol glutamate/mg protein ± 0.081 in unstimulated and KCl-stimulated samples respectively.

Figure 4.1 Tyrphostin blocks NT-3-induced ERK activity

A. This representative immunoblot shows incubation in the presence of NT-3 (20ng/ml) enhanced ERK activity (lane 2) in synaptosomes prepared from dentate gyri compared with unstimulated ERK activity (lane 1). The stimulatory effect of NT-3 was blocked in the presence of tyrphostin AG879 (100μM) in synaptosomes prepared from dentate gyri (lane 3). Incubation in the presence of tyrphostin AG879 alone did not affect ERK activity (lane 4).

B. Densitometric analysis demonstrates that incubation with NT-3 (20ng/ml) significantly stimulated ERK activation in synaptosomes prepared from dentate gyri compared with unstimulated samples (* $p<0.05$, unpaired *t*-test with Welch's correction). Incubation with NT-3 (20ng/ml) in combination with tyrphostin AG879 (100μM) blocked the NT-3-induced increase while incubation with tyrphostin AG879 alone did not affect levels of ERK activity. Results are means (± SEM) of 6 observations and expressed as arbitrary units.

A.


1
2
3
4

B.

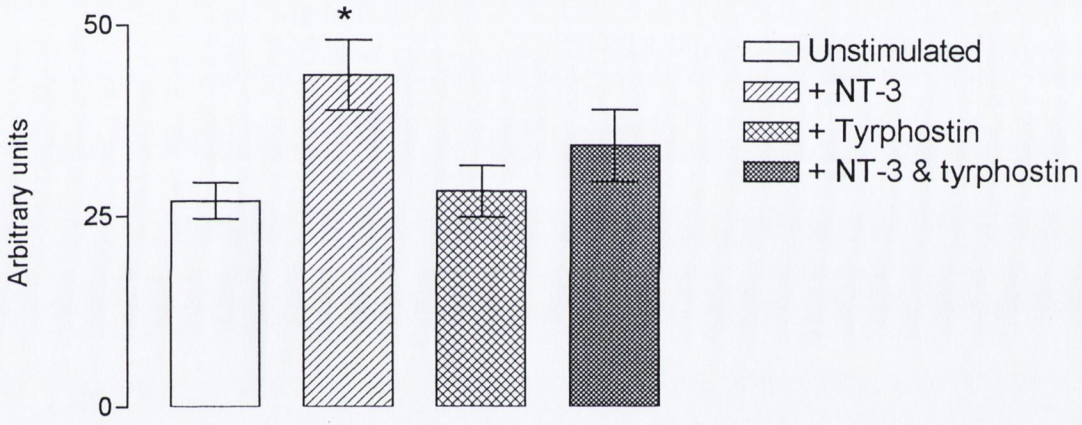

50
25
0
Arbitrary units
*
Unstimulated
+ NT-3
+ Tyrphostin
+ NT-3 & tyrphostin

Figure 4.2 Depolarisation induces NT-3 release from synaptosomes prepared from dentate gyrus

Addition of KCl (50mM) to synaptosomes prepared from dentate gyrus significantly increased NT-3 release (* $p<0.01$, unpaired *t*-test with Welch's correction). Results are presented as means (± SEM) of 6 observations and expressed as pmol NT-3/mg protein.

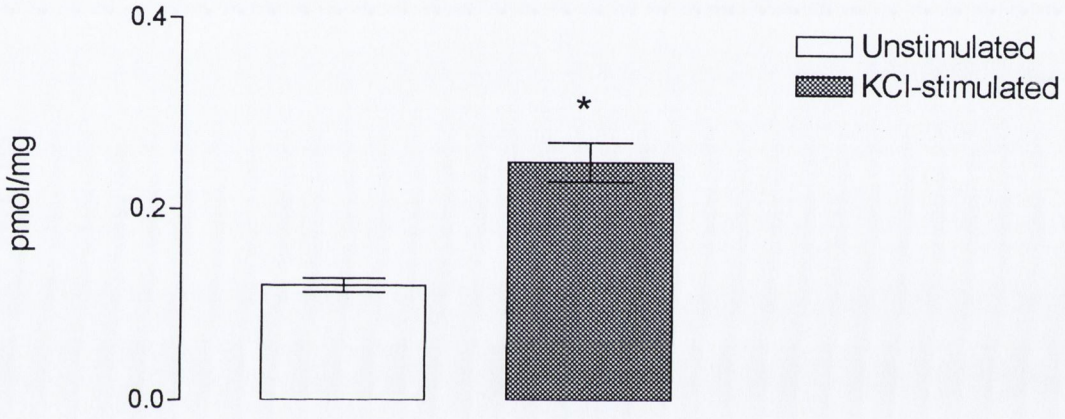

0.4
0.2
0.0
pmol/mg
*
Unstimulated
KCl-stimulated

Figure 4.3 NGF or BDNF do not cause additional enhancement of depolarisation-stimulated NT-3 release from slices prepared from dentate gyrus

KCl-induced depolarisation resulted in significantly increased NT-3 release from slices prepared from dentate gyrus (+ $p<0.01$, unpaired *t*-test with Welch's correction). Preincubation of slices with either NGF or BDNF did not result in additional enhancement of KCl-stimulated release but in the case of NGF, KCl stimulated a significant increase in NT-3 release (* $p<0.05$, unpaired *t*-test with Welch's correction). Incubation in the presence of tyrphostin AG879 blocked KCl-stimulated NT-3 release. Results are means (± SEM) of 6 observations and expressed as pmol NT-3/mg protein.

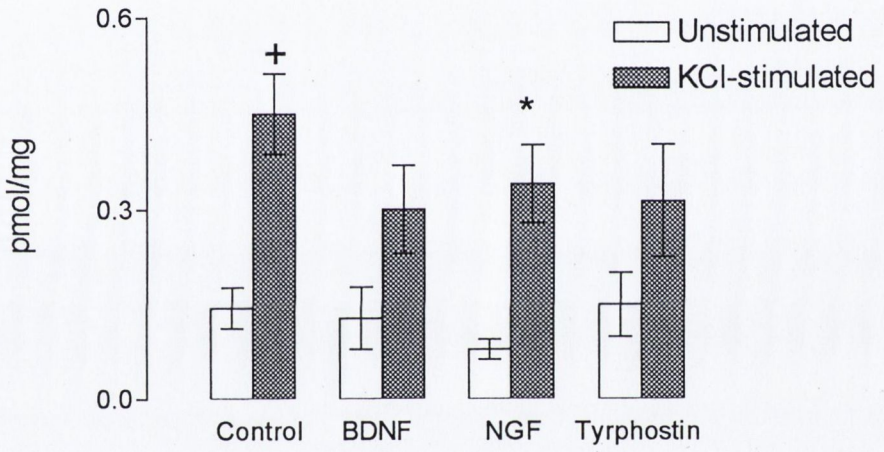

0.6
0.3
0.0
pmol/mg
+
*
Unstimulated
KCl-stimulated
Control
BDNF
NGF
Tyrphostin

Figure 4.4 NGF or BDNF do not cause additional enhancement of depolarisation-stimulated NT-3 release from synaptosomes prepared from dentate gyrus

KCl-induced depolarisation significantly increased NT-3 release from slices prepared from dentate gyrus (* $p<0.05$, unpaired *t*-test with Welch's correction). Preincubation of slices with either NGF or BDNF did not result in further enhancement of KCl-stimulated NT-3 release but in these cases, KCl stimulated a significant increase in NT-3 release (* $p<0.05$, unpaired *t*-test with Welch's correction). Incubation in the presence of tyrphostin AG879 blocked KCl-stimulated NT-3 release. Results are means (± SEM) of 6 observations and expressed as pmol NT-3/mg protein.

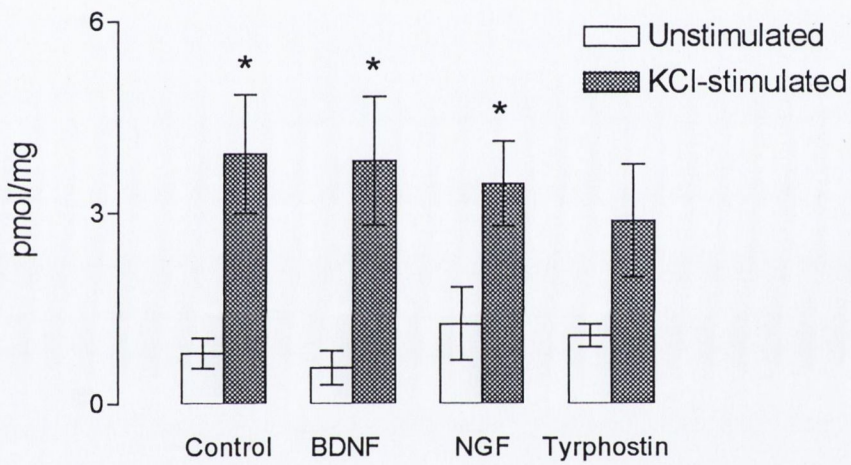

6
3
0
pmol/mg
Unstimulated
KCl-stimulated
*
*
*
Control
BDNF
NGF
Tyrphostin

Figure 4.5 NGF and NT-3 promote protein synthesis in slices prepared from hippocampus

Incubation in the presence of NGF (50ng/ml) or NT-3 (20ng/ml) significantly stimulated [^{35}S] methionine incorporation into TCA-precipitated proteins in slices prepared from hippocampus (* $p<0.05$, unpaired *t*-test with Welch's correction). Results are means (± SEM) of 6 observations and expressed as cpm/mg protein.

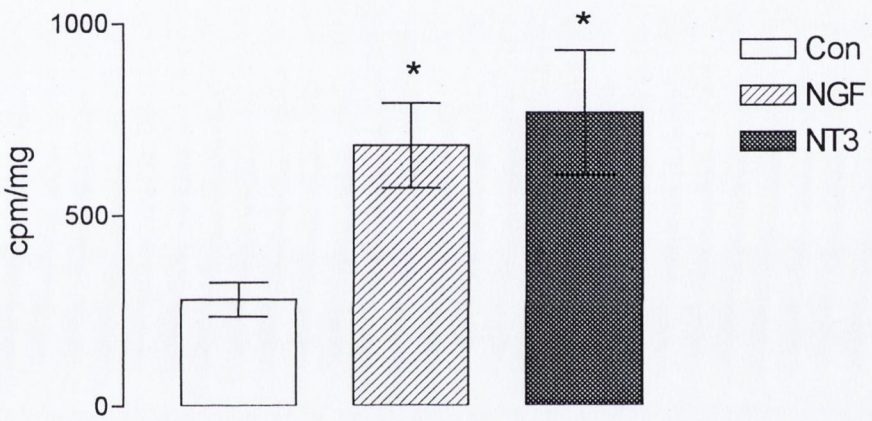

1000
500
0
cpm/mg
*
*
Con
NGF
NT3

Figure 4.6 NGF or NT-3 do not promote protein synthesis in slices prepared from entorhinal cortices

Incubation in the presence of NGF or NT-3 did not stimulate [^{35}S] methionine incorporation into TCA-precipitated proteins in slices prepared from entorhinal cortices compared with unstimulated samples ($p>0.05$ unpaired *t*-test with Welch's correction). Results are means (± SEM) of 6 observations and expressed as cpm/mg protein.

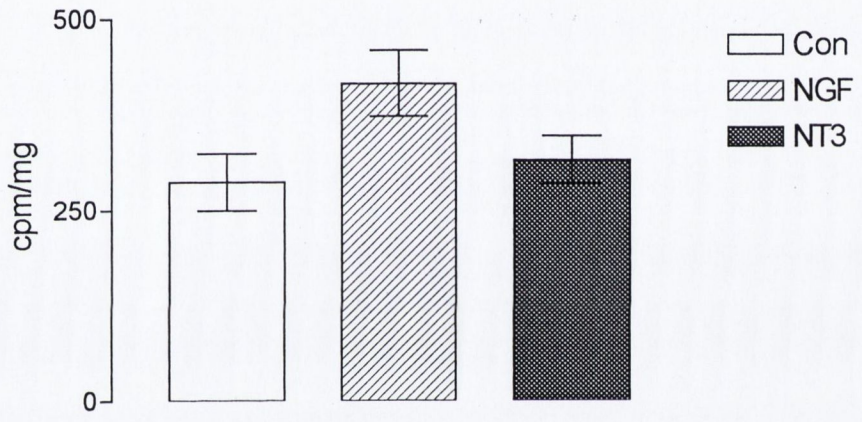

500
250
0
cpm/mg
Con
NGF
NT3

Figure 4.7 Incubation with different concentrations of NT-3 does not result in an additive effect on KCl-stimulated glutamate release

Addition of KCl (50mM) significantly stimulated glutamate release from synaptosomes prepared from dentate gyrus compared with unstimulated release from control samples (* $p<0.05$, unpaired t-test with Welch's correction). Incubation with NT-3 at 3 different concentrations (4ng/ml, 20ng/ml and 100ng/ml) had no additive effect on KCl-stimulated glutamate release but in these cases, KCl-induced depolarisation resulted in enhanced glutamate release (* $p<0.05$, unpaired *t*-test with Welch's correction). Results are means (± SEM) of 6 observations and expressed as μmol glutamate/mg protein.

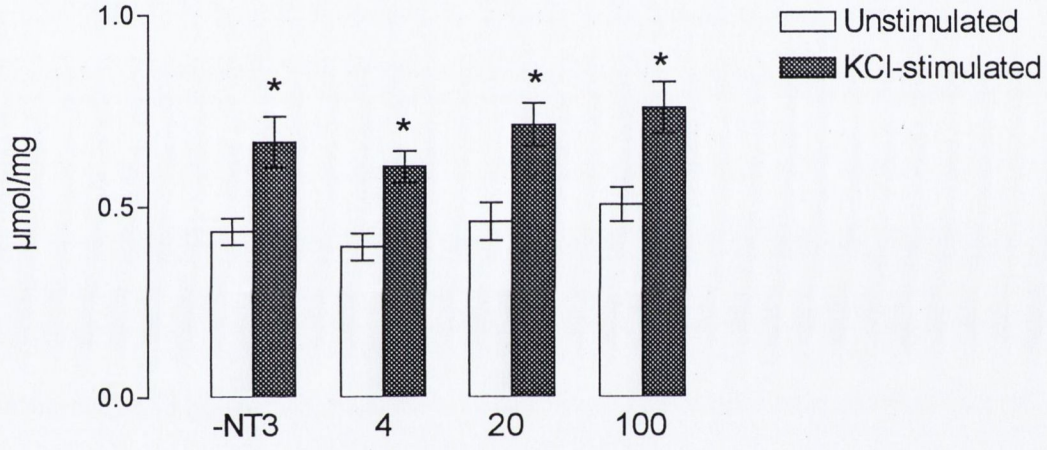

1.0
0.5
0.0
µmol/mg
*
*
*
*
-NT3
4
20
100
Unstimulated
KCl-stimulated

Figure 4.8 Incubation with different concentrations of BDNF does not cause additional increases in KCl-stimulated glutamate release

Addition of KCl (50mM) significantly stimulated glutamate release from synaptosomes prepared from dentate gyrus compared with unstimulated release from control samples (* $p<0.05$, unpaired *t*-test with Welch's correction). Incubation with BDNF at 3 different concentrations (50ng/ml, 200ng/ml and 1000ng/ml) had no additive effect on KCl-stimulated glutamate release but in these cases, addition of KCl resulted in significantly enhanced glutamate release (* $p<0.05$, unpaired *t*-test with Welch's correction). Results are means (± SEM) of 6 observations and expressed as μmol glutamate/mg protein.

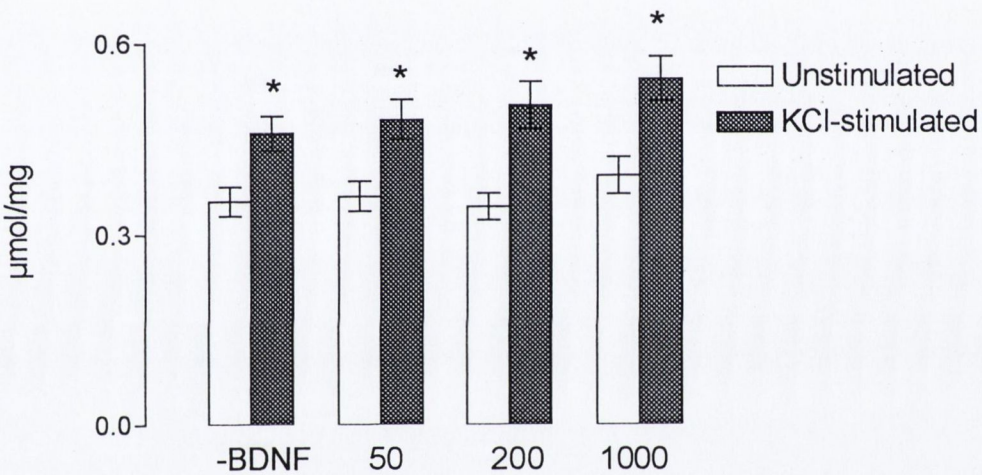
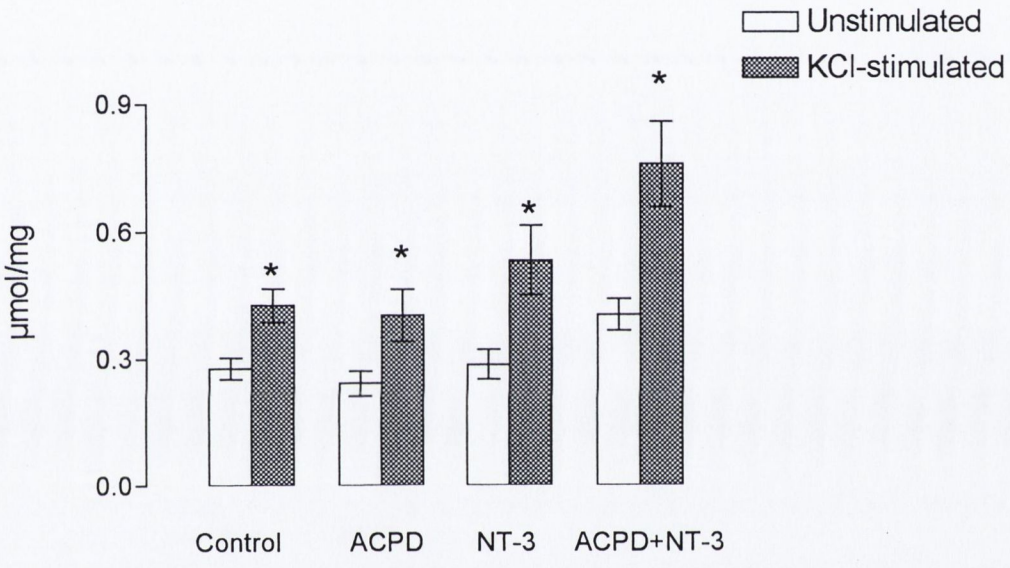

0.6
0.3
0.0
µmol/mg
*
*
*
*
Unstimulated
KCl-stimulated
-BDNF
50
200
1000

Figure 4.9 Neither NT-3 nor ACPD, alone or in combination, increase KCl-stimulated glutamate release

Addition of KCl (50mM) significantly stimulated glutamate release from synaptosomes prepared from dentate gyrus compared with unstimulated release from control samples (* $p<0.05$, unpaired t-test with Welch's correction). Incubation with NT-3 (20ng/ml) alone, APCD (50μM) alone or NT-3 (20ng/ml) and ACPD (50 μM) in combination had no additive effect on KCl-stimulated release but in these cases, KCl-induced depolarisation resulted in significantly enhanced glutamate release (* $p<0.05$, unpaired t-test with Welch's correction). Results are means (± SEM) of 6 observations and expressed as μmol glutamate/mg protein.


Unstimulated
KCl-stimulated
µmol/mg
0.9
0.6
0.3
0.0
*
*
*
*
Control
ACPD
NT-3
ACPD+NT-3

4.4 Discussion

The objectives of this series of experiments were 1) to investigate intracellular mechanisms stimulated by NT-3, 2) to analyse factors that impact on NT-3 release and glutamate release and 3) to identify mechanisms by which neurotrophins induce long-lasting changes. The data show that incubation with NT-3 stimulated ERK activity in synaptosomes prepared from dentate gyrus. This is unsurprising as NT-3, like BDNF and NGF, interacts with a receptor tyrosine kinase (Merlio *et al.*, 1992) and triggers an intracellular cascade, which is propagated by tyrosine phosphorylation (Cordon-Cardo *et al.*, 1991; Lambelle *et al.*, 1991). In the case of NT-3, it binds to its high affinity receptor, TrkC, known to be highly expressed in the dentate gyrus (Merlio *et al.*, 1992). In addition to TrkC, NT-3 binds both TrkA and TrkB with lower affinity (Klein *et al.*, 1991; Barbacid *et al.*, 1991). Activation of Trk receptors, stimulated by binding of a neurotrophin, leads to homodimerisation and subsequently to autophosphorylation of tyrosine residues in the cytosolic domain (Kaplan *et al.*, 1991a, 1991b; Jing *et al.*, 1992). This event initiates the ras/Raf/ERK pathway (Segal and Greenberg, 1996), which amplifies the signal in the cytosolic domain. This signalling cascade involves several steps dependent on tyrosine phosphorylation such as activation of ERK, which requires dual phosphorylation on tyrosine and threonine (Payne *et al.*, 1991; Her *et al.*, 1993). Other candidates for tyrosine phosphorylation following NT-3 stimulation are PLCγ (Marsh and Palfrey, 1996; Widmer *et al.*, 1993) and PI-3 kinase (Yuen and Mobley, 1999; Yang *et al.*, 2001), which are activated downstream of Trk receptors.

This NT-3-induced enhancement of ERK activity was blocked in the presence of tyrphostin AG879, suggesting that ERK activation is a direct consequence of Trk receptor activation. This is consistent with previous findings that NT-3 stimulated ERK activity in cultured hippocampal neurons (Baldelli *et al.*, 2000), in primary cortical neurons (Cavanaugh *et al.*, 2001) and oligodendrocytes (Johnson *et al.*, 2000) and also resembles NGF-induced ERK phosphorylation in synaptosomes prepared from dentate gyrus (Maguire *et al.*, 1999).

Data presented here indicate that NT-3 release from presynaptically-enriched synaptosomes prepared from dentate gyrus was induced by KCl-stimulated depolarisation. This is similar to previous findings, which showed that depolarisation-

induced release of BDNF (Goodman *et al.*, 1996; Androutsellis-Theotokis, 1996) or NGF (Blöchl and Thoenen, 1995). Similar observations were made during experiments in this lab to investigate NGF release (C. Maguire, personal communication) and BDNF release (M. Gooney, personal communication).

To investigate the capability of neurotrophins to stimulate their own release (Canossa *et al.*, 1997; Krüttgen *et al.*, 1998), the effects of BDNF, NGF and Trk receptor activation on NT-3 release from slices and synaptosomes prepared from dentate gyrus were examined. Preincubation with NGF did not augment depolarisation-induced NT-3 release from slices or synaptosomes prepared from dentate gyrus. Preincubation with BDNF resulted in different effects on NT-3 release from slices and synaptosomes. Depolarisation failed to elicit NT-3 release from slices but induced NT-3 release from synaptosomes following administration of BDNF. This indicates differential regulation of NT-3 release slices by BDNF on both sides of the synapse. BDNF-related inhibition of NT-3 release from slices, and not synaptosomes, prepared from dentate gyrus may be as a consequence of BDNF-mediated tyrosine phosphorylation of NMDA receptors (Suen *et al.*, 1997; Levine *et al.*, 1998; Lin *et al*, 1998) as application of NMDA also blocked NGF release (C. Maguire, personal communication) and BDNF release (M. Gooney, personal communication) from slices *in vitro* and because a greater number of NMDA receptors are located postsynaptically. It is also possible that differences in receptor distribution or type is responsible for these observations. This hypothesis may be supported by work on PC12 cells by Krüttgen and coworkers (1998), who suggested that BDNF binding to Trk receptors and to the pan-neurotrophin receptor, p75, a low affinity neurotrophin receptor, was responsible for neurotrophin-induced NT-3 release. In their study, REX polyclonal antibody, which blocks neurotrophin interactions with p75, abolished BDNF-stimulated NT-3 release but a role for p75 in NT-induced release was disputed by Canossa and coworkers (1997).

Several findings provide indirect evidence that NT-3 may control its own secretion. Reports that NT-3 and BDNF exist as a heterodimer (Jungbluth *et al.*, 1994; Roberson *et al*, 1995) and that NT-3 and BDNF are cosecreted (Farhadi *et al.*, 1999), in addition to reports of NT-3 induced BDNF release from hippocampal slices (Canossa *et al.*, 1997), support a self-regulatory process of NT-3 release. The results presented here

indicate that incubation with tyrphostin AG879 completely blocked depolarisation-regulated NT-3 release from both synaptosomes and slices prepared from dentate gyrus, consistent with findings that Trk receptor activation is necessary for neurotrophin release from hippocampal slices (Canossa *et al.*, 1994, 2001). Trk receptors may result in a transient downstream calcium signal by increasing intracellular calcium mobilised from intracellular stores (Jiang *et al.*, 1999) which allows neurotrophins to regulate their own release. PLCγ is the likely candidate to elicit increased calcium levels in response to Trk activation (Marsh and Palfrey, 1996; Widmer *et al.*, 1993; Vetter *et al.*, 1991) as it leads to the formation of IP_3, which can trigger calcium efflux from the endoplasmic reticulum.

It has been reported that release of NGF and BDNF from PC12 cells is sensitive to both Trk activation and metabotropic glutamate receptor activity (Canossa *et al.*, 2001) and that PLCγ acts as a downstream convergence point of these signals or a coincidence detector. It may therefore have been of interest to investigate the effects of ACPD on NT-3 release. The locus of release of NT-3 is not identified by results of these experiments and appears to be manifest on both sides of the synapse, which conflicts with the traditional concept of retrograde messengers released from a postsynaptic site and acting at a presynaptic site.

As a primary effect of neurotrophins is alteration of neuronal morphology (Ip *et al*, 1993; Lambelle and Leclerc, 2000), the effects of NGF and NT-3 on protein synthesis in hippocampus and entorhinal cortex were assessed. In the hippocampus, both neurotrophins stimulated increased protein synthesis but in the entorhinal cortex, neither NGF nor NT-3 enhanced protein synthesis. Regional differences in the effects of NGF and NT-3 on protein synthesis may be explained by the topography of distribution of their respective Trk receptors. NT-3 and its cognate receptor, TrkC are highly expressed in the hippocampus and, in particular, in the granule cells of the dentate gyrus (Zhou and Rush, 1994; Tokuyama *et al.*, 1999; Merlio *et al*, 1992) relative to other brain regions. This may explain why NT-3 failed to exert an effect on protein synthesis in the entorhinal cortex. NGF and TrkA are reported to be more diffusely expressed in the hippocampus (Maisonpierre *et al.*, 1990; Ernfors *et al.*, 1990) which may allow NGF to modulate protein synthesis in the hippocampus. Previous work has reported stimulation of calcium channel synthesis in hippocampal neurons by NGF and NT-3 and inhibition of this effect

by incubation with the protein synthesis inhibitor, anisomysin, indicating the involvement of *de novo* protein synthesis in the response (Baldelli *et al.*, 2000).

No attempt was made in the current study to identify the underlying transduction mechanism or possible protein products but it is likely that the transcription factor CREB plays a central role. It has been shown that both NGF and NT-3 stimulate phosphorylation of CREB (Ginty *et al.*, 1994; Johnson *et al.*, 2000). In turn, pCREB can act as a regulatory mechanism to facilitate transcriptional activation of immediate early genes, such as *c-fos* (Sheng *et al.*, 1990; Ginty *et al.*, 1994; Ahn *et al.*, 1998). CREB activation induces recruitment of co-activators CREB-binding protein (CBP) and p300, resulting in prominent activation of target genes (Chrivia *et al.*, 1993, Kwok *et al.*, 1994, Lundblad *et al.*, 1995). It has been shown that these coactivators regulate transcription of a wide range of target genes by bridging the interacting transcription factors to the basal transcription machinery including RNA polymerase II (Cho *et al.*, 1998; Kee *et al.*, 1996; Nakajima *et al.*, 1997). Ginty and coworkers (1994) found that NGF stimulates *c-fos* transcription in PC12 cells by phosphorylation of CREB mediated by Ras-dependent protein kinase while Xing and coworkers (1996) implicated RSK-2 in the activation of CREB by neurotrophin-initiated signalling. In addition, Kang and Schuman (1996) reported increases in protein synthesis hippocampal neurons in response to exposure to BDNF and NT-3. Taken together with previous findings, the results of the present study indicated that NGF and NT-3 induce protein synthesis in hippocampal neurons, most likely through their action on CREB.

To assess the impact of neurotrophins on short-term plasticity, the dose-dependent effects of BDNF and NT3 on glutamate release from synaptosomes prepared from dentate gyrus were analysed. This may provide a mechanism by which neurotrophins can enhance the synaptic plasticity responsible for maintenance of long-term potentiation. The data presented here show that preincubation with three different concentrations of BDNF or NT-3 did not cause an increase in depolarisation-induced glutamate release. This is in contrast with previous findings, which showed that administration of exogenous BDNF or NT-3 can stimulate glutamatergic transmission following depolarisation (Knipper *et al.*, 1994; Lessman *et al.*, 1994; Lessman and Heuman, 1998; Kim *et al.*, 1994). Several reports demonstrate rapid enhancement of synaptic transmission in

response to neurotrophins (Kang and Schuman, 1995, 1996; Kang *et al.*, 1996; Messaoudi *et al.*, 1998). Further work suggests that neurotrophins can alter synaptic vesicle docking, synaptic vesicle protein distribution (Pozzo-Miller *et al.*, 1999; Tyler and Pozzo-Miller, 2001) and synaptic vesicle protein phosphorylation (Jonanovic *et al.*, 1996). In contrast, other groups have failed to show neurotrophin-mediated increases in excitatory transmission (Figurov *et al.*, 1996; Patterson *et al.*, 1996; Göttschalk *et al.*, 1998). Differences between the current data and previous findings may be due to differences in the length of incubation. It has been reported that BDNF is a sticky molecule and that its ability to penetrate into the slices may vary depending on perfusion rates (Kang *et al.*, 1996). There are also differences in perfusion methods and incubation times. In this study, the paradigm was relatively brief exposure, thereby diminishing the response. In addition to this, there are differences in the parameters used to determine increased glutamatergic transmission. It has been suggested that postsynaptic responses are a reflection of presynaptic release (Bekkers and Stevens, 1990), whereas direct quantitation of glutamate concentration in response to depolarisation was used here. Even so it is difficult to argue that exogenous neurotrophins, acutely administered, cause similar changes to synaptic activity to activity-induced release of endogenous neurotrophins.

Previous work in this laboratory indicated that both NGF and BDNF act synergistically with ACPD, the mebatotropic glutamate receptor agonist, to enhance depolarisation-stimulated glutamate release, while incubation with NGF or BDNF does not increase activity-dependent glutamate release. This suggests neither NGF nor BDNF alone is sufficient to increase regulated glutamate release and possibly indicates that PLCγ is involved in mediating the downstream effects of activation of both tyrosine kinase and mGluR receptors (McGahon and Lynch, 1998; Kelly and Lynch, 1998). It has been suggested that PLCγ acts as a coincidence detector and its activation can give rise to the formation of IP_3 and DAG, which impact on intracellular calcium concentrations. These data, together with the findings of previous work, suggest that while neurotrophins may be necessary for glutamatergic transmission, they are not sufficient.

Because recent findings in this laboratory indicate depolarisation-induced glutamate release was further stimulated by simultaneous application of ACPD and either

NGF or BDNF, the effect of NT-3 and metabotrophic glutamate receptor activity on glutamate release was examined. Co-application of NT-3 and the mGluR receptor agonist, APCD, did not result in increased KCl-induced glutamate release in comparison to application of NT-3 or ACPD alone. This contrasts similar findings from this laboratory of the influence of BDNF and NGF on glutamate release from synaptosomes prepared from the dentate gyrus.

This study has shown that NT-3 stimulates the Ras/Raf/ERK pathway in synaptosomes prepared from the dentate gyrus and that NT-3 release is activity-dependent and may be modulated by activation of Trk receptors. It was also demonstrated that NT-3 induces both short- and long-term modifications in the hippocampus.

Chapter 5

Establishing a role for Trk receptors in LTP

5.1 Introduction

Interest in the involvement of neurotrophins in synaptic plasticity and in LTP in the hippocampus has been growing in the past few years. This is perhaps due to the marked expression of both neurotrophic factors and their cognate receptors, Trk receptors, in the hippocampus (Klein *et al.*, 1989; Merlio *et al.*, 1992, Lambelle *et al.*, 1991, Maisonpierre *et al.*, 1990; Zhou and Rush, 1994). It has been proposed by several groups that neurotrophins participate in activity-dependent modification of synaptic function because their synthesis and secretion are regulated by activity (Gall and Isackson, 1989; Zafra *et al.*, 1990; Blöchl and Thoenen, 1995; Goodman *et al.*, 1996; Wang and Poo, 1997). It has also been shown that LTP is abolished when neurotrophin function is impaired (Korte *et al.*, 1995; Patterson *et al.*, 1996; Kang *et al.*, 1996). ERK, a downstream messenger of neurotrophic signals, is increased following the induction of LTP in the CA1 region (English and Sweatt, 1996) and in the dentate gyrus (Maguire *et al.*, 1999). Inhibition of ERK has also been shown to block LTP (English and Sweatt, 1996; McGahon *et al.*, 1998) suggesting that the effect of neurotrophins of LTP are mediated by Trk receptors.

The exact locus of neurotrophin-mediated enhancement of synaptic function remains elusive. Evidence for a modulatory role for BDNF points to effects manifested on both sides of the synapse. Levine and coworkers (1995) reported BDNF-induced enhancement of synaptic function was due to postsynaptic receptor tyrosine kinase. The same group (1998) also document BDNF-mediated increases in postsynaptically-located NMDA receptor open probability which was suppressed by injection of tyrosine kinase inhibitor, K252a. BDNF-induced tyrosine phosphorylation of NMDA receptor subunit 2B was also reported by Lin and coworkers (1998), adding to the evidence of a postsynaptic role for BDNF. Other studies, however, indicate that the site of action of neurotrophins is presynaptic and act by modulating glutamate release (Knipper *et al.*, 1994, Maguire *et al.*, 1999). Kelly and coworkers (1998) suggest a synergistic action of NGF and metabotropic glutamate receptor in the enhancement of glutamate release in the dentate gyrus.

The objectives of this study were 1) to assess the impact of Trk activation on the expression of LTP at the perforant path-granule cell synapses, 2) to identify possible downstream substrates of Trk activation in the presynaptic and postsynaptic regions, 3) to investigate long-term changes in the entorhinal cortex underlying the expression of LTP.

5.2 Methods

5.2.1 Induction of LTP *in vivo*

LTP was induced *in vivo* as described in Section 2.5. Briefly, rats were anaesthetised with urethane (1.5g/kg i.p.) and secured in the head holder of a stereotaxic frame. An intracerebroventricular injection of either tyrphostin AG879 (5µl, final concentration: 100µM) or saline (5µl) was administered 30min prior to commencement of the recording period. Test shocks were applied to the perforant path every 30s for a 10min control period. This was followed by delivery of 3 trains of stimuli (250Hz for 200ms) at 30s intervals. Recording at test shock frequency resumed for a 40min period. At the end of the experimental period, rats were killed by cervical dislocation and decapitation. Tissue was sliced and stored at –80°C.

5.2.2 Assessment of tyrosine kinase activity

Synaptosomes, prepared from tetanised and untetanised dentate gyri of rats pretreated with tyrphostin AG879 or saline, were immunoprecipitated with anti-phosphotyrosine (1:25; Affiniti, UK), as described in Section 2.12. Immunocomplexes were separated on 10% SDS-polyacrylamide gels and visualised by silver staining as described in section 2.15. Protein bands were quantitated using densitometric analysis.

5.2.3 Analysis of NT-3 release

The method used is described in Section 2.10. Synaptosomes, prepared from tetanised and untetanised dentate gyri of rats pretreated with tyrphostin AG879 or saline, were preincubated in Krebs solution containing $CaCl_2$ (2mM) for 3min at 37°C under continuous oxygenation. Samples were spun at 5,000rpm for 1min and the pellet was incubated in Krebs solution containing $CaCl_2$ (2mM) for 5min at 37°C under constant oxygenation to assess unstimulated NT-3 release. The supernatant was collected following centrifugation and the pellet was incubated in Krebs solution containing KCl (50mM) for 5min at 37°C under continuous oxygenation to assess depolarisation-induced NT-3 release. Samples were spun at 5,000rpm for 1min. The supernatant was retained for

analysis of NT-3 concentration as described in Section 2.11. Protein concentrations were determined from the pellet.

5.2.4 Measurement of ERK phosphorylation

ERK activity was analysed in synaptosomes (P_2) and crude postsynaptic preparations (P_1) prepared from tetanised and untetanised dentate gyri of rats pretreated with either tyrphostin AG879 or saline. Proteins were separated on 10% SDS-polyacrylamide gels as outlined in section 2.14 and immunoblotted with anti-active ERK (1.5:1000 in TBS-T containing 2% non-fat dried milk; Promega, USA) as described in Section 2.16.

5.2.5 Measurement of CREB phosphorylation

CREB phosphorylation was assessed in synaptosomes (P_2) and postsynaptically enriched preparations (P_1) prepared from tetanised and untetanised dentate gyri of rats pretreated with either tyrphostin AG879 or saline. In some experiments, CREB phosphorylation was analysed in homogenate prepared from entorhinal cortices from the tetanised or untetanised side of the brain of rats pretreated with either tyrphostin AG879 or saline. Proteins were separated on 14% SDS-polyacrylamide gels as described in section 2.14. Separated proteins were immunoblotted with anti-phospho CREB (1:1000 in 5% BSA in TBS-T) as outlined in Section 2.16.

5.2.6 Analysis of protein synthesis

The method used to assess [^{35}S]-labelling of TCA-precipitated proteins was described in Section 2.17. Slices were prepared from entorhinal cortices of tetanised or untetanised sides of the brain of rats pretreated with either tyrphostin AG879 or saline. Slices were incubated in Krebs solution containing $CaCl_2$ (2mM), ATP (3.5mM) and [^{35}S]-methionine (0.2µl/ml) for 60min at 37°C under constant oxygenation. The reaction was terminated and [^{35}S]-methionine labelling of TCA-precipitated proteins was assessed. Protein concentrations were determined from incubated slices.

5.3 Results

5.3.1 Tyrphostin AG879 blocks the expression of LTP at the perforant path-granule cells synapses in vivo.

Figure 5.1 demonstrates that following tetanic stimulation, the epsp slope increases in rats pretreated with saline (n=6). In contrast, there was no change in the epsp slope in a group of rats pretreated with tyrphostin AG879 (n=6), following high-frequency stimulation. The mean percentage increases of the epsp slope in the 2min immediately preceding tetanic stimulation were compared with values in the 2min prior to tetanic stimulation.

5.3.2 Depolarisation-dependent NT-3 release is inhibited in tyrphostin-injected rats.

Figure 5.2 shows the effect of LTP on NT-3 release from synaptosomes prepared from tetanised and untetanised dentate gyri of rats pretreated with saline or tyrphostin AG879. Incubation in the presence of KCl (50mM) stimulated release of NT-3 from synaptosomes prepared from untetanised dentate gyri of saline-injected (* $p<0.01$, unpaired *t*-test with Welch's correction). Mean values (±SEM; n=6) were 0.086pmol NT3/mg protein ± 0.014 for unstimulated release and 0.262pmol NT3/mg protein ± 0.042 for KCl-stimulated release. In synaptosomes prepared from tetanised dentate gyri of the saline-injected group; mean values (±SEM; n=6) were 0.121pmol NT3/mg protein ± 0.013 for unstimulated release and 0.360pmol NT3/mg protein ± 0.047 for KCl-stimulated release, which represented a significant effect (* $p<0.01$, unpaired *t*-test with Welch's correction). KCl failed to elicit NT-3 release from synaptosomes prepared from untetanised or tetanised dentate gyri of rats pretreated with tyrphostin AG879. Mean values (±SEM; n=6) were 0.086pmol NT3/mg protein ± 0.022 for unstimulated release and 0.169pmol NT3/mg protein ± 0.038 for KCl-stimulated release from synaptosomes prepared from untetanised dentate gyri. In the case of NT-3 release from synaptosomes prepared from tetanised tissue, NT-3 concentrations (±SEM; n=6) were 0.169pmol NT3/mg protein ± 0.038 and 0.042pmol NT3/mg protein ± 0.027and 0.140pmol NT3/mg protein ± 0.047.

5.3.3 Tyrosine kinase activity is increased following LTP but this effect is blocked by tyrphostin AG879.

Gel electrophoresis and silver staining were employed to assess tyrosine phosphorylation of proteins in synaptosomes prepared from untetanised and tetanised tissue. Densitometric data from 4 consistently present and most prominent bands of apparent molecular weights 40kDa, 47kDa, 73kDa and 86kDa were pooled to give an estimate of total tyrosine kinase activity in synaptosomes prepared from dentate gyri of saline- and tyrphostin AG879-treated rats. Figure 5.3 indicates the effect of LTP on tyrosine kinase activity in synaptosomes prepared from untetanised and tetanised dentate gyri of rats pre-injected with saline or tyrphostin AG879. Tyrosine kinase activity was significantly stimulated in synaptosomes prepared from dentate gyri of saline-injected rats following tetanic stimulation (* $p<0.05$, unpaired *t*-test with Welch's correction); mean values (± SEM; n=6) were 8.71 pmol/mg ± 1.30 and 15.52 pmol/mg ± 1.77. In contrast, tetanus failed to enhance tyrosine kinase activity in synaptosomes prepared from dentate gyri of rats preinjected with tyrphostin AG879: mean values (± SEM; n=6) were 10.65 pmol/mg ± 2.77 and 13.11 pmol/mg ± 2.13.

5.3.4 Tyrphostin AG879 inhibits LTP-induced increases in ERK activity in the presynaptic region.

ERK activity was increased by tetanic stimulation in synaptosomes prepared from tetanised compared with untetanised dentate gyri of saline-treated rats as shown in one sample immunoblot (Figure 5.4A). This effect was blocked in synaptosomes prepared from tetanised dentate gyri compared with untetanised dentate gyri of tyrphostin AG879-treated rats. Analysis of densitometric data indicates a significant enhancement of ERK activity in synaptosomes following tetanic stimulation (* $p<0.05$, unpaired *t*-test with Welch's correction); mean values (± SEM; n=6; in arbitrary units) were 16.041 ± 0.948 and 21.580 ± 2.049 in samples prepared from untetanised and tetanised tissue respectively. The tetanus-induced effect was blocked in tissue prepared from tyrphostin AG879-pretreated rats; mean values (± SEM; n=6; in arbitrary units) were 15.653 ± 2.439 and 20.724 ± 4.591 in samples prepared from untetanised and tetanised tissue respectively.

5.3.5 Tyrphostin AG879 inhibits LTP-induced increases in ERK activity in the postsynaptic region.

In postsynaptically enriched preparations from the dentate gyrus, ERK activity was increased following tetanic stimulation compared with untetanised samples from saline-treated rats as shown in Figure 5.5. In contrast, ERK was not stimulated following tetanus in postsynaptic preparations compared with postsynaptic preparations from untetanised dentate gyri of tyrphostin AG879-treated rats. Densitometric data demonstrate a tetanus-associated enhancement of ERK activity in saline-injected rats (* $p<0.05$, unpaired *t*-test with Welch's correction). Mean values (± SEM; n=6; in arbitrary units) were 25.950 ± 1.996 and 37.836 ± 3.639 for samples prepared from untetanised and tetanised dentate gyri respectively. The effect of the tetanic stimulation on ERK activity was completely inhibited in postsynaptic preparations from tyrphostin AG879-injected rats. Mean values (± SEM; n=6) were 28.194 arbitrary units ± 5.127 and 30.657 arbitrary units ± 4.490 for samples prepared from untetanised and tetanised dentate gyri respectively.

5.3.6 Tyrphostin AG879 blocks LTP-associated increases in CREB phosphorylation in the presynaptic region.

Figure 5.6A shows a sample immunoblot indicating changes in CREB phosphorylation following high-frequency stimulation. CREB phosphorylation was increased following high-frequency stimulation in synaptosomes prepared from tetanised compared with untetanised dentate gyri of saline-injected rats. In contrast, there was no change in CREB activity in synaptosomes prepared from tetanised compared with untetanised dentate gyri of tyrphostin AG879-treated rats. Densitometric data from western immunoblot experiments indicate a significant LTP-associated enhancement of CREB activity in synaptosomes prepared from tetanised compared with untetanised dentate gyri of saline-pretreated rats (* $p<0.01$, unpaired *t*-test with Welch's correction). In this group, mean values (±SEM; n=6; in arbitrary units) were 13.989 ± 1.557 and 26.158 ± 2.481 in synaptosomes prepared from untetanised and tetanised dentate gyri

respectively. In synaptosomes prepared from dentate gyri of tyrphostin AG879-pretreated rats, tetanus failed to induce a change in CREB phosphorylation. Mean values (±SEM; n=6; in arbitrary units) in this group were 15.125 ± 3.208 and 17.514 ± 3.368 in synaptosomes prepared from untetanised and tetanised dentate gyri respectively.

5.3.7 Tyrphostin AG879 blocks LTP-associated increases in CREB phosphorylation in the postsynaptic region.

A sample immunoblot (Figure 5.7) shows changes in CREB phosphorylation in response to tetanic stimulation. In the saline-treated group, CREB activity was enhanced in postsynaptically enriched preparations from tetanised dentate gyri compared with untetanised dentate gyri. In the tyrphostin AG879-treated group, there was no change in CREB activity in postsynaptic preparations from tetanised dentate gyri compared with postsynaptic preparations from untetanised dentate gyri. Densitometric data indicated a significant increase in CREB phosphorylation in postsynaptic preparations obtained from tetanised dentate gyri compared with postsynaptic preparations obtained from untetanised dentate gyri of saline-injected rats (* $p<0.05$, unpaired *t*-test with Welch's correction). Mean values (±SEM; n=6; in arbitrary units) were 13.940 ± 1.145 and 17.173 ± 0.802 in postsynaptic preparations from untetanised and tetanised dentate gyri respectively. Pretreatment with tyrphostin AG879 blocked the tetanus-induced increase in CREB phosphorylation. Mean values (±SEM; n=6; in arbitrary units) were 12.692 ± 1.027 and 13.43 ± 1.286 in postsynaptic preparations prepared from untetanised and tetanised dentate gyri respectively.

5.3.8 Tyrphostin abolishes LTP-enhanced CREB phosphorylation in the entorhinal cortex.

CREB phosphorylation was assessed in homogenate prepared from entorhinal cortices from the side of the brain that received tetanic stimulation (ipsilateral) and the unstimulated side (contralateral). In the saline-treated group, CREB phosphorylation in homogenates prepared from the ipsilateral side was increased compared with the contralateral side as shown in a representative immunoblot (Figure 5.8A). In the tyrphostin AG879-treated group, tetanic stimulation failed to increase CREB

phosphorylation from unstimulated levels. Analysis of CREB phosphorylation by densitometry shows a significant increase in CREB activation in homogenates prepared from ipsilateral entorhinal cortices compared with the contralateral entorhinal cortices of the saline-treated group (* $p<0.05$, unpaired *t*-test with Welch's correction). Mean values (±SEM; n=6; in arbitrary units) were 16.260 ± 1.481 and 21.711 ± 1.860 in homogenates prepared from ipsilateral and contralateral entorhinal cortices respectively. Preinjection with tyrphostin AG879 blocked tetanus-associated CREB phosphorylation. In the tyrphostin AG879-treated group, mean values (±SEM; n=6; arbitrary units) were 12.289 ± 2.656 and 13.228 ± 2.172 in homogenates prepared from ipsilateral and contralateral entorhinal cortices respectively.

5.3.9 Tyrphostin AG879 inhibits LTP-associated increases in protein synthesis in the entorhinal cortex.

Protein synthesis was assessed as [^{35}S]-methionine labelling of TCA-precipitated proteins in slices ipsilateral and contralateral entorhinal cortex of saline- and tyrphsotin-AG879 treated rats following high-frequency stimulation. Protein synthesis was significantly increased in the ipsilateral entorhinal cortex compared with the contralateral side (* $p<0.05$, unpaired *t*-test with Welch's correction) of saline-treated rats as shown in Figure 5.9. Mean values (± SEM; n=6) were 264.200 cpm/mg protein ± 32.401 and 523.717 cpm/mg protein ± 92.727 in contralateral and ipsilateral entorhinal cortices respectively. In contrast, there was no change in protein synthesis in slices prepared from ipsilateral compared with contralateral entorhinal cortices of tyrphostin-AG879-treated rats; mean values (± SEM; n=6) were 236.583 cpm/mg protein ± 36.000 and 319.667 cpm/mg protein ± 44.473 respectively.

5.3.10 Protein synthesis in the hippocampus is not increased following LTP.

Protein synthesis was assessed in slices prepared from untetanised and tetanised hippocampal slices from saline- and tyrphostin-AG897-treated rats. There was no significant change in protein synthesis, measured as [^{35}S]-methionine incorporation into TCA-precipitated proteins, in untetanised or tetanised hippocampal tissue prepared from

saline- or tyrphostin-AG879-treated rats. In the saline-injected group, mean values (± SEM; n=6) were 192.902 cpm/mg protein ± 25.610 and 255.200 cpm/mg protein ± 38.471 in untetanised and tetanised samples respectively. In the tyrphostin-AG879-injected group, mean values (± SEM; n=6) were 190.604 cpm/mg protein ± 26.090 and 225.200 cpm/mg protein ± 24.633 in slices prepared from untetanised and tetanised hippocampi respectively.

Figure 5.1 Tyrphostin blocks the expression of LTP at the perforant path-granule cells synapses *in vivo*

Expression of LTP at the perforant path-granule cell synapses was inhibited by intracerebroventricular injection of tyrphostin AG879. Test shocks were delivered at 30s intervals followed by delivery of 3 trains of tetanus at 30s intertrain intervals (indicated by an arrow). Results are expressed as mean percentage changes in epsp slope with reference to the epsp slope in the 2min period immediately before tetanisation of 6 observations for each group. SEM values are shown for every 10^{th} response.

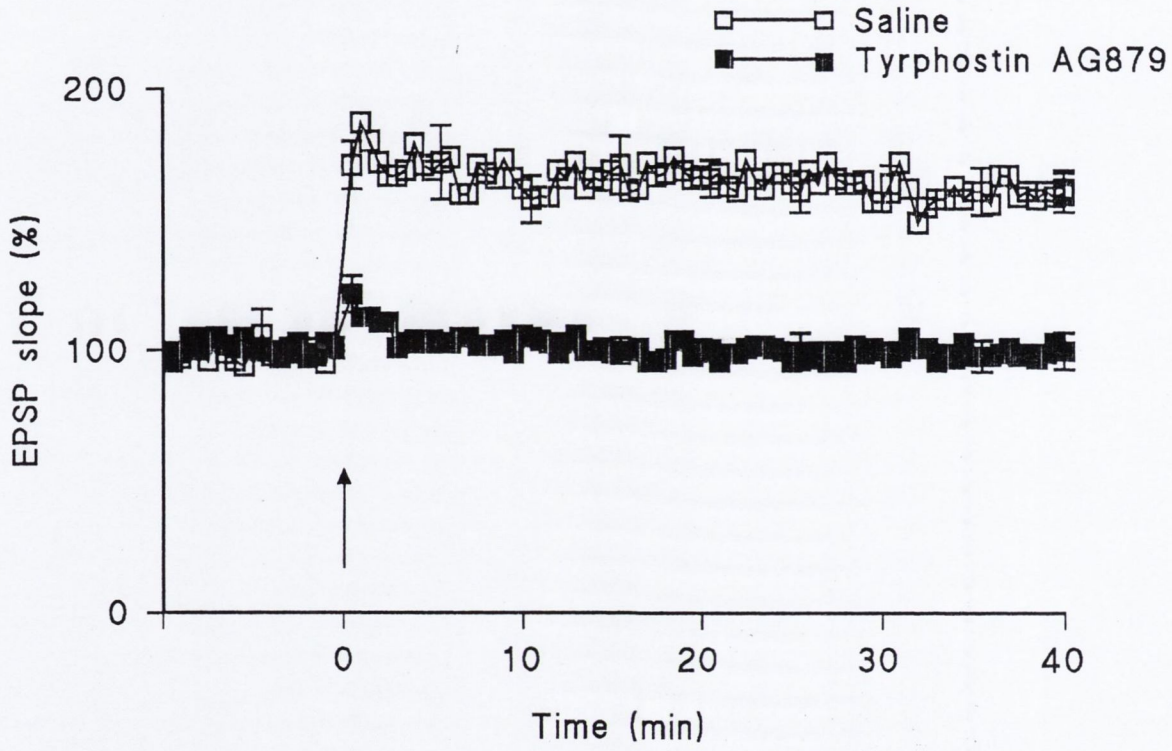

Saline
Tyrphostin AG879
200
100
0
EPSP slope (%)
0
10
20
30
40
Time (min)

Figure 5.2 Depolarisation-dependent NT-3 release is inhibited in tyrphostin AG879-injected rats

Addition of KCl (50mM) to synaptosomes prepared from untetanised and tetanised dentate gyri from saline-treated rats significantly increases NT-3 release (** $p<0.01$, unpaired *t*-test with Welch's correction). Incubation in the presence of KCl (50mM) fails to elicit increased NT-3 release from synaptosomes prepared from dentate gyri of tyrphostin AG879-treated rats. Results are means (± SEM) of 6 observations and expressed as pmol NT-3/mg protein.

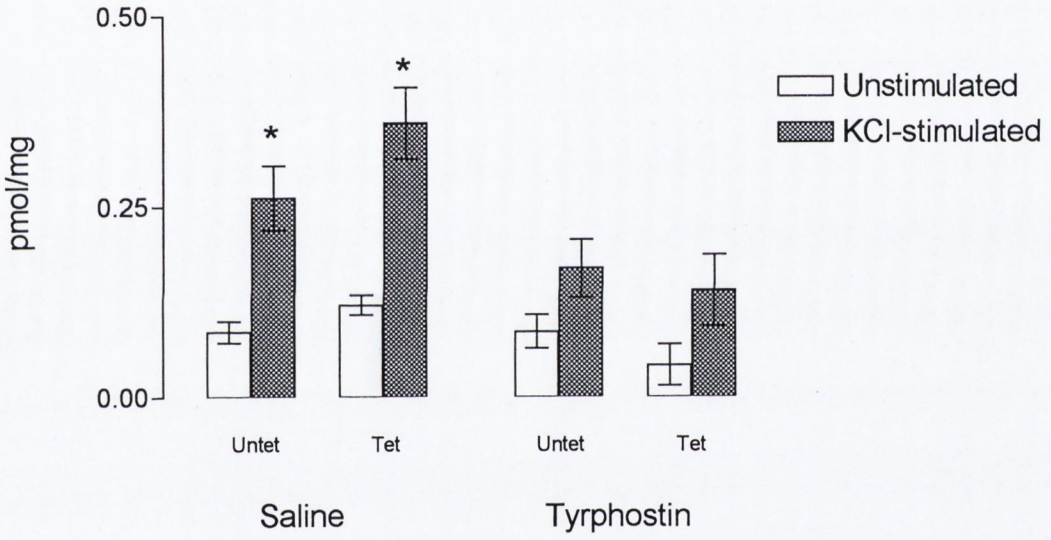

0.50
0.25
0.00
pmol/mg
*
*
Unstimulated
KCl-stimulated
Untet
Tet
Untet
Tet
Saline
Tyrphostin

Figure 5.3 Tyrosine kinase activity is increased following LTP but this effect is blocked by tyrphostin AG879

Densitometric data obtained from silver staining experiments were pooled to give a value for total tyrosine kinase activity in synaptosomes prepared from tetanised and untetanised tissue from saline- and tyrphostin AG879-injected rats. Tyrosine phosphorylation was significantly enhanced in synaptosomes prepared from tetanised dentate gyri of saline treated rats compared with synaptosomes prepared from untetanised dentate gyri (* $p<0.05$, unpaired *t*-test with Welch's correction). This tetanus-induced increase was not observed in synaptosomes prepared from tyrphostin AG879-treated rats. Results are means (± SEM) of 6 observations and expressed as arbitrary units.

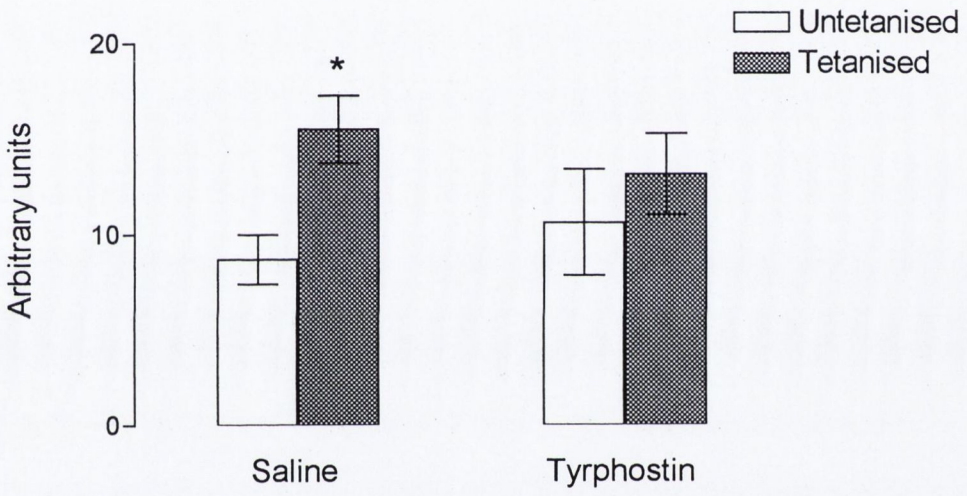
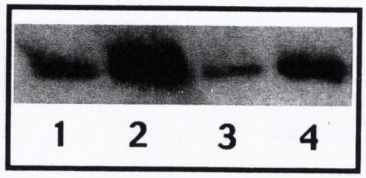

Untetanised
Tetanised
20
10
0
Arbitrary units
*
Saline
Tyrphostin

Figure 5.4 Tyrphostin AG879 inhibits LTP-induced increase in ERK activity in the presynaptic region.

A. Following the induction of LTP in rats pretreated with saline, phosphorylation of ERK was greater in synaptosomes prepared from dentate gyrus in tetanised tissue (lane 2) compared with untetanised tissue (lane 1). This tetanus-induced increase in ERK phosphorylation was blocked in synaptosomes prepared from tetanised (lane 4) and untetanised (lane 3) dentate gyri of rats pretreated with tyrphostin AG879.

B. Analysis of densitometric data indicates that ERK phosphorylation was significantly increased following tetanisation in synaptosomes prepared from dentate gyrus of rats pretreated with saline (* $p<0.05$, unpaired *t*-test with Welch's correction). The LTP-associated increase was blocked in synaptosomes prepared from the dentate gyri of rats pretreated with tyrphostin AG879. Results are expressed as arbitrary units and are means (± SEM) of 6 observations.

A.


1
2
3
4

B.

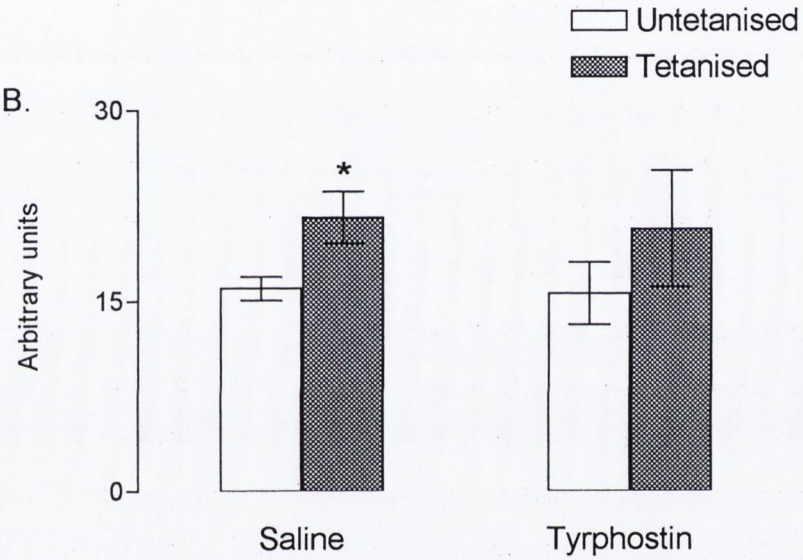
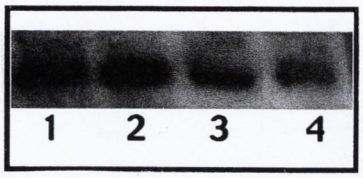

Untetanised
Tetanised
30
15
0
Arbitrary units
*
Saline
Tyrphostin

Figure 5.5 Tyrphostin AG879 inhibits LTP-induced increase in ERK activity in the postsynaptic region.

A. Following the induction of LTP in rats pretreated with saline, phosphorylation of ERK was greater in postsynaptically enriched preparations from tetanised (lane 2) compared with untetanised (lane 1) dentate gyri of saline-treated rats. This tetanus-induced increase in ERK phosphorylation was blocked in postsynaptically enriched preparations from tetanised (lane 4) compared with untetanised dentate gyri (lane 3) of tyrphostin AG879-treated rats.

B. Analysis of densitometric data indicates that ERK phosphorylation was significantly increased following tetanisation in postsynaptically enriched preparations prepared from tetanised dentate gyri compared with synaptosomes prepared from untetanised dentate gyri of rats pretreated with saline (* $p<0.05$, unpaired *t*-test with Welch's correction). The LTP-associated increase in ERK activity was blocked in postsynaptic samples prepared from the dentate gyri of rats preinjected with tyrphostin AG879. Results are expressed as arbitrary units and are means (± SEM) of 6 observations.

A.


1
2
3
4

B.

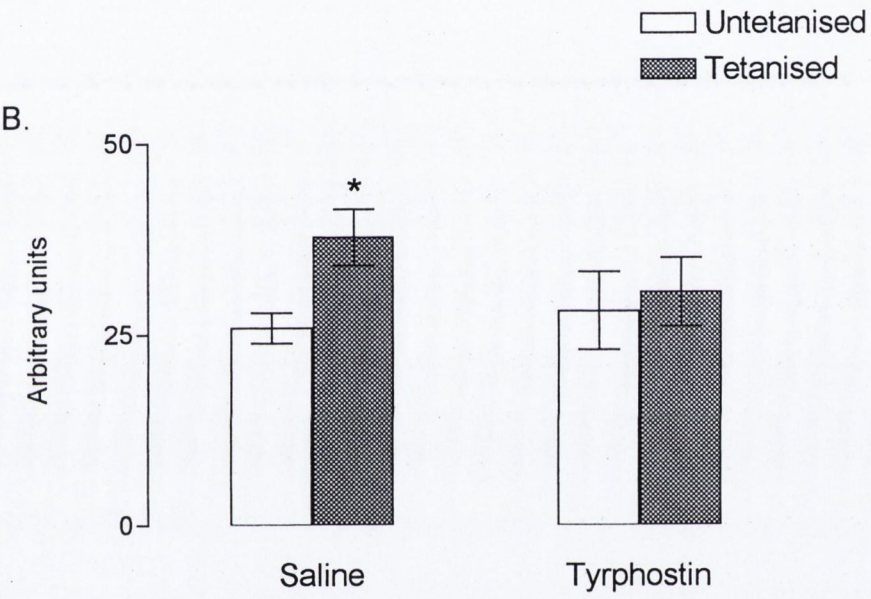

Untetanised
Tetanised
50
25
0
Arbitrary units
*
Saline
Tyrphostin

Figure 5.6 Tyrphostin AG879 blocks LTP-associated increase in CREB phosphorylation in the presynaptic region.

A. Phosphorylation of CREB was greater in synaptosomes prepared from dentate gyri of tetanised (lane 2) compared with untetanised (lane 1) dentate gyri of saline-injected rats as shown in this representative immunoblot. In contrast, pretreatment with tyrphostin AG879 blocked the tetanus-associated increase in CREB phosphorylation in synaptosomes prepared from tetanised dentate gyri (lanes 4) compared with those prepared from untetanised dentate gyri (lane 3).

B. Analysis of densitometric data indicates that in saline-injected rats, mean values of CREB phosphorylation were significantly increased in synaptosomes prepared from dentate gyri (* $p<0.01$, unpaired *t*-test with Welch's correction). This effect was absent from samples prepared from tyrphostin AG879-pretreated rats. Results are given as arbitrary units and are expressed as means (± SEM) of 6 observations.

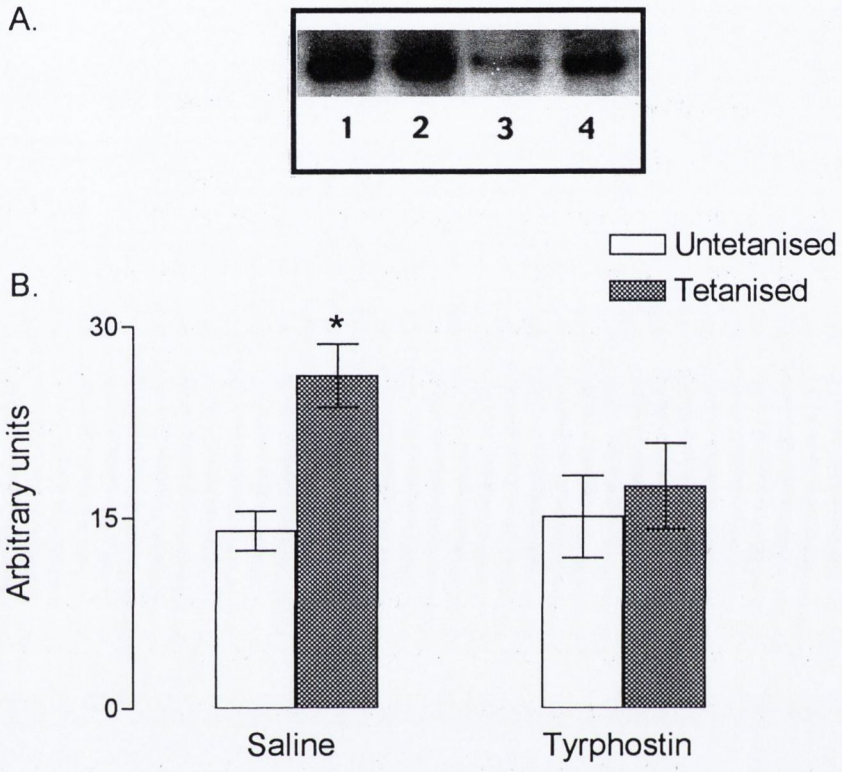
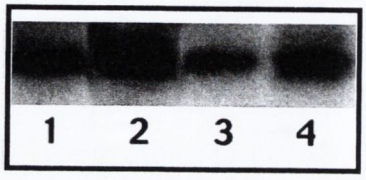

A.
1
2
3
4
B.
Untetanised
Tetanised
Arbitrary units
30
15
0
*
Saline
Tyrphostin

Figure 5.7 Tyrphostin AG879 blocks LTP-associated increase in CREB phosphorylation in the postsynaptic region.

A. Phosphorylation of CREB was greater in postsynaptically enriched preparations from tetanised (lane 2) compared with untetanised (lane 1) dentate gyri of saline-injected rats as shown in this representative immunoblot. In contrast, pretreatment with tyrphostin AG879 blocked the tetanus-associated increase in CREB phosphorylation in postsynaptically enriched preparations from tetanised dentate gyri (lanes 4) compared with those prepared from untetanised dentate gyri (lane 3) of tyrphostin AG879-injected rats.

B. Analysis of densitometric data indicates that in saline-injected rats, mean values of CREB phosphorylation were significantly increased in postsynaptically enriched preparations from dentate gyrus (* $p<0.05$, unpaired *t*-test with Welch's correction) following tetanic stimulation. This effect was absent from postsynaptically enriched preparations from tyrphostin AG879 pretreated rats. Results are given as arbitrary units and are expressed as means (± SEM) of 6 observations

A.


1
2
3
4

B.

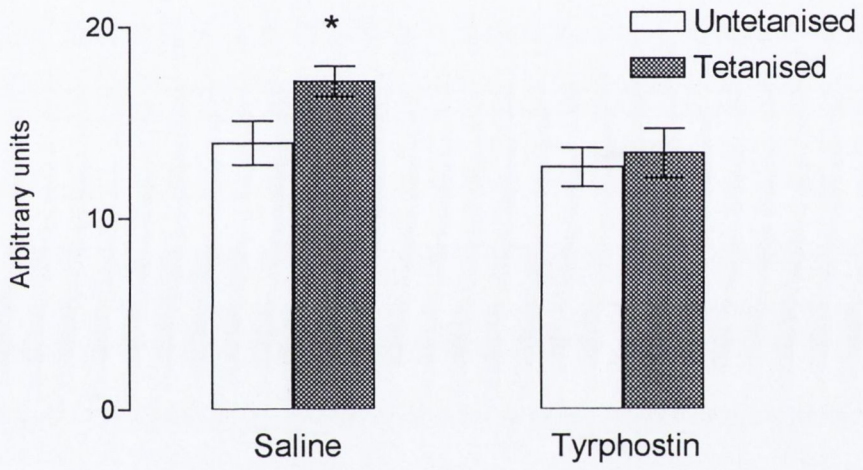
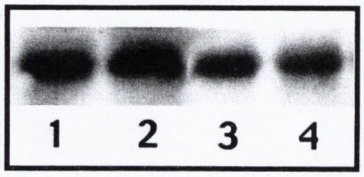

20
10
0
Arbitrary units
*
Untetanised
Tetanised
Saline
Tyrphostin

Figure 5.8 Tyrphostin abolishes LTP-enhanced CREB phosphorylation in the entorhinal cortex.

A. Following tetanic stimulation, phosphorylation of CREB was greater in homogenates prepared from the ipsilateral entorhinal cortices (lane 2) compared with samples prepared from the contralateral entorhinal cortices (lane 1) of saline-injected rats as shown in this representative immunoblot. Pretreatment with tyrphostin AG879-blocked increased CREB activity in homogenates prepared from ipsilateral (lane 4) compared with contralateral (lane 3) entorhinal cortices.

B. Analysis of densitometric data indicates that in saline-injected rats, mean values of CREB phosphorylation were significantly increased in homogenates prepared from entorhinal cortex (* $p<0.05$, unpaired *t*-test with Welch's correction). This effect was absent from samples prepared from tyrphostin AG879-pretreated rats. Results are given as arbitrary units and are expressed as means (± SEM) of 6 observations.

A.


1
2
3
4

B.

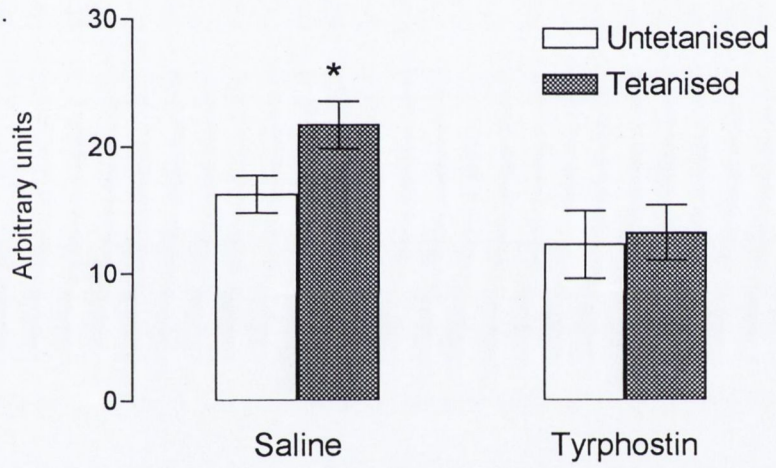

30
20
10
0
Arbitrary units
*
Untetanised
Tetanised
Saline
Tyrphostin

Figure 5.9 Tyrphostin AG879 inhibits LTP-associated increase in protein synthesis in the entorhinal cortex.

Protein synthesis was assessed as [^{35}S]-methionine labelling of proteins in slices prepared entorhinal cortex from tetanised and untetanised sides of the brain of saline- and tyrphostin AG879-injected rats. Protein synthesis was increased following tetanic stimulation in saline-treated rats (* $p<0.05$, unpaired *t*-test with Welch's correction). In contrast, there was no change in protein synthesis samples prepared the entorhinal cortex following tetanisation of the dentate gyrus of the tyrphostin AG879-treated group. Results are expressed as cpm/mg protein and are means (± SEM) of 6 observations.

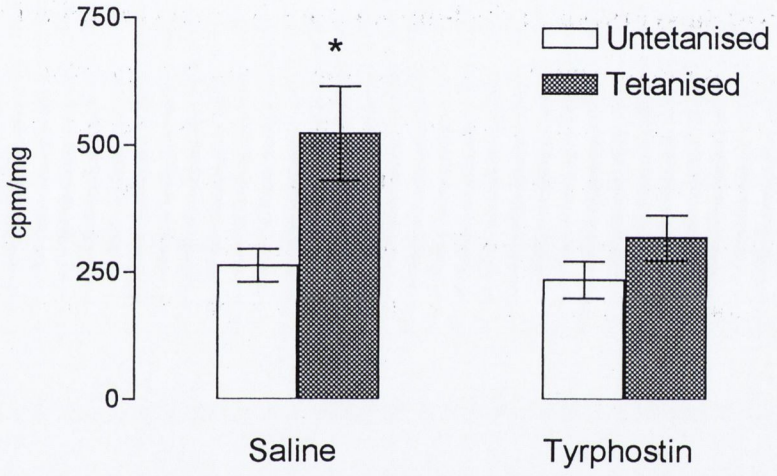

750
500
250
0
cpm/mg
*
Untetanised
Tetanised
Saline
Tyrphostin

Figure 5.10 Protein synthesis in the hippocampus is not increased following LTP. Protein synthesis was assessed as [^{35}S]-methionine labelling of proteins in slices prepared from untetanised and tetanised hippocampi of saline- and tyrphostin AG879-injected rats following tetanus. Protein synthesis was not increased following the induction of LTP in either the saline- or tyrphostin AG879-injected groups ($p>0.05$, unpaired *t*-test with Welch's correction).. Results are expressed as cpm/mg protein and are means (± SEM) of 6 observations.

5.4 Discussion

The aim of this study was to investigate the contribution of Trk receptors to long-term potentiation in the perforant path-granule cell synapses and to elucidate the intracellular signalling mechanism underlying such involvement. The Trk inhibitor, tyrphostin AG879, was used to assess impact on LTP of tyrosine kinase activity initiated by activation of Trk receptors. The data presented here indicates that LTP is inhibited by pretreatment with tyrphostin AG879. Following analysis of intracellular phosphorylation events, it was found that pretreatment with tyrphostin AG879 blocked increases in activation of several intracellular substrates that accompany LTP. In the dentate gyrus, increases in tyrosine kinase, ERK activity and CREB activity were reported both pre- and post-synaptically following LTP. In addition, pretreatment with tyrphostin AG879 suppressed LTP-induced changes in ERK and CREB activity on both sides of the synapse. In the entorhinal cortex, LTP enhanced CREB activity and simultaneously stimulated protein synthesis but treatment with tyrphostin AG879 abolished these responses.

Intracerebroventricular injection of tyrphostin AG879 resulted in a complete blockade of the expression of LTP in the dentate gyrus, consistent with previous findings that tyrphostin AG879 inhibits LTP (Maguire *et al.*, 1999). It also confirms other findings that general tyrosine kinase inhibitors block the expression of LTP in both the CA1 region (O'Dell *et al.*, 1991a; Huang and Hsu, 1999) and the dentate gyrus (Abe and Saito, 1993, McGahon and Lynch, 1997). In addition, it is supported by the results presented in Chapter 3. Involvement of tyrosine receptor kinase, Trk, in the expression of LTP has been proposed by several reports, which indicates a role for neurotrophic factors in LTP and is suggested by the high levels of expression of neurotrophins and their receptors in the hippocampus (Klein *et al.*, 1989; Hofer *et al.*, 1990; Phillips *et al.*, 1990; Wetmore *et al.*, 1990, Merlio *et al.*, 1992, Lambelle *et al.*, 1991, Maisonpierre *et al.*, 1990). Strong evidence of a role for neurotrophins in the underlying biochemical events of LTP at the perforant path-granule cell synapses is provided by many reports citing modulatory function of neurotrophins in glutamate release (Teyler and Pozzo-Miller, 2001; Pozzo-Miller *et al.*, 1999), a central feature of LTP in the perforant path-granule cell synapses.

To investigate the effect of expression of LTP on neurotrophin activity, NT-3 release was assessed in synaptosomes prepared from the dentate gyrus of saline- and tyrphostin AG879-treated rats. Addition of KCl to synaptosomes prepared from untetanised dentate gyri stimulated NT-3 release, consistent with data presented in Chapter 4 and with other reports of activity-controlled neurotrophin release (b *et al.*, 2000, Blochl and Thoenen, 1996; Goodman *et al.*, 1996). The depolarising pulse did not result in greater release of NT-3 from synaptosomes prepared from tetanised dentate gyri. This pattern contrasts with that reported from this laboratory for the release of NGF following LTP (Kelly *et al.*, 2000). In the tyrphostin AG879-treated group, KCl-stimulation of NT-3 release was completely abolished suggesting activation of Trk receptors is an essential prerequisite of NT-3 release from presynaptic terminals. Involvement of tyrosine kinase in NT-3-stimulated release of BDNF or NGF has previously been suggested by work demonstrating the inhibitory effect of tyrosine kinase inhibitor, K252a, on release from hippocampal neurons (Canossa *et al.*, 1997) and by results presented in Chapter 4.

To assess the impact of tyrphostin AG879-treatment on intracellular signalling cascades, tyrosine kinase activity was analysed in synaptosomes prepared from dentate gyri of rats pretreated with saline or tyrphostin AG879. Results presented here indicate an LTP-associated rise in tyrosine kinase activity presynaptically. This confirms findings presented in Chapter 3 and those reported previously (Maguire *et al.*, 1999). Tyrosine kinase activity was not elevated in the tyrphostin AG879-treated group, indicating that the Trk receptor was responsible for transducing synaptic events into intracellular enhancement of tyrosine kinase signalling pathways. The inhibitory influence of tyrphostin AG879 on tyrosine kinase activity may be twofold; it may act directly on the Trk receptors thus eliminating intracellular tyrosine phosphorylation cascades while also abolishing Trk-mediated release of NT-3 or other neurotrophins (Canossa *et al.*, 1997; Krüttgen *et al.*, 1998).

To elucidate the signalling mechanism and to identify possible loci of action of tyrosine kinase, Western immunoblotting experiments were conducted on pre- and postsynaptic preparations to examine components of the intracellular cascade triggered by Trk activation. In the presynaptic region, activities of two possible downstream

messengers, ERK and CREB, were examined. In the saline-treated group, ERK phosphorylation in synaptosomes was stimulated by expression of LTP. This is supported by findings in this laboratory (McGahon and Lynch, 1998; Maguire *et al.*, 1999) and is consistent with previous findings suggesting that ERK activity is necessary for the expression of LTP in the CA1 region (English and Sweatt, 1996) and in the dentate gyrus (McGahon and Lynch, 1998). In contrast, pretreatment with tyrphostin AG879 abolished the LTP-associated increases in ERK activity in presynaptic terminals, confirming reports that LTP-induced enhancements in ERK activity are mediated by Trk receptor activity (Maguire *et al.*, 1999). Similarly, ERK activation was increased in postsynaptic preparations, prepared from tetanised dentate gyrus, compared with untetanised dentate gyrus of the saline-treated group. Treatment with tyrphostin AG879 blocked the LTP-associated enhancement of ERK activity in the postsynaptic region.

Activation of ERK is most likely to result from a cascade triggered by Trk receptor, leading to the activation of ras (Wood *et al.*, 1992; Thomas *et al.*, 1992). However, another possible mechanism is by activation of PLCγ, a tyrosine kinase substrate known to interact with the phosphotyrosine residues in the cytosolic domain of the Trk receptor (Vetter *et al.*, 1991). Important second messengers, IP_3 and DAG, are products of PLCγ and it has been well documented that stimulation of protein kinase C by DAG is a consequence of PLCγ activation (Kaplan and Stephens, 1994). It has been suggested that PKC phosphorylates ERK on threonine residues (Stratton *et al.*, 1999), which is necessary but not sufficient for ERK activation (Payne *et al.*, 1991; Her *et al.*, 1993). The functional consequences of ERK activation following LTP have not been fully explored but it has been suggested that ERK may be a convergence point for several intracellular pathways and may therefore serve to amplify or co-ordinate several discrete signals. The effects of ERK activity may be immediate such as enhancement of glutamate release by phosphorylation of synapsin 1 (Jonanovic *et al.*, 1996) or ERK may serve as a conduit to reinforce long-term changes such as protein synthesis, thought necessary for the persistence of LTP (Krug *et al*, 1984; Mullany and Lynch, 1997), perhaps through phosphorylation of the transcription factor CREB (Nguyen and Kandel, 1999; Schulz *et al.*, 1999).

Analysis of phosphoCREB in synaptosomes prepared from dentate gyrus of the saline-injected group indicated that CREB activity is increased following tetanisation. Administration of tyrphostin AG879 blocked the activation of CREB in response to LTP-inducing stimuli. Increases in CREB activity presented in the present study confirm earlier reports that CREB activity is necessary for the expression of LTP (Schulz *et al.*, 1999; Davis *et al.*, 2000). Parallel enhancement of both ERK and CREB phosphorylation in the pre- and postsynaptic regions of the dentate gyrus indicate a functional coupling of both events. The outcome of increased CREB activity in presynaptic terminals is unknown but it is possible that it may induce local protein synthesis as suggested by findings presented by Kang and Schuman (1996), who reported that NT-3 and BDNF stimulate local protein synthesis in hippocampal neurons, presumably by activation of ERK and CREB. The functional consequence of activation of transcription factors may be the initiation of synaptogenesis, known to accommodate synaptic changes accompanying LTP (Buchs and Miller, 1996; Toni *et al.*, 1999).

Postsynaptic components of the inhibitory mechanism of LTP by tyrphostin AG879 were also considered because previous reports have suggested a postsynaptic involvement of tyrosine kinase in LTP (Huang and Hsu, 1999). In the postsynaptic region, ERK activity was increased following the expression of LTP in the saline-treated group. A parallel increase was not observed in the tyrphostin AG879-treated group. At least two possible stimuli for ERK activity in the postsynaptic region exist; ERK activity may be a direct consequence of Trk activation or alternatively, ERK activation may be a consequence of NMDA receptor activation (Rosen *et al.*, 1994; Kurino *et al.*, 1995; Brambilla *et al.*, 1997) and may thus consolidate postsynaptic events triggered by glutamate release. Direct activation of ERK, mediated by Trk, may be a result of increased activity-dependent NT-3 release. It is also possible that increased ERK activity is a downstream result of modulation of NMDA receptor activity by tyrosine kinase. NMDA receptors have previously been identified as a substrate for tyrosine kinase (Rostas *et al.*, 1996; Rosenblum *et al.*, 1995) and their activity is modulated by BDNF (Suen *et al.*, 1997; Levine *et al.*, 1998; Lin *et al*, 1998).

The GTP-binding protein, ras, which is active in its GTP-bound form, is the likely link between the action of the NMDA receptor and ERK. SynGAP, which retards

inactivation of ras (Chen *et al.*, 1998), is located in a macromolecular complex in the postsynaptic density with NMDA receptor and PSD-95. CaM kinase II phosphorylates and thus inactviates SynGAP activation (Chen *et al.*, 1998). Together, SynGAP and CAM kinase II constitute a high proportion of the post-synaptic density (Chen *et al.*, 1998; Kennedy *et al.*, 1983) and therefore activation of ERK may be a principal consequence of NMDA activity. One result of persistent activation of ras is increased phosphorylation of ERK downstream, which ultimately leads to induction of IEGs (Vanhoutte *et al.*, 1999, Xia *et al.*, 1996, Treisman, 1995), an event which is considered to contribute to the maintenance of LTP (Jeffrey *et al.*, 1990; Richardson *et al.*, 1992).

One target of ERK is the constitutively-expressed transcription factor CREB. The data obtained in this study indicate that CREB activation was enhanced in the postsynaptic region of preparations obtained from saline-treated rats following tetanisation but this effect was inhibited by pretreatment with tyrphostin AG879. Concomitant increases in phosphorylation of CREB on Ser133 and dual phosphorylation of ERK in the postsynaptic region suggest a coupling of the phosphorylation events. This suggestion is supported by other reports, which indicate involvement of CREB activation in the maintenance of LTP in the hippocampus (Schulz *et al.*, 1999; Davis *et al.*, 2000). ERK-mediated CREB activation is most likely due to phosphorylation of Rsk by ERK, which in turn phosphorylates CREB (Xing *et al.*, 1998). The neurotrophin-stimulated ras/Raf/ERK path is not the sole means by which activation of CREB can be achieved. Inputs from PKA and cAMP may also modify CREB activity following the induction of LTP but in view of inhibition of LTP-associated enhancement of CREB activity by tyrphostin AG879, it is probable that in this case, CREB activity is a direct consequence of Trk activation. It has been demonstrated that CREB induces morphological changes in synaptic spines, in response to stimulation by BDNF (Murphy and Segal, 1997), and this may serve as a mechanism underlying synaptic remodelling necessary for the maintenance of LTP (Fifkova and and Van Harreveld, 1977; Geinisman *et al.*, 1993; Engert and Bonhoffer, 1999; Buchs and Miller, 1996; Toni *et al.*, 1999).

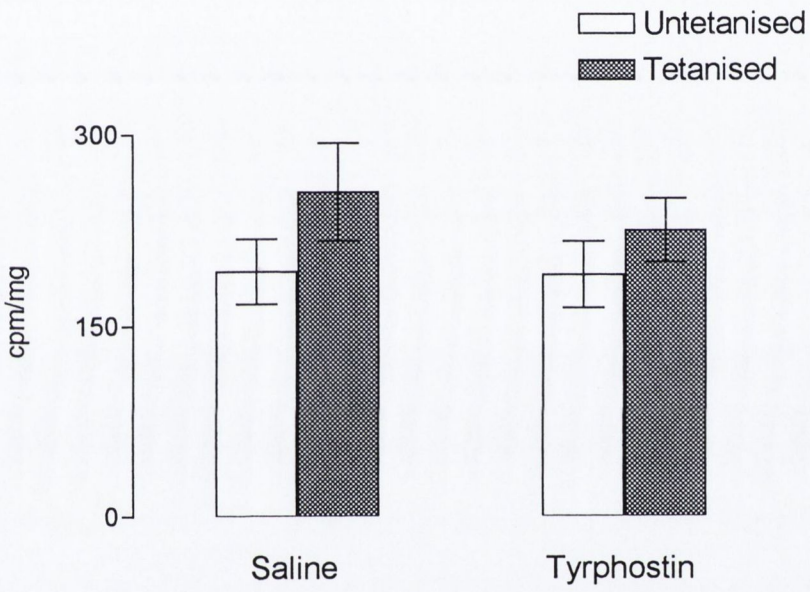
Following tetanisation, changes in CREB phosphorylation and protein synthesis were assessed in the entorhinal cortex, where the cell bodies of the perforant path reside. Data presented in this study indicate that CREB phosphorylation was increased in

samples prepared from the saline-treated group as a consequence of the delivery of LTP-inducing tetanus to the perforant path but a similar trend was not demonstrated in samples prepared from the tyrphostin AG879-treated group. These findings concur with results presented in Chapter 3, showing an LTP-associated increase in CREB activation that was blocked by tyrosine kinase inhibition. Previous studies have reported the involvement of CREB in the signalling events underpinning LTP (Schulz *et al.*, 1999; Davis *et al.*, 2000). CREB has also been identified as a mediator of NGF-stimulated induction of *c-fos* (Ginty *et al.*, 1994). This indicates possible overlap of the signalling pathways employed by LTP and neurotrophins and adds further credence to evidence that neurotrophins are involved in the expression of LTP.

One effect of phosphoCREB is likely to be provision of newly synthesised proteins as an outcome of the induction of IEGs (Ginty *et al.*, 1994; Sheng and Greenberg, 1990; Ginty *et al.*, 1992) and it is interesting to note that molecules implicated in the expression of LTP, such as BDNF (Hughes *et al.*, 1993) and ERK (Thomas *et al*, 1994) are products of IEGs. To determine a parallel between CREB phosphorylation and protein synthesis resulting from the expression of LTP in the dentate gyrus, *de novo* protein synthesis was examined in the entorhinal cortex. Incorporation of [^{35}S] methionine into newly synthesised proteins was elevated in samples prepared from ipsilateral entorhinal cortex of saline-injected rats compared with the contralateral entorhinal cortex. In samples prepared from entorhinal cortex of tyrphostin AG879-injected rats, delivery of tetanus to the perforant path failed to elicit enhancement of protein synthesis. Other groups have reported that protein synthesis is an integral component of the maintenance phase of LTP (Krug *et al.*, 1984; Mullany *et al.*, 1997) and that protein synthesis inhibitors block the stabilisation of LTP (Otani *et al.*, 1989; Krug *et al.*, 1984, Mullany *et al.*, 1997). The absence of an increase in protein synthesis in the tyrphostin AG879-treated group may be a direct result of the absence of Trk signalling and activation of its resultant signalling molecules, such as ERK and CREB, known to impact on protein synthesis (Xia *et al.*, 1996; Vanhoutte *et al.*, 1999). Neurotrophins and their receptors have previously been linked to changes in synaptogenesis (Murphy and Segal, 1999; Vicario-Abejon *et al.*, 1998; Martinez *et al.*, 1998), a process likely to require synthesis of new proteins. Therefore it seems reasonable to suggest that such

neurotrophin-mediated signalling events may underlie structural modifications following the induction of LTP at perforant path-granule cell synapses. In contrast, tetanus failed to stimulate protein synthesis in samples prepared from the hippocampus of the saline- or tyrphostin AG879-treated groups. It may reflect the retrograde transport of the neurotrophin-Trk complex (Bhattacharyya *et al.*, 1997) in the entorhinal cortex, rather that a feedforward mechanism in the trisynaptic loop of the hippocampal circuit. It has previously been proposed that neurotrophin effects are mediated by long-range retrograde signalling to the soma, where changes in gene expression are induced (Ehlers *et al.*, 1995). The signalling requires autophosphorylation of specific tyrosine residues on the Trk receptors, followed by receptor endocytosis and retrograde transport to the cell body (Korshing *et al.*, 1983; DiStefano *et al.*, 1992).

This study demonstrated Trk-mediated signalling events play a substantial role in the biochemical events underlying the expression of LTP in the dentate gyrus. Such signalling events involve downstream messengers such as ERK and CREB and occur on both sides of the synapse. Corresponding changes in the entorhinal cortex such as increases in CREB and protein synthesis are also shown to rely on Trk-dependent signalling but in the absence of such signals, tetanus failed to induce robust LTP.


Untetanised
Tetanised
300
150
0
cpm/mg
Saline
Tyrphostin

Chapter 6

Investigating a possible role for NT-3 in LTP

6.1 Introduction

Evidence implicates the involvement of neurotrophins, particularly BDNF in synaptic plasticity. Neurotrophin-3 (NT-3) has been suggested to play a role in short-term modulation of synaptic transmission. Initial evidence of a modulatory function for NT-3 was provided by Lohof and coworkers (1993). Since then, NT-3 has been reported to participate in short-term enhancement of synaptic plasticity at central synapses. Fast actions of NT-3 on synaptic transmission in the CNS were first reported by Kim and coworkers (1994), who suggested that NT-3 potentiates synaptic transmission in developing cortical neurons by inhibiting GABAergic transmission. Other groups have reported involvement of NT-3 in presynaptically-mediated enhancement of transmission in the perforant path-granule cell synapses of the dentate gyrus (Kokaia *et al.*, 1998). NT-3 knockout mice show aberrant synaptic transmission and this deficit is reversed by application of recombinant NT-3 (Kokaia *et al.*, 1998; Asztely *et al.*, 2000). This rescue effect is similar to that observed in BDNF knockout mice (Patterson *et al.*, 1996).

Evidence supporting a role for NT-3 in LTP in the hippocampus is more controversial. NT-3 has been reported to elicit long-lasting changes in synaptic transmission in the hippocampus that resemble LTP (Kang and Schuman 1995). However, it was also found that antibodies to NT-3 do not affect the expression of LTP (Chen *et al.*, 1999a). Patterson and coworkers (1992) found that induction of LTP leads to an increase in mRNA levels of BDNF and NT-3 in the CA1 region of the hippocampus but this contrasts with reports that induction of LTP in the dentate gyrus leads to a decrease in NT-3 mRNA (Castren *et al.*, 1993). More recently, Ma and coworkers (1999) have disputed the involvement of NT-3 in LTP in the CA1 region. However, the high levels of NT-3 distribution which are found in the granule cells of the dentate gyrus (Zhou and Rush, 1994; Tokuyama *et al.*, 1999) may result in differential regulation of synaptic plasticity in different hippocampal areas.

The aims of this study were 1) to explore the possibility of participation of NT-3-mediated signalling in the expression of LTP at the perforant path-granule cell synapses and 2) to assess the effect of exogenous NT-3 on downstream second messengers of the Trk signalling cascade involved in LTP.

6.2 Methods

6.2.1 Induction of LTP *in vivo*

The method used was described in Section 2.5. Rats were anaesthetised with urethane (1.5g/kg i.p.) and placed in a head holder of a stereotaxic frame. An intracerebroventricular injection of either NT-3 (5µl; final concentration: 20ng/ml) or saline (5µl) was administered 30min prior to recording. Test shocks were delivered every 30s for a 10min control period. This was followed by delivery of 3 trains of high frequency stimuli (250Hz for 200ms) at 30s interval. Recording at test shock frequency resumed for a 40min period. At the end of the experimental period, rats were killed by cervical dislocation and decapitation. Tissue was sliced and stored at –80°C.

6.2.2 Analysis of NT-3 release

The methods used for collection of samples for analysis of NT-3 release are outlined in detail in Section 2.10. Synaptosomes were prepared from tetanised and untetanised dentate gyri of rats pretreated with NT-3 or saline and preincubated in Krebs solution containing $CaCl_2$ (2mM) for 3min at 37°C under constant oxygenation. Synaptosomes were then centrifuged at 5,000rpm for 1min and the supernatant discarded. To investigate unstimulated release, the pellet was resuspended in Krebs solution containing $CaCl_2$ (2mM) and incubated for 5min at 37°C under constant oxygenation. Synaptosomes were centrifuged at 5,000rpm for 1min and the supernatant retained for analysis of NT-3 concentration. To investigate depolarisation-induced NT-3 release, the pellet was resuspended in Krebs solution containing KCl (50mM) and incubated at 37°C for 5min under constant oxygenation. The samples were spun at 5,000rpm and the supernatant was recovered. The pellet was retained for determination of protein concentrations and the supernatants were analysed for NT-3 concentrations as described in Section 2.11.

6.2.3 Measurement of ERK phosphorylation

Synaptosomes (P_2) and crude postsynaptic preparations (P_1), prepared from tetanised and untetanised dentate gyri of rats pretreated with NT-3 or saline, were analysed for ERK activation. Proteins were separated on 10% SDS-polyacrylamide gels and immunoblotted with anti-active ERK (1.5:1000 in TBS-T containing 2% non-fat dried milk; Promega, USA) as described in Section 2.16.

6.2.4 Measurement of CREB phosphorylation

CREB phosphorylation was measured in synaptosomes (P_2) and crude postsynaptic preparations (P_1) from tetanised and untetanised dentate gyri of rats pretreated with either NT-3 or saline. In some experiments, CREB phosphorylation was analysed in homogenates prepared from entorhinal cortices from either tetanised or untetanised dentate gyri of rats pretreated with either NT-3 or saline. Proteins were separated on 14% SDS-polyacrylamide gels as described in Section 2.14. Separated proteins were immunoblotted with anti-phospho CREB (1:1000 in 5% BSA in TBS-T) as outlined in Section 2.16.

6.2.5 Analysis of protein synthesis

The method used to assess [^{35}S]-labelling of TCA-precipitated proteins was described in Section 2.17. Slices were prepared from entorhinal cortices of tetanised or untetanised sides of the brain of rats pretreated with either NT-3 or saline. Slices were incubated in Krebs solution containing $CaCl_2$ (2mM), ATP (3.5mM) and [^{35}S]-methionine 0.2µl/ml) for 60min at 37°C under constant oxygenation. The reaction was terminated and [^{35}S]-methionine labelling of TCA-precipitated proteins was assessed. Protein concentrations were determined from incubated slices.

6.3 Results

6.3.1 Expression of LTP is not enhanced by pretreatment with NT-3 in the perforant path-granule cells synapses in vivo.

The slope of the epsp recorded from the granule cell layer of the dentate gyrus increased following delivery of tetanic stimulation to the perforant path of saline- and NT-3-treated rats (n=6). The mean percentage increases of the epsp slope in the 2min immediately preceding tetanic stimulation were compared with values in the 2min prior to tetanic stimulation.

6.3.2 Depolarisation causes NT-3 release from synaptosomes prepared from tetanised and untetanised dentate gyri.

Addition of KCl (50mM) stimulated NT-3 release from synaptosomes prepared from untetanised dentate gyri of saline-treated rats (*$p<0.01$, unpaired *t*-test with Welch's correction), as shown in Figure 6.2. Mean values (±SEM; n=6) were 0.086pmol NT3/mg protein ± 0.014 for unstimulated release and 0.262pmol NT3/mg protein ± 0.042 for KCl-stimulated release. Tetanisation increased the KCl-induced response in the saline-injected group (*$p<0.01$, unpaired *t*-test with Welch's correction), mean values (±SEM; n=6) were 0.121pmol NT3/mg protein ± 0.013 for unstimulated release and 0.360pmol NT3/mg protein ± 0.047 for KCl-stimulated release. Injection of NT-3 resulted in a further increase in KCl-induced NT-3 release from synaptosomes prepared from untetanised dentate gyri (* $p<0.01$, unpaired *t*-test with Welch's correction). Mean values (±SEM; n=6) were 0.099pmol NT3/mg protein ± 0.021 for unstimulated release and 0.300pmol NT3/mg protein ± 0.031 for KCl-stimulated release. Addition of KCl to synaptosomes prepared from tetanised dentate gyri did not cause further increases in depolarisation-stimulated NT-3 release (*$p<0.01$, unpaired *t*-test with Welch's correction). In this case, NT-3 concentrations were 0.128pmol NT3/mg protein ± 0.025 for unstimulated release and 0.339pmol NT3/mg protein ± 0.049 for KCl-stimulated release.

6.3.3 LTP enhances ERK activity in the presynaptic region.

ERK activity was stimulated in synaptosomes prepared from tetanised compared with untetanised dentate gyri of saline- and NT-3-treated rats, as shown in one sample immunoblot (Figure 6.3A). Figure 6.3B shows that ERK phosphorylation was enhanced in synaptosomes prepared from the dentate gyri of saline- and NT-3-treated rats following tetanus (* $p<0.05$, unpaired *t*-test with Welch's correction). In the saline-treated groups, mean values (± SEM; n=6; in arbitrary units) were 16.041 ± 0.948 and 21.580 ± 2.049 in samples prepared from untetanised and tetanised dentate gyri respectively. Pretreatment with NT-3 did not cause additional stimulation of ERK activity (* $p<0.05$, unpaired *t*-test with Welch's correction). In this case, mean values (± SEM; n=6) were 16.609 ± 1.401 and 25.460 ± 3.001 in synaptosomes prepared from untetanised and tetanised dentate gyri respectively.

6.3.4 LTP enhances ERK activity in the postsynaptic region.

As shown in one sample immunoblot (Figure 6.4A), ERK activity was enhanced following high-frequency stimulation in postsynaptic samples prepared from the dentate gyri of saline- and NT-3-treated rats. Following delivery of tetanic stimulation, densitometric data, shown in Figure 6.4B, indicate that mean ERK activity was significantly stimulated in the postsynaptic area of the dentate gyrus of rats preinjected with saline or NT-3 (* $p<0.05$, unpaired *t*-test with Welch's correction). In the saline-pretreated group, mean values (± SEM; n=6) were 23.005 arbitrary units ± 3.845 and 38.284 arbitrary units ± 4.894 for samples prepared from untetanised and tetanised dentate gyri respectively. Pretreatment with NT-3 did not result in any further enhancement of ERK activity (* $p<0.05$, unpaired *t*-test with Welch's correction). Mean values (± SEM; n=6; in arbitrary units) for this group were 23.005 ± 3.845 and 38.284 ± 4.894 for samples prepared from untetanised and tetanised dentate gyri respectively.

6.3.5 LTP promotes CREB phosphorylation in the presynaptic region.

CREB phosphorylation in synaptosomes prepared from dentate gyri of saline- and NT-3-treated rats was enhanced by tetanic stimulation as shown in one sample immunoblot (Figure 6.5A). Mean data obtained from densitometric analysis confirm an

LTP-associated increase in CREB activity in synaptosomes prepared from saline- and NT-3-treated rats (* $p<0.05$, unpaired *t*-test with Welch's correction), as shown in Figure 6.5B. In saline-treated group, mean values (±SEM; n=6; in arbitrary units) were 13.989 ± 1.557 and 26.158 ± 2.481 in synaptosomes prepared from untetanised and tetanised dentate gyri respectively. ERK activity was not further increased by pretreatment with NT-3 (* $p<0.05$, unpaired *t*-test with Welch's correction). In this group, mean values (±SEM; n=6; in arbitrary units) were 17.597 ± 2.096 and 29.598 ± 4.41 in synaptosomes prepared from untetanised and tetanised dentate gyri respectively.

6.3.6 LTP promotes CREB phosphorylation in the postsynaptic region.

LTP promotes CREB phosphorylation in postsynaptic preparations from the dentate gyri of saline- and NT-3-injected rats, as shown in one sample immunoblot (Figure 6.6A). Mean data obtained from densitometric analysis, shown in Figure 6.6B, indicate CREB activity was significantly increased in postsynaptic preparation from dentate gyri following tetanus (* $p<0.05$, unpaired *t*-test with Welch's correction). Mean values (±SEM; n=6; in arbitrary units) were 13.940 ± 1.145 and 17.173 ± 0.802 in postsynaptic preparations prepared from untetanised and tetanised dentate gyri of saline-treated rats respectively. Injection with NT-3 prior to high-frequency stimulation did not result in further stimulation of CREB (* $p<0.05$, unpaired *t*-test with Welch's correction). In this group, mean values (±SEM; n=6; in arbitrary units) were 15.558 ± 1.345 and 19.783 ± 0.994 in postsynaptic preparations prepared from untetanised and tetanised dentate gyri respectively.

6.3.7 LTP stimulates CREB phosphorylation in the entorhinal cortex.

Following tetanus, CREB activity was enhanced in ipsilateral compared contralateral entorhinal cortices, as shown in one sample immunoblot (Figure 6.7A). Mean data obtained from densitometric analysis confirm an LTP-induced increase in CREB phosphorylation in slices prepared from entorhinal cortices of saline- and NT-3-injected rats (* $p<0.05$, unpaired *t*-test with Welch's correction), as shown in Figure 6.7B. Mean values (±SEM; n=6; in arbitrary units) were 16.260 ± 1.481 and 21.711 ± 1.860 in slices prepared from ipsilateral and contralateral entorhinal cortices respectively of

saline-treated rats. Treatment with NT-3 prior to delivery of high-frequency stimulation did not result in additional stimulation of CREB activity (* $p<0.05$, unpaired *t*-test with Welch's correction). In this group, CREB activity (±SEM; n=6; in arbitrary units) was 13.914 ± 1.282 in slices prepared from ipsilateral entorhinal cortices compared with 18.981 ± 1.099 contralateral entorhinal cortices.

6.3.8 LTP increases protein synthesis in the entorhinal cortex.

Mean values for [^{35}S]-methionine incorporation into TCA-precipitated proteins were increased in slices prepared from ipsilateral entorhinal cortices, compared with the contralateral side, following tetanic stimulation (* $p<0.05$, unpaired *t*-test with Welch's correction). Mean values were 264.201cpm/mg protein ± 32.401 and 523.717 cpm/mg protein ± 92.728 in slices of contralateral and ipsilateral entorhinal cortices prepared from saline-treated rats respectively. There was no additional increase in tetanus-related protein synthesis in the NT-3 treated group (* $p<0.05$, unpaired *t*-test with Welch's correction). In this case, mean values (±SEM; n=6) were 311.567 cpm/mg protein ± 49.550 and 545.317 cpm/mg protein ± 92.185 in slices prepared from contralateral and ipsilateral entorhinal cortices respectively.

6.3.9 Protein synthesis in the hippocampus is not changed by LTP.

Following high-frequency stimulation, there was no significant increase in mean incorporation of [^{35}S]-methionine into TCA-precipitated proteins in slices prepared from the ipsilateral or contralateral hippocampus of saline- or NT-3-preinjected rats. In the saline-injected group, mean values (±SEM; n=6) were 192.901 cpm/mg protein ± 25.622 and 255.263 cpm/mg protein ± 38.408 in contralateral and ipsilateral entorhinal cortices respectively. In the case of NT-3-injected rats, mean values (±SEM; n=6) were 307.010 cpm/mg protein ± 59.200 and 277.961 cpm/mg protein ± 59.498 in contralateral and ipsilateral entorhinal cortices respectively.

Figure 6.1 Expression of LTP is not affected by pre-injection with NT-3 in the perforant path-granule cells synapses

Expression of LTP at the perforant path-granule cell synapses was induced by delivery of tetanus in saline-treated rats. Intracerebroventricular injection of NT-3 (20ng/ml) 30min prior to tetanisation did not result in enhancement of the epsp slope. Test shocks were delivered at 30s intervals followed by delivery of 3 trains of tetanus at 30s intertrain intervals (indicated by an arrow). Results are expressed as mean percentage changes in epsp slope with reference to the epsp slope in the 2min period immediately before tetanisation. SEM values are shown for every 10^{th} response.

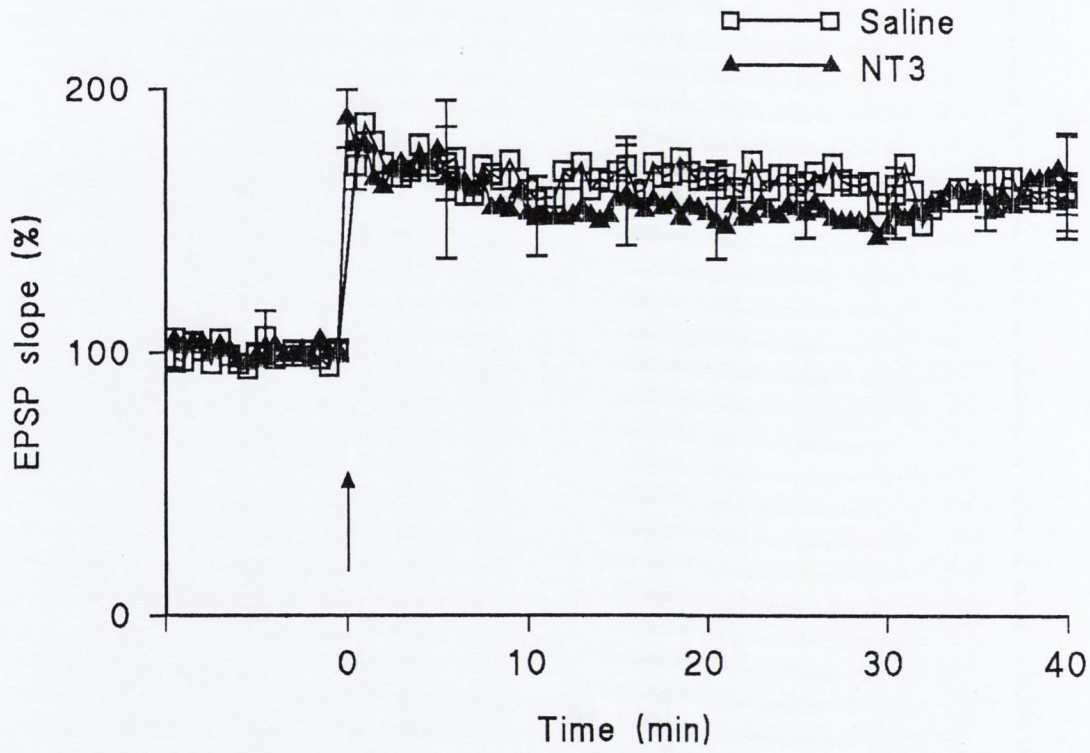

Saline
NT3
200
100
0
EPSP slope (%)
0
10
20
30
40
Time (min)

Figure 6.2 Depolarisation causes NT-3 release from synaptosomes prepared from tetanised and untetanised dentate gyri

Addition of KCl (50mM) to synaptosomes prepared from untetanised dentate gyri from saline-injected rats (*$p<0.01$, unpaired *t*-test with Welch's correction). KCl induced NT-3 release from synaptosomes prepared from tetanised dentate gyri of saline rats (* $p<0.01$, unpaired *t*-test with Welch's correction). Preinjection with NT-3 did not result in an additional effect on NT-3 release from synaptosomes from either untetanised or tetanised dentate gyri but in these cases, the LTP-associated increase in NT-3 release was also observed (*$p<0.01$, unpaired *t*-test with Welch's correction). Results are means (± SEM) of 6 observations and expressed as pmol NT3/mg protein.

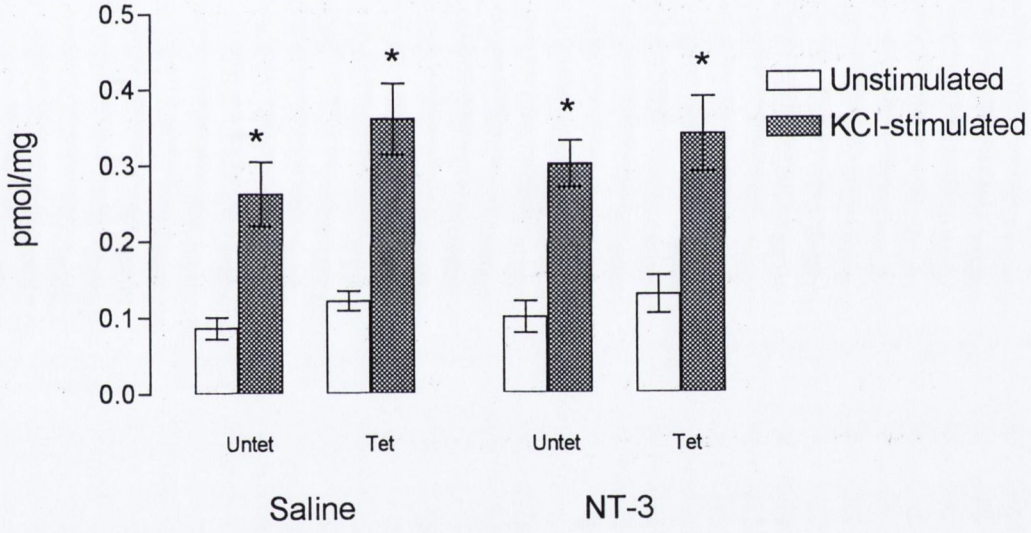

0.5
0.4
0.3
0.2
0.1
0.0
pmol/mg
*
*
*
*
Unstimulated
KCl-stimulated
Untet
Tet
Untet
Tet
Saline
NT-3

Figure 6.3 LTP enhances ERK activity in the presynaptic region

A. Following the induction of LTP in rats pretreated with saline, phosphorylation of ERK was greater in synaptosomes prepared from tetanised (lane 2) compared with untetanised (lane 1) dentate gyri of saline-treated rats. This tetanus-induced increase in ERK phosphorylation was also present in synaptosomes prepared from tetanised (lane 4) compared with untetanised (lane 3) dentate gyri of rats pretreated with NT-3.

B. Analysis of densitometric data indicates that ERK phosphorylation was significantly increased following tetanisation in synaptosomes prepared from dentate gyri of rats pretreated with saline (* $p<0.05$, unpaired *t*-test with Welch's correction). The LTP-associated increase was also observed in synaptosomes prepared from the dentate gyri of rats preinjected with NT-3 (* $p<0.05$, unpaired *t*-test with Welch's correction) but pretreatment with NT-3 had no additive effect. Results are means (± SEM) of 6 observations and expressed as arbitrary units.

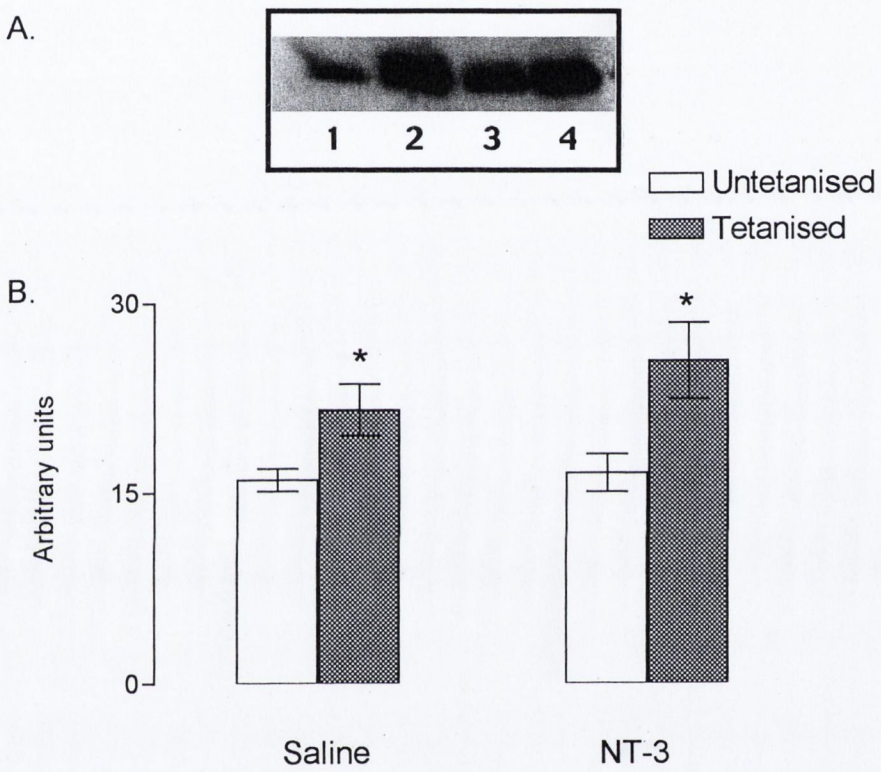
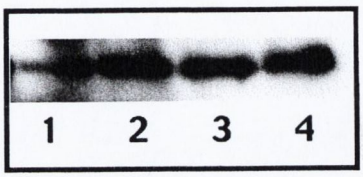

A.
1
2
3
4
Untetanised
Tetanised
B.
30
15
0
Arbitrary units
*
*
Saline
NT-3

Figure 6.4 LTP enhances ERK activity in the postsynaptic region

A. Following the induction of LTP in rats pretreated with saline, phosphorylation of ERK was greater in postsynaptically enriched preparations tetanised (lane 2) compared with untetanised (lane 1) dentate gyri. This tetanus-induced increase in ERK phosphorylation was also observed in postsynaptic preparations from tetanised (lane 4) compared with untetanised (lane 3) dentate gyri of rats pretreated with NT-3.

B. Analysis of densitometric data indicates that ERK phosphorylation was significantly increased following tetanisation in postsynaptically enriched preparations of tetanised compared with untetanised dentate gyri of rats pretreated with saline (* $p<0.05$; Student t-test for unpaired means). The LTP-associated increase was also observed in synaptosomes prepared from the dentate gyri of rats preinjected with NT-3 (* $p<0.05$, unpaired *t*-test with Welch's correction) but pretreatment with NT-3 had no additional effect. Results are expressed as arbitrary units and are means (± SEM) of 6 observations.

A.


1
2
3
4

B.

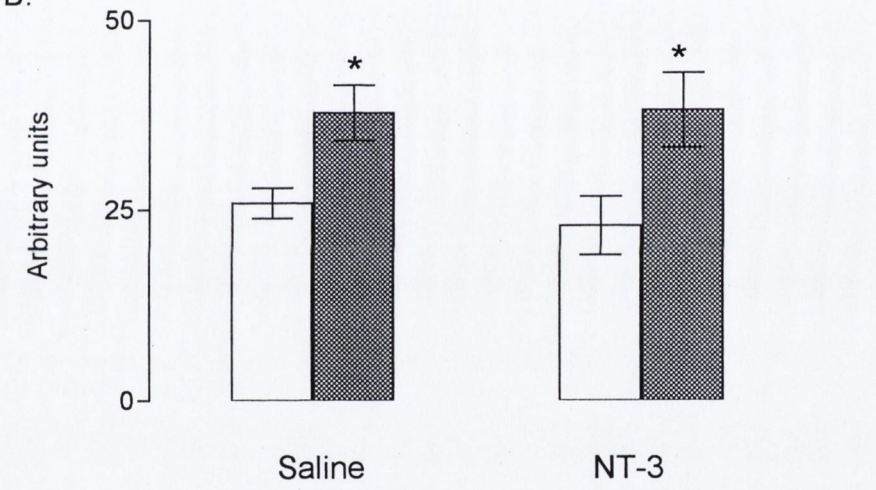
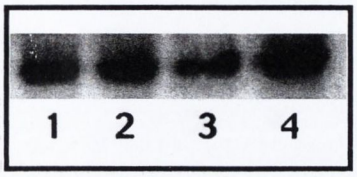

Untetanised
Tetanised
Arbitrary units
50
25
0
*
*
Saline
NT-3

Figure 6.5 LTP promotes CREB phosphorylation in the presynaptic region

A. Phosphorylation of CREB was greater in synaptosomes prepared tetanised (lane 2) compared with untetanised (lane 3) dentate gyri of saline-injected rats following tetanisation as shown in this representative immunoblot (lanes 1 and 2). Pretreatment with NT-3 did not result in increases in CREB activity following tetanus in postsynaptic preparations from tetanised dentate gyri (lanes 4) compared with postsynaptic preparations from untetanised dentate gyri (lane 3).

B. Analysis of densitometric data indicates that in saline-injected rats, mean values of CREB phosphorylation were significantly increased in synaptosomes prepared from tetanised dentate gyrus compared with untetanised dentate gyri (* $p<0.05$, unpaired *t*-test with Welch's correction). Preinjection with NT-3 30min prior to delivery of tetanus failed to have an additive effect on the LTP-associated increase in CREB phosphorylation but an LTP-induced increase in CREB phosphorylation was observed in this group (* $p<0.05$, unpaired *t*-test with Welch's correction). Results are given as arbitrary units and are expressed as means (± SEM) of 6 observations.

A.


1
2
3
4

B.

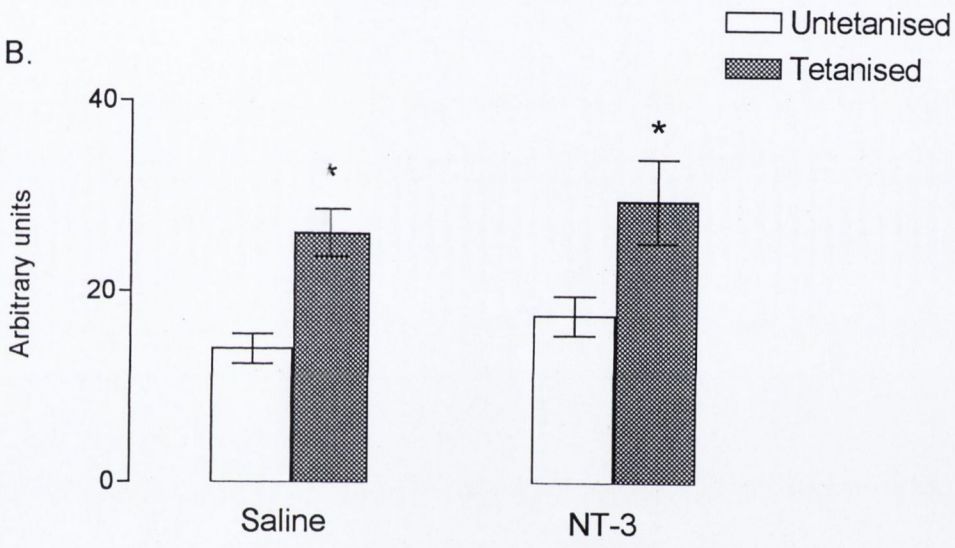
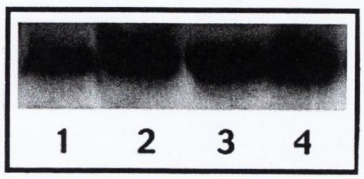

Untetanised
Tetanised
Arbitrary units
40
20
0
*
*
Saline
NT-3

Figure 6.6 LTP promotes CREB phosphorylation in the postsynaptic region

A. Phosphorylation of CREB was greater in postsynaptically enriched preparations from tetanised (lane 2) compared with untetanised (lane 1) dentate gyri of saline-injected rats following tetanisation as shown in this representative immunoblot. Intracerebroventricular injection of NT-3 did not result in enhancement of CREB phosphorylation in postsynaptic preparations from tetanised (lane 4) compared with untetanised (lane 3) of dentate gyri.

B. Analysis of densitometric data indicates that in saline-injected rats, mean values of CREB phosphorylation were significantly increased in postsynaptically enriched preparations from dentate gyrus following tetanic stimulation (* $p<0.05$, unpaired *t*-test with Welch's correction). The LTP-associated increase was also observed in synaptosomes prepared from the dentate gyri of rats preinjected with NT-3 (* $p<0.05$, unpaired *t*-test with Welch's correction) but pretreatment with NT-3 had no additional effect. Results are given as arbitrary units and are expressed as means (± SEM) of 6 observations

A.


1
2
3
4

B.

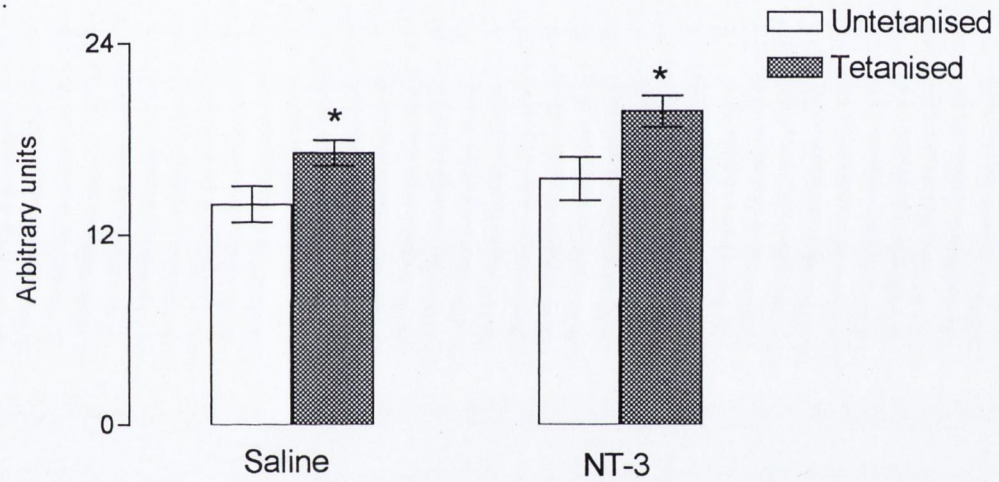
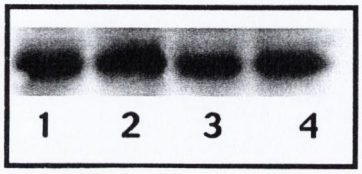

Untetanised
Tetanised
Arbitrary units
24
12
0
*
*
Saline
NT-3

Figure 6.7 LTP stimulates CREB phosphorylation in the entorhinal cortex

A. Phosphorylation of CREB was greater in homogenates prepared from ipsilateral (lane 2) compared with CREB phosphorylation in homogenates prepared from entorhinal cortices from the untetanised brain side (lane 1). Preinjection with NT-3 did not stimulate the tetanus-associated increase in CREB phosphorylation in ipsilateral entorhinal cortices (lanes 4) compared with CREB activity in homogenate prepared from the contralateral side (lane 3).

B. Analysis of densitometric data indicates that in saline-injected rats, mean values of CREB phosphorylation were significantly increased in homogenates prepared from entorhinal cortices of the tetanised side of the brain (* $p<0.05$, unpaired *t*-test with Welch's correction) compared with those prepared from entorhinal cortices from the contralateral side. Intracerebroventricular injection of NT-3 did not elicit additional phosphorylation of CREB but the LTP-associated increase was observed in this group (* $p<0.05$, unpaired *t*-test with Welch's correction). Results are given as arbitrary units and are expressed as means (± SEM) of 6 observations.

A.


1
2
3
4

B.

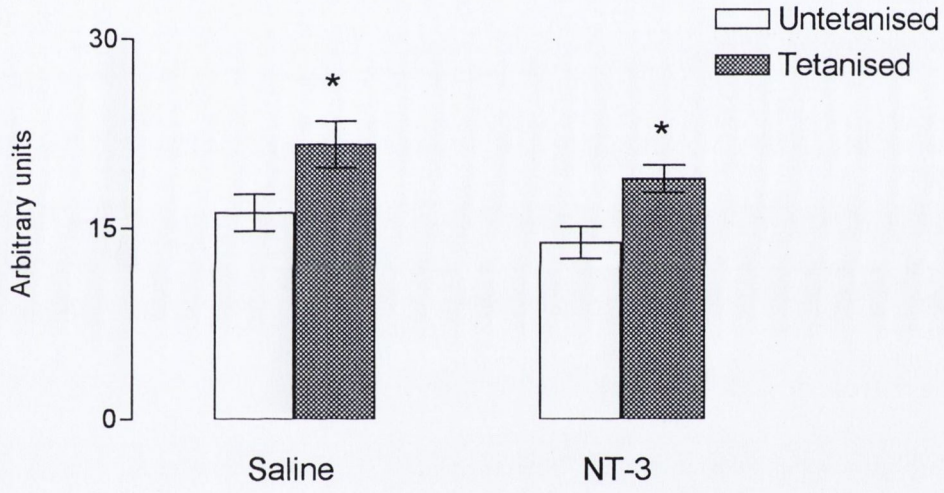

Untetanised
Tetanised
Arbitrary units
30
15
0
*
*
Saline
NT-3

Figure 6.8 LTP increases protein synthesis in the entorhinal cortex

Protein synthesis was assessed as [^{35}S]-methionine labelling of proteins prepared from slices of entorhinal cortex from tetanised and untetanised sides of the brain of saline- and NT-3-injected rats. Protein synthesis was increased following the induction of LTP (* $p<0.05$, unpaired *t*-test with Welch's correction) in saline-treated rats. Preinjection with NT-3 did not result in any additional increase in protein synthesis following tetanus but expression of LTP resulted in a significant increase in protein synthesis in this group (* $p<0.05$, unpaired *t*-test with Welch's correction). Results are expressed as cpm/mg protein and are means (± SEM) of 6 observations.

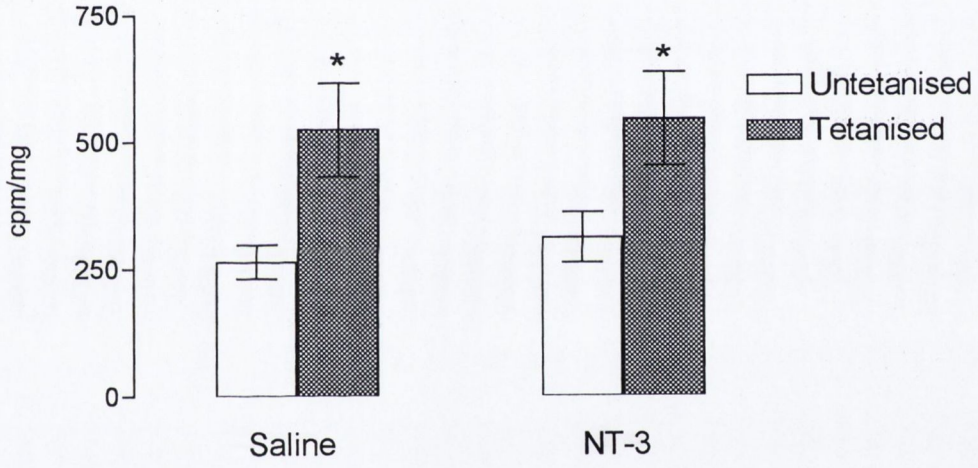
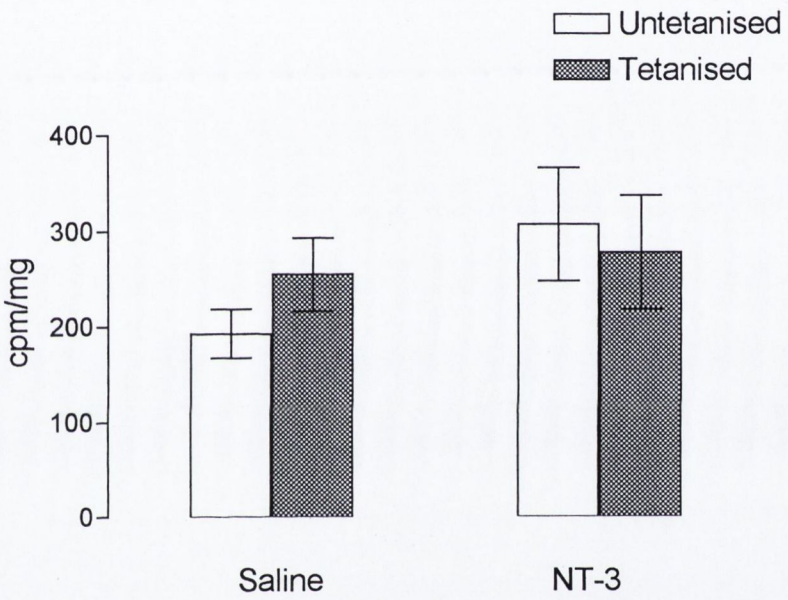

750
500
250
0
cpm/mg
*
*
Untetanised
Tetanised
Saline
NT-3

Figure 6.9 Protein synthesis in the hippocampus is not changed by LTP

Protein synthesis was assessed as [^{35}S]-methionine labelling of proteins in slices prepared from hippocampus from the ipsilateral and contralateral sides of the brain of saline- and NT-3-injected rats following tetanus. Protein synthesis was not increased following the induction of LTP in either the saline- or NT-3-injected groups ($p>0.05$, unpaired *t*-test with Welch's correction). Results are expressed as cpm/mg protein and are means (± SEM) of 6 observations.


Untetanised
Tetanised
400
300
200
100
0
cpm/mg
Saline
NT-3

6.4 Discussion

The objective of this study was to investigate a possible modulatory effect of NT-3 on LTP at the perforant path-granule cell synapses. This was achieved by administration of an intracerebroventricular injection of NT-3 prior to the delivery of tetanus to the perforant path and by the subsequent analysis of the signalling cascade triggered by NT-3.

Tetanic stimulation delivered to the perforant path resulted in successful induction of LTP in the dentate gyrus in saline- and NT-3-treated rats. In the NT-3-treated group, the magnitude of the increase in the epsp slope was not greater than that in the saline-treated group. This suggests that application of exogenous NT-3 does not enhance the expression LTP in the dentate gyrus. This is consistent with the similar findings in the CA1 reported by Long and coworkers (1999). In their study, the expression of LTP in the CA1 region of the hippocampus was not hampered in NT-3 conditional knockouts. There are, however, notable differences in NT-3 and TrkC expression between both regions. The granule cell layer of the dentate gyrus exhibits higher expression of NT-3 and its receptor, TrkC (Zhou and Rush, 1994; Tokuyama *et al.*, 1999; Merlio *et al*, 1992). Previous work in this laboratory on the effects of exogenous NGF on the expression of LTP is comparable to the results of this study (C. Maguire, unpublished observations); it was found that preinjection with NGF failed to stimulate further enhancements in LTP.

Analysis of KCl-induced NT-3 release from untetanised and tetanised tissue presented here indicate that tetanisation did not result in a greater response to depolarisation in the saline-treated group. This is in contrast to observations of LTP-associated increases in NGF and BDNF release has been reported in this laboratory. It also eliminates the possibility that LTP-induced release of either BDNF or NGF does not result in stimulation of NT-3 release. This is in contrast with results presented in Chapter 4, suggesting neurotrophin-induced NT-3 release *in vitro*, and with previous reports of neurotrophin-induced neurotrophin release (Canossa *et al.*, 1997; Krüttgen *et al.*, 1998). The current finding that tetanisation did not result in a further increase in KCl-stimulated NT-3 release may be due to saturation resulting from expression of LTP.

Phosphorylation of ERK presynaptically was investigated to assess the intracellular impact of additional NT-3 release induced by LTP. ERK activity was increased following LTP in both experimental groups but no additional increase was observed in the NT-3-treated groups. Previously, ERK has been identified as a pivotal signalling molecule in the underlying events of LTP in the CA1 region (English and Sweatt, 1996) and the dentate gyrus (McGahon and Lynch, 1998; Maguire *et al.*, 1999). While on the surface, the findings presented in this study appear to contrast with results of *in vitro* experiments presented in Chapter 4 and from other work (Marsh and Palfrey, 1996; Baldelli *et al*, 2000), indicating a stimulatory effect of NT-3 on ERK phosphorylation, the effect of NT-3 on ERK activation following the expression of LTP is likely to be occluded by an already elevated level of ERK activity in response to LTP. The effect of preinjection of NT-3 on ERK activity is similar to the effect of preinjection of NGF (C. Maguire, unpublished observations); pretreatment with neither neurotrophin caused additional enhancement of ERK activity.

Following examination of ERK activity in saline- and NT-3-treated groups, CREB activity in the presynaptic terminal was measured to investigate any increases in phosphorylation mediated by ERK. Phosphorylation of CREB on Ser133 was enhanced by the expression of LTP in both groups but pretreatment with NT-3 did not result in greater activation of CREB. This is not surprising, as there was no increase in ERK activity, which may have had a knock-on effect on CREB activity.

Data shown here indicate an LTP-associated enhancement of ERK activity in the postsynaptic area in both experimental groups. Such an increase may be the downstream consequence of NMDA receptor activation (Rosen *et al.*, 1994; Kurino *et al.*, 1995) or postsynaptically located Trk phosphorylation (Wood *et al.*, 1992; Thomas *et al.*, 1992). Pretreatment with NT-3 did not stimulate additional increases in ERK phosphorylation following expression of LTP. Comparable results are presented for CREB phosphorylation in postsynaptic preparations. Expression of LTP at the perforant path-granule cell synapses resulted in enhanced phosphorylation of CREB in postsynaptic preparations from the saline-treated group but not in preparations from the tyrphostin AG879-treated group. These data indicate that application of exogenous NT-3 does not modulate postsynaptic mechanisms of LTP expression in the dentate gyrus. It may be

argued that such intracellular mechanisms are saturated and further increases in such substrates would not be of any functional consequence.

Investigation of changes in the entorhinal cortex resulting from expression of LTP in the dentate gyrus indicates alterations that may contribute to the robust nature of LTP. Analysis of phosphorylation of CREB in homogenates prepared from ipsilateral entorhinal cortex, compared with the contralateral entorhinal cortex, demonstrates increased phosphorylation of CREB on Ser133 following expression of LTP in both groups. This event may provide a means of transducing transient phosphorylation events into long-lasting changes necessary for synaptic remodelling by stimulating protein synthesis (Murphy and Segal, 1999). LTP-associated increases in phosphorylation of CREB were not stimulated further by preinjection with NT-3.

Likewise, protein synthesis in the entorhinal cortex was stimulated in both experimental groups, possibly as a direct consequence of CREB activation. Augmented synthesis of new proteins may accommodate modifications necessary to maintain enhancements of synaptic efficacy required for the late phase of LTP (Mullany and Lynch, 1997; Buchs and Miller, 1996; Toni *et al.*, 1999). Treatment with NT-3 prior to the delivery of tetanus failed to induce further increases in protein synthesis. This may be due to saturation of LTP or may reflect the low expression of TrkC receptors in the entorhinal cortex (Merlio *et al.*, 1992). In contrast, tetanisation failed to increase protein synthesis in the hippocampus in either experimental group. This indicates that the effects of tetanus may be localised to the dentate gyrus and entorhinal cortex.

The data presented indicate that the administration of exogenous NT-3 does not enhance the expression of LTP in the dentate gyrus nor does it modulate specific markers of Trk-mediated signalling such as ERK or CREB in the pre- or postsynaptic regions at the perforant path-granule cell synapses following tetanisation. Furthermore, injection of NT-3 did not modify LTP-associated increases in CREB phosphorylation and protein synthesis in the entorhinal cortex.

VII General discussion

The objective of this study was to examine the role that neurotrophins, in particular NT-3, might play in the dentate gyrus and to elucidate the mechanisms underlying any such modulation, with specific interest in identifying a role for tyrosine kinase. Several observations led to the establishment of this project. Firstly, tyrosine kinase inhibitors have been reported to block the expression of LTP in the CA1 region (Huang and Hsu, 1999; O'Dell *et al.*, 1991a) and in the dentate gyrus (McGahon and Lynch, 1997; Abe and Saito, 1993). Furthermore, several tyrosine kinase substrates have been implicated in the signalling mechanisms underlying the expression of LTP (English and Sweatt, 1996; Mullany and Lynch, 1997; Lauri *et al.*, 1999). Secondly, increased glutamate release is central to the expression of LTP in the perforant path-granule cell synapses (McGahon and Lynch, 1998; Dolphin *et al.*, 1982) and high levels of tyrosine kinase substrates are detected in synaptic vesicles (Pang *et al.*, 1988). Thirdly, evidence has accumulated from many studies indicating that neurotrophins are involved in short-term modulation of synaptic transmission (Knipper *et al.*, 1994, Kelly *et al.*, 1998), in addition to reports that neurotrophins impact on LTP in the hippocampus (Korte *et al.*, 1995; Patterson *et al.*, 1996; Kang *et al.*, 1997; Maguire *et al.*, 1999). The working hypothesis therefore was that tyrosine kinase activity stimulated by neurotrophins may impact on glutamate release and thus, may alter the expression of LTP in the perforant path-granule cell synapses. In addition, neurotrophins have been reported to induce structural alterations in neurons such as synaptogenesis (Murphy and Segal, 1999; Martinez *et al.*, 1998; Vicario-Abejon *et al.*, 1998) and neurite outgrowth (Lambelle and Leclerc, 2000; Corbit *et al.*, 1999) so the possibility that neurotrophin-mediated signalling may result in the stabilisation of LTP, through stimulation of protein synthesis, was also examined.

The results presented here indicate a strong case for the involvement of tyrosine kinase activity on both sides of the synapse in the expression of LTP at the perforant path-granule cell synapses. In addition, the present data suggest a role for tyrosine kinase in events immediately following tetanisation such as phosphorylation of calcium channel

subunits, which modulates channel activity, as well as long-lasting effects mediated by changes in protein synthesis.

The following findings support these views:

- The tyrosine kinase inhibitor, genistein, and the Trk inhibitor, tyrphostin AG879, blocked the expression of LTP in the dentate gyrus and inhibited tyrosine phosphorylation of substrates such as ERK and CREB, which are known to play a role in LTP.

- NT-3 release was regulated in an activity-dependent and was modulated by the activity of Trk.

- LTP was associated with changes in protein phosphorylation in both pre- and post-synaptic regions.

The results presented demonstrate that the LTP-associated process of glutamate release is inhibited by tyrosine kinase inhibitor, genistein. The expression of LTP in the dentate gyrus is blocked by preinjection with genistein and further analysis reveals that genistein also blocked the LTP-induced increases in tyrosine phosphorylation of several proteins. It may be therefore suggested that the blockade of tyrosine kinase activity resulted in the failure to sustain LTP. In addition, genistein suppressed the LTP-associated increase in ERK activity in synaptosomes prepared from the dentate gyrus, while tyrosine phosphorylation of the α_1 subunit of voltage operated calcium channels was enhanced by the expression of LTP, an effect that was also occluded by treatment with genistein. The functional consequences of channel subunit phosphorylation have yet to be determined but it is likely that it may modulate channel kinetics, as the α_1 subunit is the pore-forming subunit. In this manner, tyrosine kinase may permit greater calcium entry into the presynaptic terminal, directly facilitating glutamate release. In addition, it has been suggested that the α_1 subunit interacts with syntaxin and SNAP-25 (Rettig *et al.*, 1997), suggesting direct interplay between voltage-operated calcium channels and

constituent proteins of the synaptic vesicle membrane, which may influence glutamate release.

ERK activity in the presynaptic terminal was enhanced by LTP but this effect was abolished by genistein, presumably because ERK activity relies in part on tyrosine phosphorylation for activation (Payne *et al.*, 1991; Her *et al.*, 1993). The LTP-induced increase in ERK activity proposed by present data may also impact on glutamate release by mediating the phosphorylation of synapsin I (Jonanovic *et al.*, 1996) and thereby promoting migration of the synaptic vesicle to the active site, in preparation for release. Alternatively, ERK may induce long-term changes by phosphorylation of the transcription factor CREB, mediated by RSK (Xing *et al.*, 1996) and thereby transduce transient synaptic activity into enduring changes in gene expression, necessary for the maintenance phase of LTP (Jeffrey *et al.*, 1990; Richardson *et al.*, 1992)

In this study, the effects of NT-3 *in vitro* were analysed to investigate its possible involvement in signalling cascades, which might contribute to synaptic plasticity in the dentate gyrus. Data presented show that NT-3 release in synaptosomes prepared from the dentate gyrus is stimulated by depolarisation and suggests that NT-3 release may be regulated by neurotrophins. This is in agreement with previous reports (Canossa *et al.*, 1997; Kruttgen *et al.*, 1998), because in the presence of tyrphostin AG879, KCl-stimulated NT-3 release was abolished. Analysis of the effects of neurotrophins on protein synthesis indicated that both NGF and NT-3 stimulated protein synthesis in the hippocampus and that neither resulted in enhanced protein synthesis in the entorhinal cortex. The regional differences in the effects on NGF and NT-3 may correspond with the distribution of NGF and NT-3 and their receptors, TrkA and TrkC (Zhou and Rush, 1994; Tokuyama *et al.*, 1999; Merlio *et al*, 1992; Maisonpierre *et al.*, 1990; Ernfors *et al.*, 1990).

Analysis of the effects of BDNF and NT-3 on glutamate release indicates that incubation in the presence of neurotrophins alone is not sufficient to enhance glutamate release from synaptosomes prepared from dentate gyrus. This contradicts previous findings, which demonstrated that the application of neurotrophins stimulates glutamatergic transmission in the hippocampus (Kang and Schuman, 1996; Knipper *et al.*, 1994). Differences in the results presented in this study may be accounted for by

regional differences between the CA1 region and the dentate gyrus and also by methodological differences. Further examination of the role of NT-3 in glutamate release from synaptosomes prepared from the dentate gyrus indicates that NT-3, alone or in combination with ACPD, does not increase depolarisation-triggered glutamate release. This scheme is in contrast to that presented for NGF (Kelly and Lynch, 1998), in which it is proposed that PLCγ acts as a coincidence detector of simultaneous activation of mGluR and Trk.

Evidence is presented which supports the notion that Trk plays a pivotal role in the expression of LTP at the perforant path-granule cell synapses and that this involvement is evident in the pre- and postsynaptic regions. The finding that preinjection of tyrphostin AG879 prior to the delivery of tetanus completely blocks LTP in the dentate gyrus is supported by previous work in this laboratory (Maguire *et al.*, 1999). Under these circumstances, the failure of tetanus to induce LTP at these synapses is correlated with the absence of LTP-induced tyrosine phosphorylation. Similar findings show that activation of tyrosine kinase is involved in LTP (Lauri *et al.*, 1999) and mounting evidence that neurotrophins participate in biochemical events underlying the expression of LTP adds weight to this argument (Kelly *et al.*, 1998; Maguire *et al.*, 1999; Korte *et al.*, 1995; Patterson *et al.*, Kang *et al.*, 1997). Identification of pre- and post-synaptic substrates of downstream cascades triggered by LTP, highlight possible overlapping signal transduction by LTP and neurotrophins. ERK and CREB are identified as targets of LTP, and LTP-linked activity of both ERK and CREB is occluded by inhibition of Trk activity. Previous work has pinpointed the activity of ERK and CREB as important to signalling mechanisms underpinning LTP (English and Sweatt, 1996; Davis *et al.*, 2000). Both protein messengers may be critical for the translation of transient synaptic events into long-term alterations in synaptic efficacy by stimulation of gene expression (Vanhoutte *et al.*, 1999; Ginty *et al.*, 1994, Xia *et al.*, 1996). This may be important in the process of synaptic remodelling that governs the persistence of LTP (Toni *et al.*, 1999; Buchs and Miller, 1996).

The stimulus for increased ERK and CREB phosphorylation may be enhanced levels of neurotrophins released in response to tetanic stimulation at the perforant path-granule cell synapses. Previously, it has been demonstrated that neurotrophins may

control neurotrophin release through the action of Trk receptors (Canossa *et al.*, 1997; Kruttgen *et al.*, 1998), and together with the present observations that NGF and BDNF fail to increase depolarisation-induced NT-3 release, it seems likely that NT-3 may act to increase its own release. Augmented neurotrophins levels may then stimulate Trk (Lambelle *et al.*, 1991; Condon-Cardo *et al.*, 1991) activation and trigger the ras/Raf/ERK cascade, culminating in the activation of CREB and ultimately in transcription of *c-fos* (Ginty *et al.*, 1994).

In addition to changes in the dentate gyrus, this study demonstrates changes in the entorhinal cortex following delivery of tetanus to the perforant path. Genistein blocked LTP-associated changes in CREB activation and protein synthesis in the entorhinal cortex. Similarly, injection of tyrphostin AG879 inhibited LTP-induced enhancements in CREB phosphorylation and protein synthesis. This indicates that changes in protein synthesis to support the expression of LTP are mediated by the activity of Trk and that the primary stimulus may be the LTP-induced increases in neurotrophin release. It is considered that retrograde transport of Trk receptors (Bhattacharyya *et al.*, 1997) to the cell bodies of the perforant path is responsible for stimulation of CREB and protein synthesis in the entorhinal cortex. It has been shown that neurotrophins induce both CREB activation (Ginty *et al.*, 1994) and changes in neuronal morphology that necessitate protein synthesis (Baldelli *et al.*, 2000; Lambelle and Leclerc, 2000) under certain experimental conditions so it is likely that neurotrophins act in a similar manner during the expression of LTP.

A multiplicity of changes accompany LTP at perforant path-granule cell synapses and evidence presented here indicates that signalling cascades initiated by neurotrophins may count in part for changes observed in the pre- and post-synaptic regions. The data presented here may serve as a template for future work to further characterise the involvement of NT-3 in the mechanisms supporting the expression of LTP in the dentate gyrus.

Further work

- The paradigm to investigate the impact of NT-3 on the expression of LTP in the dentate gyrus does not address the role of endogenous NT-3 in the expression of LTP. It would therefore, be of interest to investigate LTP in NT-3 knockout mice or in the presence of antibodies to chelate endogenous NT-3.

- At peripheral synapses, NT-3 has been shown to modulate transmitter release by mobilising calcium from intracellular store. It would be of interest to investigate the possibility that NT-3 may act through PLCγ to stimulate cytosolic calcium levels in hippocampal neurons and to determine whether elevations in calcium may lead to increased glutamate release at hippocampal synapses.

- Mechanisms underlying neurotrophin release are not well understood and it would be beneficial to investigate the detailed mechanisms modulating NT-3 release and whether it stimulates its own release. In addition, analysis of the corelease of NT-3 and BDNF may further the understanding of interplay between neurotrophins in the expression of LTP.

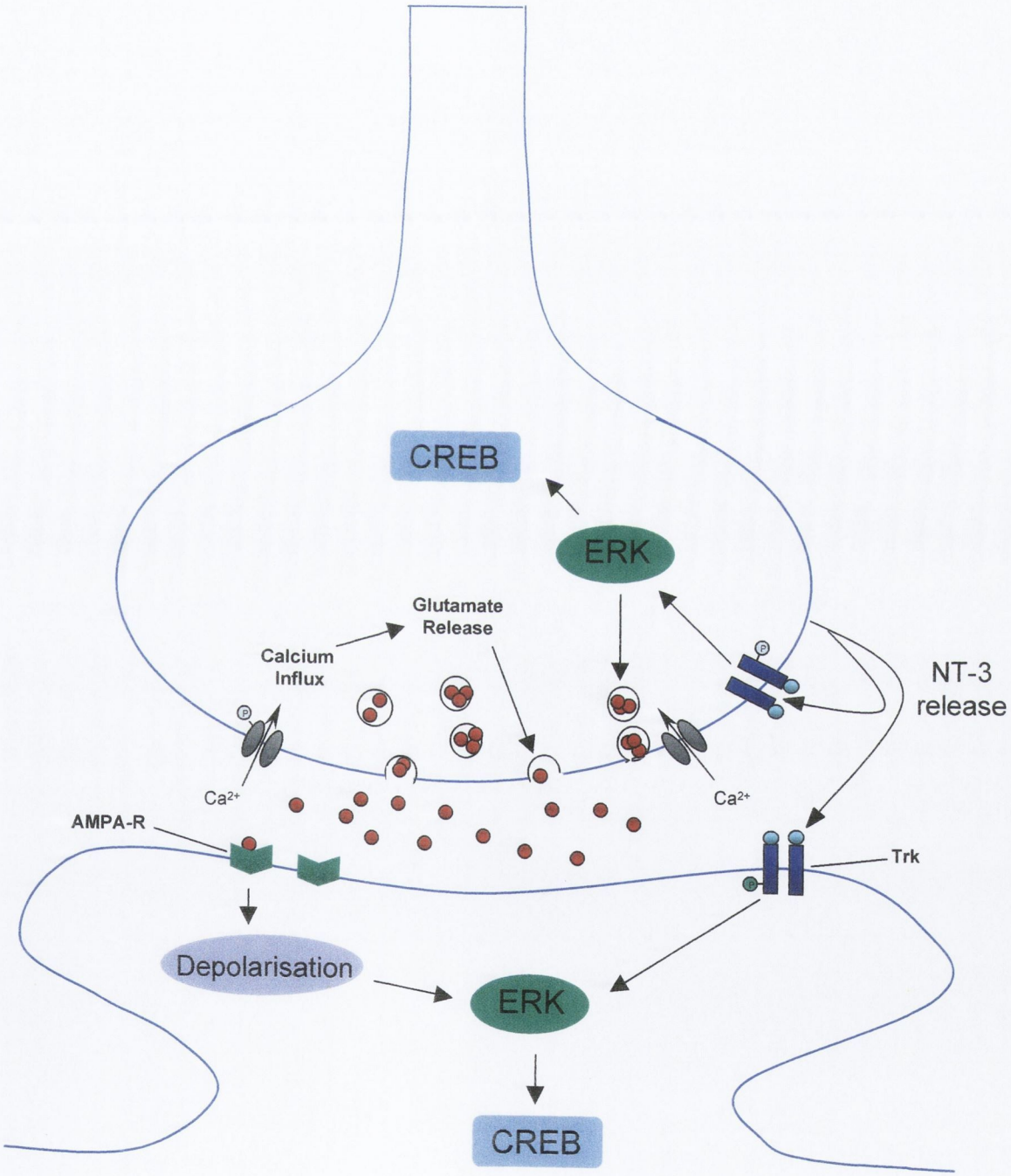
- Previous research has identified links between activity and neurotrophin expression. It would therefore be of interest to examine the effects of LTP on expression of NT-3 and its receptor, TrkC. Furthermore, as it has been shown that LTP stimulates NT-3 release in this study, it would be of considerable interest to examine the effects of LTP on the phosphorylation of TrkC in the dentate gyrus.

- Because deficits in neurotrophins and their downstream messengers have been linked to failure to execute hippocampal learning tasks and because of the putative involvement of neurotrophins in LTP, it would be of interest to correlate Trk activation in the expression of LTP and in hippocampus-dependent learning tasks.

- Increased protein synthesis has been demonstrated by several groups as a consequence of LTP-inducing tetanus. The products of protein synthesis in most cases have not been identified. Examination of targets of protein synthesis in the expression of LTP may provide a greater understanding of processes that reinforce initial changes in synaptic transmission.

Figure VI.I Intracellular signalling molecules involved in the modulation of LTP at the perforant path-granule cell synapses by neurotrophins

Increased glutamate release is a central feature of the expression of LTP at the perforant path-granule cell synapses. Phosphorylation of voltage-operated calcium channels (VOCC) α_1 subunit by tyrosine kinase may modulate glutamate release by altering calcium influx into the presynaptic terminal. Activation of ERK may facilitate exocytosis of glutamate-containing vesicles by phosphorylation of synapsin-1 and thus freeing the vesicles from the actin meshwork. Alternatively, ERK may also impact on gene expression by phosphorylating CREB. Increased ERK phosphorylation resulting from the expression of LTP, may be triggered by interaction of Trk with a neurotrophin at the presynaptic or postsynaptic terminal. In addition, AMPA receptors may contribute to ERK activation by triggering depolarisation of the postsynaptic membrane.


CREB
ERK
Glutamate
Release
Calcium
Influx
NT-3
release
Ca2+
Ca2+
AMPA-R
Trk
Depolarisation
ERK
CREB

VIII Bibliography

Abe, K. and Saito, H. (1993) Tyrosine kinase inhibitors, herbimycin and lavendustin A, block formation of long-term potentiation in the dentate gyrus in vivo. *Brain Research,* **621**, 167-170.

Abe, K., Ishiyama, J. and Saito, H. (1992) Effects of epidermal growth factor and basic fibroblast growth factor on generation of long-term potentiation in the dentate gyrus of fimbria-fornix-lesioned rats. *Brain Research*, **593**, 335-338.

Abe, K., Xie, F. J. and Saito, H. (1991) Epidermal growth factor enhances short-term potentiation and facilitates induction of long-term potentiation in rat hippocampal slices. *Brain Research,* **547**, 171-174.

Adams, P., Anderson, A., Varga, A., Dinley, K., Cook, R., Pfaffinger, P. J. and Sweatt, J.D. (2000) The A-type potassium channel Kv4.2 is a substrate for the nitrogen-activated protein kinase ERK. *Journal of Neurochemistry,* **75**, 2277-2287.

Adler, E.M., Augustine, G.J., Duffy, S.N. and Charlton, M.P. (1991) Alien intracellular calcium chelators attenuate neurotransmitter release at the squid giant synapse. *Journal of Neuroscience*, **11**, 1496-1507.

Ahn, S., Olive, M., Aggarwal, S., Krylov, D., Ginty, D and Vinson. C. (1998) A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of *c-fos. Molecular and Cellular Biology,* **18**, 967-977.

Akers, R.F., Lovinger, D.M., Colley, P.A., Linden, D.J. and Routtenberg, A. (1986) Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. *Science,* **231**, 587-589.

Akiyama, T., Ischida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. and Fukani, Y. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *Journal of Biological Chemistry,* **262**, 5592-5595.

Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C.J. and Crowley, S. (1994) Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. *EMBO Journal,* **13**, 1610-1619.

Alford, S., Frenguelli, B.G., Schofield, J.G. and Collingridge, G.L. (1992) Characterization of Ca^{2+} signals induced in hippocampal CA1 neurons by symapathetic nervous system. *Journal of Physiology,* **469**, 693-716.

Andersen, P., Sundberg, S.H., Sveen, O. and Wigstorm, H. (1977) Specific long-lasting potentiation of synaptic transmission in hippocampal slices. *Nature*, **266**, 736-737.

Andersen, P.O. (1987) Properties of hippocampal synapses of importance for integration and memory. In *New insights into synaptic function,* eds. Edelman, G. M., Gall, W.E. and Cowen., P. New York: Neuroscience Foundation/Wiley.

Anderson, K.D., Alderson, R.F., Altar, C.A., DiStefano, P.S., Corcoran, T.L., Lindsay, R.M., and Wiegland, S.J. (1995) Differential distribution of exogenous BDNF, NGF and NT-3 in the brain corresponds to the relative abundance and distribution of high-affinity and low-affinity neurotrophin receptors. *Journal of Comparative Neurology,* **357**, 296-317.

Androutsellis-Theotokis, A., McCormack, W.J., Bradford, H.F., Stern, G.M. and Pliego-Rivero, F.B. (1996) The depolarisation-induced release of [^{125}I]BDNF from brain tissue. *Brain Research,* **743**, 40-48.

Angenstein, F., Riedel, G., Reymann, K. G. and Staak, S. (1994) Hippocampal long-term potentiation in vivo induces translocation of protein kinase C gamma. *Neuroreport,* **5**, 381-384.

Aniksztejn. L. and Ben-Ari, Y. (1991) Novel form of long-term potentiation produced by K^+ channel blocker in the hippocampus. *Nature,* **349**, 67-69.

Annaert, W. and De Stooper, B. (1999) Presenilins: molecular switches between proteolysis and signal transduction. *Trends in Neuroscicnce,* **22**, 439-443.

Applegate, M.D., Kerr, D.D. and Landfield, P.W. (1987) Redistribution of synaptic vesicles during long-term potentiation in the hippocampus. *Brain Research,* **401**, 401-406.

Arnoult, C., Lemos, J.R. and Florman, H.M. (1997) Voltage-dependent modulation of t-type calcium channels by protein tyrosine phosphorylation. *EMBO Journal,* **16**, 1593-1599.

Ashcroft, M., Stephens, R. M., Hallberg, B., Downward, J. and Kaplan, D. R. (1999) The selective and inducible activation of endogenous PI 3-kinase PC12 cells results in efficient NGF-mediated survival but defective neurite outgrowth. *Oncogene*, **18**, 4586-4597.

Asztely, F., Kokaia, M., Olofsdotter, K., Örtegren, U. and Lindvall, O. (2000) Afferent-specific modulation of short-term synaptic plasticity by neurotrophins in dentate gyrus. *European Journal of Neuroscience,* **12**, 662-669.

Augustine, G.J., Charlton, M.P. and Smith, S.J. (1987) Calcium action in synaptic transmitter release. *Annual Review of Neuroscience*, **10**, 633-693.

Bading, H. and Greenberg, M. E. (1991) Stimulation of protein tyrosine phosphorylation by NMDA receptor activation. *Science,* **253**, 912-914.

Bailey, C. H. and Kandel, E.R. (1993) Structural changes accompanying memory storage. *Annual Review of Physiology,* **55**, 397-426.

Baldelli, P., Forni, P.E. and Carbone, E. (2000) BDNF, NT-3 and NGF induce distinct new Ca^{2+} channel synthesis in developing hippocampal neurons. *European Journal of Neuroscience,* **12**, 4017-4032.

Balkowiec, A. and Katz, D.M. (2000) Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA *in situ. Journal of Neuroscience,* **20**, 7417-7423.

Barbacid, M (1994) The trk family of neurotrophin receptors. *Journal of Neurobiology,* **25**, 1386-1403.

Barbacid, M., Lambelle, F., Pulido, D. and Klein, R. (1991) The trk family of tyrosine protein kinase receptors. *Biochim. Biophys. Acta.,* **1072**, 115-127.

Barde, Y-A., Edgar, D., and Thoenen, H. (1982) Purification of a new neurotrophic factor from mammalian brain. *EMBO Journal,* **1**, 549-553.

Barker, P.A. (1998) p75(NTR): a study in contrasts. *Cell Death Differ,* **5**, 346-356.

Barlett, S.E., Reynolds, A.J., Weible, M., Heydon, K. and Hendry, I.A. (1999) In symapthetic but not sensory neurons, phosphoinositide-3 kinase is important for NGF-dependent survival and the retrograde transport of ^{125}I-NGF. *Brain Research,* **761**, 257-262.

Barnekow, A., Jahn, R. and Schartl, M. (1990) Synaptophysin: A substrate for the protein tyrosine kinase pp60c-src in intact synaptic vesicles. *Oncogene,* **5**, 1019-1024.

Baron, C., Benes, C., Van Tan, H., Fagard, R. and Roisin, M.P. (1996) Potassium chloride enhances mitogen-activated protein kinase activity in rat hippocampal slices. *Journal of Neurochemistry,* **66**, 1005-1010.

Baudry, M., Oliver, M., Creager, R., Wieraszko, A. and Lynch, G. (1980) Increase in glutamate receptor following repetitive electrical stimulation in hippocampal slices. *Life Sciences,* **27**, 325-330.

Baumert, M., Takei, K., Hartinger, J., Burger, P.M., Fischer Von Mollard, G., Maycox, Pr., De Camilli, P. and Jahn, R. (1990) P29: A novel tyrosine-phosphorylated membrane protein present in small clear vesicles of neurons and endocrine cells. *Journal of Cellular Biology*, **110**, 1285-1294.

Bekkers, JM. and Stevens, CF. (1990) Presynaptic synaptic mechanism for long-term potentiation in the hippocampus. *Nature,* **346**,724-729.

Benedetti, M., Levi, A. and Chao, M.V. (1993) Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. *Proceedings of the National Academy of Sciences,* **90**, 7859-7863.

Bennett, M.K., Erondu, N.E. and Kennedy, M.B. (1983) Purification and characterisation of a calmodulin-dependent kinase that is highly concentrated in the brain. *Journal of Biological Chemistry,* **258**, 12735-12744.

Berardi, N., Lodovichi, C., Caleo, M., Pizzorusso, T. and Maffei, L. (1999) Role of neurotrophins in neural plasticity: what we learn from the visual cortex. *Restorative Neurology and Neuroscience,* **15**, 125-136.

Berkemeier, L.R., Winslow, J.W., Kaplan, D.R., Nikolics, K., Goeddel, D.V., and Rosenthal, A. (1991) Neurotrophin-5: A novel neurotrophic factor that activates trk and trkB. *Neuron,* 7, 857-866.

Berninger, B., Garcia, D. E., Inagaki, N., Hahnel, C. and Lindholm, D. (1993) BDNF and NT-3 induce intracellular Ca^{2+} elevation in hippocampal neurons. *Neuroreport,* **4**, 1303-1306.

Berninger, B., Garcia, DE., Inagaki, N., Hahnel, C., Lindholm, D. (1993) BDNF and NT-3 induce intracellular Ca^{2+} elevation in hippocampal neurons. *Neuroreport,* **4**, 1303-1306.

Berridge, M. (1993) Inositol triphosphate and calcium signaling. *Nature,* **361**, 315-325.

Bhattacharyya, A., Watson, F.L., Bradlee, T.A., Pomeroy, S.L., Stiles, C.D. and Segal, R.A. (1997) Trk receptors function as a rapid retrograde signal carriers in the adult nervous system. *Journal of Neuroscience*, **17**, 7007-7016.

Bibel, M., Hoppe, E. and Barde, Y-A. (1999) Biochemical and functional interactions between the neurotrophin receptors trk and p75(NTR). *EMBO Journal,* **18**, 616-622.

Biffo, S., Offenhauser, N., Carter, B.D. and Barde, Y.A. (1995) Selective binding and internalisation by truncated receptors limits the availability of BDNF during development. *Development,* **121**, 2461-2470.

Bito, H., Deisseroth, K. and Tsien, R. W. (1996) CREB phosphorylation and dephosphorylation: A Ca^{2+}- and stimulus duration-dependent switch for hippocampal gene expression. *Cell,* **87**, 1203-1214.

Blenis, J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proceeding of the National Academy of Sciences,* **90**, 5889-5892.

Bliss, T. V. P. and Collingridge, G. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, **361**, 31-39 .

Bliss, T. V. P. and Lømo, T. (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetised rabbit, following stimulation of the perforant path. *Journal of Physiology,* **232**, 331-356.

Bliss, T.V.P., Douglas, R.M., Errington, M.L. and Lynch, M.A. (1986) Correlation between long-term potentiation and release of endogenous amino acids from dentate gyrus of anaesthetized rats. *Journal of Physiology*, **337**, 391-408.

Blitzer, R.D., Wong, T., Nouranifar, R., Iyengar, R. and Landau, E.M. (1995) Postsynaptic cAMP pathway gates early LTP in hippocampal CA1 region. *Neuron,* **15**, 1403-1414.

Blöchl, A. and Thoenen, H. (1995) Characterisation of Nerve Growth Factor (NGF) release from hippocampal neurons: evidence for a constitutive and unconventional sodium-dependent regulated pathway. *European Journal of Neuroscience,* **7**, 1220-1228.

Blum, S., Moore, A. N., Adams, F. and Dash, P. K. (1999) A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. *Journal of Neuroscience*, **19**, 3535-3544.

Boixal, C., Tessier, S., Pansard, Y., Lang-Lazdunski, L., Mercadier, J-J., Hatem, S.N. (2000) Tyrosine kinase and protein kinase C regulate L-type Ca^{2+} current co-operatively in human atrial myocytes. *American Journal of Physiology Heart and Circulation Physiology,* **278**, 670-676.

Bonni, A., Ginty D. D., Dudeck, H. and Greenberg, M. E. (1995) Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. *Molecular and Cellular Neuroscience*, **6**, 168-183.

Bortolotto, Z.A. and Collingridge, G.L. (1994) Characterization of LTP induced by the activation of glutamate receptors in area CA1 of the hippocampus. *Neuropharmacology,* **32**, 1-9.

Bortolotto, Z.A., Bashir, Z.I., Davies, C.H. and Collingridge, G.L. (1993) Metabotropic glutamate receptors activate a molecular switch which regulates the induction of long-term potentiation. *Nature*, **368**, 740-743.

Bourtchuladze, R., Frenfuelli, B., Blendy, J., Coiffi, D., Schutz, G. and Silva, A.J. (1994) Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell,* **79**, 59-68.

Bradford, M.M., (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. *Analytical Biochemistry,* **72**, 248.

Brambilla, R., Gnesutta, N., Minichiello, L., White, G., Roylance, A.J., Herron, C., Ramsey, M., Wolfer, D.P., Cestari, V., Rossi-Arnaud, C., Grant, S.G.N., Chapman, P.F., Lipp, H-P., Sturani, E and Klein, R. (1997) A role for the Ras signalling pathway in synaptic transmission and long-term memory. *Nature*, **390**, 281-286.

Bramham, C.R., Southard, T., Sarvey, J.M., Herkenham, M. and Brady, L.S. (1996) Unilateral LTP triggers bilateral increases in hippocampal neurotrophin and trk receptor mRNA expression in behaving rats: evidence for interhemispheric communication. *Journal of Comparative Neurology,* **368**, 371-382.

Bruno, V., Copani, A., Knopfel, T., Kuhn, R., Casabona, G., Dell'Albani, P., Condorelli, D.F. and Nicoletti, F. (1995) Activation of metabotropic glutamate receptors coupled to inositol phospholipid hydrolysis amplifies NMDA-induced neuronal degeneration in cultured cortical cells. *Neuropharmacology,* **34**, 1089-1098.

Buchs, P-A. and Muller, D. (1996) Induction of long-term potentiation is associated with major ultrastructural changes of activated synapses. *Proceedings of the National Academy of Sciences,* **93**, 8040-8045.

Canevari, L., Richter-Levin, G. and Bliss, T.V.P. (1994) LTP in the dentate gyrus is associated with a persistent NMDA receptor-dependent enhancement of synaptosomal glutamate. *Brain Research,* **667**, 115-117.

Canossa, M., Gärtner, A., Campana, G., Inagaki, N. and Thoenen, H. (2001) Regulated secretion of neurotrophins by metabotropic glutamate group I (mGluRI) and Trk receptor activation is mediated by phospholipase C signalling pathways. *EMBO Journal,* **20**, 1640-1650.

Canossa, M., Griesbeck, O., Berninger, B., Campana, G., Kolbeck, R. and Thoenen, H. (1997) Neurotrophin release by neurotrophins: Implications for activity dependent neuronal plasticity. *Proceedings of the National Academy of Sciences,* **94**, 13279-13286.

Canossa, M., Rovelli, G. and Shooter, E. M. (1996) Transphosphorylation of the Neurotrophin Trk Receptors. *Journal of Biological. Chemistry,* **271**, 5812-5818.

Carroll, R.C., Beattie, E., Xia, H., Luscher, C., Altschuler, J., Nicoll, R.A., Malenka, R.C. and von Zastrow, M. (1999) Dynamin dependent endocytosis of AMPA receptors. *Proceedings of the National Academy of Sciences,* **96**, 14112-14117.

Carter, B.D., Kaltschmidt, C., Kaltschmidt, B., Offenhauser, N., Bohm-Matthaei, R., Baeuerle, P.A. and Barde, Y-A. (1996) Selective activation of NF-κB by nerve growth factor through the neurotrophin receptor p75. *Science,* **272**, 542-545.

Castrén, E., Pitkänen, M., Sirviö, J., Parsadanian, A., Lindhom, D., Thoenen, H. and Riekkinen, P.J. (1993) The induction of LTP increases BDNF and NGF mRNA but decreases NT-3 mRNA in the dentate gyrus. *Neuroreport,* **4**, 895-898.

Castrén, E., Zafra, F., Thoenen, H. and Lindholm, D. (1992) Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. *Proceeding of the National Academy of Sciences,* **89**, 9444-9448.

Catterall, W.A. (1991) Functional subunit structure of voltage gated calcium channels. *Science,* **253**, 1499-1500.

Cavanaugh, J. E., Ham, J., Hetman, M., Poser, S., Yan, C. and Xia, Z. (2001) Differential regulation of mitogen-activated protein kinases ERK1/2 and ERK5 by neurotrophins, neuronal activity and cAMP in neurons. *Journal of Neuroscience* , **21**, 434-443.

Cavus, I. and Teyler, T. (1996) Two forms of long-term potentiation in Area CA1 activate different signal transduction cascades. *Journal of Neurophysiology,* **76**, 3038-3047.

Chapman, E.R., Hanson, P.I., An, S. and Jahn, R. (1995) SNAP-25, a t-SNARE, which binds to both syntaxin and VAMP via domains that may form coiled coils, *Journal of Biological Chemistry,* **269**, 27427-27432.

Chen, G., Kolbeck, R., Barde, Y-. A., Bonhoeffer, T. and Kossel, A. (1999a) Relative contribution of endogenous neurotrophins in hippocampal long-term potentiation. *Journal of Neuroscience* , **19**, 7983-7990.

Chen, Y.A., Scales, S.J., Patel, S.M., Doung, Y.C. and Scheller, R.H. (1999b) SNARE complex formation is triggered by Ca^{2+} and drives membrane fusion. *Cell,* **97**, 165-174.

Chen, H-J., Rojas-Soto, M., Oguni, A. and Kennedy, M.B. (1998) A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. *Neuron,* **20**, 895-904.

Chen, L. and Huang, L. Y. (1992a) Protein kinase C reduces Mg2+ block of NMDA-receptor channels as a mechanism of modulation. *Nature,* **356**, 521-523.

Chen, R. H., Sarnecki, C. and Blenis, J. (1992b) Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Molecular and Cellular Biology,* **12**, 915-927.

Chetkovich, D.M., Gray, R., Johnston, D. and Sweatt, J.D. (1991) N-methyl-D-asparate receptor activation increases cAMP levels and voltage-gated channel activity in area CA1 of hippocampus. *Proceeding of the National Academy of Sciences,* **88**, 6467-6471.

Cho, H., Orphanides, G., Sun, X., Yang, X.J., Ogryzko, V., Lees, E., Nakatani, Y. and Reinberg, D. (1998). A human RNA polymerase II complex containing factors that modify chromatin structure. *Molecular and Cellular Biology,* **18**, 5355-5363.

Chrivia, J.C., Kwok, R.P., Lamb, N., Hagiwara, M., Montminy, M.R. and Goodman, R.H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature,* **365**, 855-859.

Clark G.D., Happel L.T., Zorumski C.F. and Bazan N.G. (1992) Enhancement of hippocampal excitatory synaptic transmission by platelet-activating factor. *Neuron,* **9**, 1211-1216.

Clements, M. P., Bliss, T. V. P. and Lynch, M. A. (1991) Increase in Arachidonic acid concentration in a post synaptic membrane fraction following the induction of long-term potentiation in the dentate gyrus. *Neuroscience,* **45**, 379-389.

Cohen, G., Ren, R. and Baltimore, D. (1995) Modular binding domains in signal transduction proteins. *Cell,* **80**, 237-248.

Collingridge, G. L. and Lester, R. A. (1989) Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacological Reviews,* **41**, 143-210.

Collingridge, G.L., Kehl, S.J. and McLennan, H. (1983) Excitatory amino acids in synaptic transmission in the Schaffer-collateral commissural pathway of the rat hippocampus. *Journal of Physiology,* **334**, 33-46.

Collins, D.R. and Davis, S.N. (1993) Co-administration of (1S, 3R)-1-aminocyclopentane-1,3,dicarboxylic acid and arachidonic acid potentiates synaptic transmission in the hippocampus. *European Journal of Pharmacology,* **240**, 325-326.

Corbit, K.C., Foster, D.A. and Rosner, M.R. (1999) Protein kinase Cdelta mediates neurogenic but not mitogenic activation of mitogen-activated protein kinase in neuronal cells. *Molecular and Cellular Biology,* **19**, 4209-4218.

Cordon-Cardo, C., Tapley, P., Jing, S., Nanduri, V., O'Rourke, E., Lambelle, F., Kovary, K., Klein, R., Jones, K.R., Reichardt, L.F. and Barbacid, M. (1991) The *trk* tyrosine protein kinase mediates the mitogenis properties of nerve growth factor and neurotrophin-3. *Cell,* **66**, 173-183.

Crews, C., Alessandrini, A. and Erikson, E. (1992) The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science,* **258**, 478-480.

Crowley, C., Spenser, S.D., Nishimura, M.C., Chen, K.S., Pitts-Meek, S., Armanini, M.P., Ling, G.H., McMahon, S.B., Sheldon, D.L., Levinson, A.D., and Phillips, H.S. (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain neurons. *Cell,* **76**, 1001-1011.

Cunningham, M. E. and Greene, L. A. (1998) A function-structure model for NGF activated TRK. *EMBO Journal,* **17**, 7282-7293.

Curtis, D.R., Phillis, J.W. and Watkins, J.C. (1960) the excitation and depression of spinal neurons by certain amino acids. *Journal of Physiology,* **150**, 656-682.

Davies, S. N., Lester, R. A., Reymann, K. G. and Collingridge, G. L. (1989) Temporally distinct pre- and post-synaptic mechanisms maintain long-term potentiation. *Nature* **338**, 500-503.

Davis, A.F., Bai, J., Fasshauser, D., Wolowick, M.J., Lewis, J.L. and Chapman, E.R. (1999) Kinetics of synaptotagmin responses to Ca^{2+} and assembly with the core SNARE complexes onto membranes. *Neuron*, **24**, 363-376.

Davis, S., Vanhoutte, P., Pages, C., Caboche, J. and Laroche, S. (2000) The MAPK/ERK cascade targets both Elk-1 And cAMP Response Element Binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. *Journal of Neuroscience,* **20**, 4563-4572.

Desmond, N.L. and Levy, W.B. (1988) Synaptic interface surface area increases with long-term potentiation in the hippocampal dentate gyrus. *Brain Research,* **453**, 500-503.

Desmond, N.L. and Levy, W.B. (1990) Morphological correlates of long-term potentiation imply the modification of existing synapses, not synaptogenesis, in the hippocampal dentate gyrus. *Synapse,* **5**, 139-143.

Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M., Gout, I., Totty, N.F., Truong, O., Vicendo, P. and Yonezawa, K. (1993) PI3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein serine kinase activity. *EMBO Journal,* **13**, 522-533.

Dichter, M.A., Tischler, A.S. and Greene, L.A. (1977) Nerve growth factor-induced increase in electrical excitability and acetlycholine sensitivity of a rat pheochromocytoma cell line. *Nature*, **268**, 501-504.

Dikic, I, Batzer, A.G., Blaikie, P., Obermeier, A., Ullrich, A., Schlessinger, J. and Margolis, B. (1995) Shc binding to nerve growth factor receptor is mediated by the phosphotyrosine interaction domain. *Journal of Biological Chemistry,* **270**, 15125-15129.

Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A. and Schlessinger, J. (1996) A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383**, 547-550.

DiStefano, P.S., Friedman, B., Radziejewski, C., Schick, C.M., Lindsay, R.M. and Wiegand, S.J. (1992) The neurotrophins BDNF, NT-3 and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. *Neuron,* **8**, 983-993.

Dobrowsky, R.T., Werner, M.H., Castellino, A.M., Chao, M.V. and Hannun, Y.A. (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. *Science,* **265**, 1596-1599.

Dolphin, A.C. and Scott, R.H. (1989a) Modulation of Ca^{2+}-channel currents in sensory neurons by pertussis toxin-sensitive G-proteins. *Ann. N. Y. Acad. Sci.,* **560**, 387-390.

Dolphin, A.C. and Scott, R.H. (1989b) Interaction between calcium channel ligands and guanine nucleotides in cultured rat sensory and sympathetic neurones. *Journal of Physiology*, **413**, 271-288.

Dolphin, A.C., Errington, M.L., and Bliss, T.V.P. (1982) Long-term potentiation of the perforant path in vivo is associated with increased glutamate. *Nature,* **297**, 496-498.

Dragunow, M., B Eilharz, E., Mason, B., Lawlor, P., Abraham, W., Gluckman, P. (1993) Brain-Derived Neurotrophic Factor expression after long-term potentiation. *Neuroscience Letters,* **160**, 232-236.

Dudar, J.D., (1974) In vitro excitation of hippocampal pyramidal cell dendrites by glutamic acid. *Neuropharmacology,* **13**, 1083-1089.

Duffy, C., Teyler, T.J. and Shashoua, V.E. (1981) Long-Term Potentiation in the hippocampal slice: evidence for stimulated secretion of newly synthesised proteins. *Science*, **212**, 1148-1151.

Durand, G. M., Kovalchuk, Y. and Konnerth, A. (1996) Long-term potentiation and functional synapse induction in developing hippocampus. *Nature,* **381**, 71-75.

Ehlers, M., Kaplan, D., Price, D. and Koliatsos, V. (1995) NGF-stimulated retrograde transport of trkA in the mammalian nervous system. *Journal of CellularBiology,* **130**, 149-156.

Ehlers, M.D. (2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron,* **28**, 511-525.

Elkabes, S., Dreyfus, C. F., Schaar, D. G. and Black, I. B. (1994) Embryonic sensory development: local expression of neurotrophin-3 and target expression of nerve growth factor. *Journal of Compative Neurology,* **341**, 204-213.

Engert, F. and Bonhoffer, T. (1999) Dendritic spine changes associated with hippocampal long-term plasticity. *Nature*, **399**, 66-70.

English, J.D. and Sweatt, J.D. (1996) Activation Of p42 mitogen-activated protein kinase in hippocampal long-term potentiation. *Journal of Biological Chemistry,* 40, 244329-24332.

Ernfors, P., Bengzon, J., Kokaia, Z., Persson, H. and Lindvall, O. (1991) Increased levels of messenger RNAs for neurotrophic factor in the brain during kindling epileptogenesis. *Neuron*, 7, 165-176.

Ernfors, P., Lee, K.F., Kucera, J. and Jaenisch, R. (1994) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb propriceptive afferents. *Cell,* 77, 503-512.

Ernfors, P., Wetmore, C., Olson, L. and Personn, H. (1990) Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron,* 5, 511-526.

Erondu, N. E. and Kennedy, M. B. (1985) Regional distribution of Type II Ca^{2+}/calmodulin-dependent protein kinase in rat brain. *Journal of Neuroscience*. **5**, 3270-3277.

Errington, M.L., Lynch, M.A., and Bliss, T.V.P (1987) Long-term potentiation in the dentate gyrus: induction and increased glutamate release are blocked by D(-) aminophosphovalerate. *Neuroscience,* **20**, 279-284.

Fanger, G.R., Jones, J.R. and Maue, R.A. (1995) Differential regulation of neuronal sodium channel expression by endogenous and exogenous tyrosine kinase receptors expressed in rat pheochromocytoma cells. *Journal of Neuroscience*, **15**, 202-213.

Farhadi, H.F., Mowla, S.J., Petrecca, K., Morris, S.J., Seidah, N.G. and Murphy, R.A. (2000) Neurotrophin-3 sorts to the constitutive secretory pathway of hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with brain-derived neurotrophic factor. *Journal of Neuroscience*, **20**, 4059-4068.

Fazeli, M.S., Corbet, J., Dunn, M.J., Dolphin, A.C. and Bliss, T.V.P (1993) Changes in protein synthesis accompanying long-term potentiation in vivo. *Journal of Neuroscience,* **13**, 1346-1353.

Gianotti, C., Nunzi, M.G., Gispen, W.H. and Corradetti, R. (1992) phosphorylation of the presynaptic protein B-50 (Gap-43) is increased during electrically induced long-term potentiation. *Neuron,* **8**, 843-848.

Ginty, D. D., Bonni, A. and Greenberg, M. E. (1994) Nerve growth factor activates a ras-dependent protein kinase that stimulates c-*fos* transcription via phosphorylation of CREB. *Cell*, 77, 713-725.

Gomez, T.M. and Spitzer, N.C. (1999) In vivo regulation of axon extension and pathfinding by growth cone calcium transient. *Nature*. **397**, 350-355.

Gonzalez, G.A. and Montminy, M.R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell,* **59**, 675-680.

Gonzalez, G.A., Menzel, P., Leonard, J., Fischer, W.H. and Montminy, M.R. (1991) Characterization of motifs which are critical for activity of the Cyclic AMP-Responsive Transcription Factor CREB. *Molecular and Cellular Biology,* **11**, 1306-1312.

Goodman, L.J., Valverde, J., Lim, F., Geschwind, M.D., Federoff, H.J., Geller, A.I. and Hefti, F. (1996) Regulated release and polarised localisation of brain-derived neurotrophic factor in hippocampal neurons. *Molecular and Cellular Neuroscience*, 7, 222-238. .

Göttschalk, W., Pozzo-Miller, L., Figurov, A. and Lu, B. (1998) Presynaptic modulation of synaptic transmission and plasticity by Brain-Derived Neurotrophic factor in the developing hippocampus. *Journal of Neuroscience*, **18**, 6830-6839.

Götz, R., Köster, R., Winkler, C., Raulf, F., Lottspeich, F., Schartl, M. and Thoenen, H. (1994). Neurotrophin-6 us a new member of the nerve growth factor family. *Nature*, **372**, 266-269.

Grant, S.G.N., O'Dell, T.J., Karl, K.A., Stein, P.L., Soriano, P. and Kandel, E.R. (1992) Impaired long-term potentiation, spatial learning and hippocampal development in fyn mutant mice. *Science,* **258**, 1903-1910.

Gray, EG and Whittaker VP. (1962) The isolation of nerve endings from brain: an electron-microscopic study of cell fragmentation derived by homogenization and centrifugation. Journal of Anatomy, **96**, 79-87.

Greengard, P., Valtorta, F., Czernik, A. and Benfenati, F. (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science,* **259**, 780-785.

Griesbeck, O., Canossa, M., Campana, G., Gärtner, A., Hoener, M.C., Nawa, H., Kolbeck, R. and Thoenen, H. (1999) Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. *Microscopy Research and Technique,* **45**, 262-275.

Grigoriev, P.A., Berg, A., Schlegel, R. and Grafe, U. (1997) Differences in ion permeability of an artificial bilayer membrane caused by ampullosporin and bergofungin, new 15-membered peptaibol-type antibiotics. *Bioelectrochemistry and Bioenergetics,* **44**, 155-158.

Grimes, M.L., Zhou, J., Beattie, E.C., Yuen, E.C., Hall, D.E., Valletta, J.S., Topp, K.S., LaVail, J.H., Bunnett, N.W. and Mobley, W.C. (1996) Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signalling endosomes. *Journal of Neuroscience,* **16**, 7950-7964.

Grover, L.M. and Teyler, T.J. (1990) Two component of LTP induced by different patterns of afferent activation. *Nature,* **347**, 477-479.

Haan, E.A. and Bowen, D.M. (1981) Protection of neocortical tissue prisms from freeze-thaw injury by dimethyl sulphoxide. *Journal of Neurochemistry,* **37**, 243-246.

Halegoua, S., Armstrong, R.C. and Kremer, N.E. (1996) Dissecting the mode of action of a neuronal growth factor. *Current Topics in Microbiological Immunology,* **165**, 119-170.

Hall, J., Thomas, K.L. and Everitt, B.J. (2000) Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nature Neuroscience*, **3**, 533-535.

Hanson, P.I., Heuser, J.E. and Jahn, R. (1997) Neurotransmitter release - 4 years of SNARE complexes. *Current Opinion in Neurobiology,* 7, 310-315.

Harris, E.W. and Cotman, C.W. (1986) Long-term potentiation of the guinea pig mossy fibre responses is not blocked by N-methyl-D-aspartate antagonists. *Neuroscience Letters*, **70**, 132-137.

Harvey, J. and Collingridge, G.L. (1992) Thapsigargin blocks the induction of long-term potentiation in rat hippocampal slices. *Neuroscience Letters,* **139**, 197-200.

Hell, J.W., Yokoyama, C.T., Breeze, L.J., Chavkin, C. and Catterall, W.A. (1995) Phosphorylation of presynaptic and postsynaptic calcium currents by cAMP-dependent protein kinase in hippocampal neurons. *EMBO Journal,* **14**, 3036-3044.

Hendry, I., Stöckel, K., Thoenen, H. and Iversen, L. (1974) The retrograde axonal transport of nerve growth factor. *Brain Research,* 103-121. .

Her, J.H., Lakhani, S., Zu, K., Vila, J., Dent, P., Sturgill, T.W. and Weber, M.J. (1993) Dual phosphorylation and autophosphorylation in mitogen-activated protein (MAP) kinase activation. *Biochemical Journal*, **296**, 25-31.

Hill, C.E., Jelinek, H., Hendry, I.A., McLennan, I.S. and Rush, R.A. (1988) Destruction by anti-NGF of autonomic, sudomotor neurons and subsequent hyperinnervation of the foot pad by sensory fibres. *Journal of Neuroscience Research,* **19**, 474-482.

Hipskind, R.A., Baccarini, M. and Nordheim, A. (1994) Transient activation of Raf-1, MEK and ERK2 coincides kinetically with ternary complex factor phosphorylation and immediate-early gene promotor activity in vivo. *Molecular and Cellular Biology*, **14**, 6219-6231.

Hjorth-Simonsen, A. and Jeune, B. (1972) Origin and termination of the hippocampal perforant path in the rat studied by silver impregnation. *Journal of Comparative Neurology*, **144**, 215-232.

Hoener, M.C. (2000) Role played by sodium in activity-dependent secretion of neurotrophins - revisited. *European Journal of Neuroscience*, 3096-3106.

Hofer, M, Pagliusi, S.R., Hohn, A., Leibrock, J. and Barde, Y-A. (1990) Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO Journal,* **9**, 2459-2464.

Hofmann, F., Biel, M. and Flockerzi, V. (1994) Molecular basis for Ca^{2+} channel diversity. *Annual Review of Neuroscience,* **17**, 399-418.

Hollmann, M. and Heinemann, S. (1994) Cloned glutamate receptors. *Annual Review of Neuroscience,* **17**, 31-108.

Hu, G.Y., Hvalby, O., Walaas, S.I., Albert, K.A., Skjeflo, P., Andersen, P. and Greengard, P. (1987) Protein kinase C injection into hippocampal pyramidal cells elicits features of long-term potentiation. *Nature,* **328**, 426-429.

Huang, C. C. and Hsu, K. S. (1999a) Protein tyrosine kinase is required for the induction of long-term potentiation in the rat hippocampus. *Journal of Physiology,* **520.3**, 783-796.

Huang, E.J., Wilkinson, G.A., Farinas, I., Backus, C., Zang, K., Wong, S.L., Reichardt, L.F. (1999b) Expression of Trk receptors in the developing mouse trigeminal ganglion: in vivo evidence fro NT-3 activation of TrkA and TrkB in addition to TrkC. *Development.* **126**, 2191-2203.

Huang, Y.Y., Colley, P.A. and Routtenberg, A. (1992a) Postsynaptic then presynaptic protein kinase C activity may be necessary for long-term potentiation. *Neuroscience,* **49**, 819-827.

Huang, J., Nasr, M., Kim, Y. and Matthews, H.R. (1992b) Genistein inhibits protein histidine kinase. *Journal of Biological Chemistry,* **267**, 15511-15515.

Huang, Y.Y. and Kandel, E.R. (1994) Recruitment of long-lasting and protein kinase A-dependent long-term potentiation in the CA1 region of the hippocampus requires repeated tetanization. *Learning and Memory,* **1**, 74-82.

Huang, Y.Y. and Malenka, R.C. (1993) Examination of TEA-induced synaptic enhancement in area CA1 of the hippocampus: the role of voltage-dependent Ca^{2+} channels in the induction of LTP. *Journal of Neuroscience,* **3**, 568-576.

Huettner, J.E. (1990) Glutamate receptor channels in rat DRG neurons: activation of kainate and quisqualate and blockade of desensitisation by Con A. *Neuron,* **5**, 255-266.

Hughes, P., Beilharz, E., Gluckman, P. and Dragunow, M. (1993) Brain-derived neurotrophic factor is induced as an immediate early gene following *N*-methyl-*D*-aspartate receptor activation. *Neuroscience,* **57**, 319-328.

Hunter, T. and Cooper, J.A. (1985) Protein-tyrosine kinases. *Annual Review of Biochemistry,* **54**, 897-930.

Hurst, H. C., Masson, M., Jones, N. C. and Lee, K. A. (1990) The cellular transcription factor CREB corresponds to activating transcription factor 47 (ATF-47) and forms complexes with a group of polypeptides related to ATF-43. *Molecular and Cell Biology,* **10**, 6192-6203.

Husi, H. and Grant, S. G. (2001) Isolation of 2000-kDa complexes of N-methyl-D-aspartate receptor and postsynaptic density 95 from mouse brain. *Journal of Neurochemistry,* **77**, 281-291.

Impey, S., Obrietan, K., Wong, S.T., Poser, S., Yano, S., Wayman, G., Deloulme, J.C., Chan, G. and Storm, D.R. (1998) Cross talk between ERK And PKA is required for Ca^{2+} stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron,* **21**, 869-883.

Ip, F.C.F., Cheung, J. and Ip, N.Y. (2001) The expression profiles of neurotrophins and their receptors in rat and chicken tissues during development. *Neuroscience Letters,* **301**, 107-110.

Ip, N.Y., Ibanez, C.F., Nye, S.H., McClain, J., Jones, P.F., Gies, D.R., Belluscio, L., Le Beau, M.M., Espinosa, R. and Squinto, S.P. (1992) Mammalian neurotrophin-4: structure, chromasomal localisation, tissue distribution and receptor specificity. *Proceedings of the National Academy of Sciences,* **89**, 3060-3064.

Ip, N.Y., Li, Y., Yancopoulos, G.D. and Lindsay, R.M. (1993) Cultured hippocampal neurons show responses to BDNF, NT-3 and NT-4, but not NGF. *Journal of Neuroscience*, **13**, 3394-3405.

Isaac, J. T., Nicoll, R. A. and Malenka, R. C. (1995) Evidence for silent synapses: Implications for the expression of LTP. *Neuron,* **15**, 427-434.

Isaackson, P.J., Ullrich, A., and Bradshaw, R.A. (1984) Mouse 7S nerve growth factor: Complete sequence of a cDNA coding for the alpha-subunit precursor and its relationship to serine proteases. *Biochemistry,* **23**, 5997-6002.

Ishiyama, J., Saito, H. and Abe, K. (1991) Epidermal growth factor and basic fibroblast growth factor promote the generation of long-term potentiation in the dentate gyrus of anaesthetized rats. *Neuroscience Research,* **12**, 403-411.

Izumi, Y., Clifford, D.B. and Zorumski, C.F. (1991) 2-amino-3-phosphonopropionate blocks the induction and maintenance of long-term potentiation in hippocampal slices. *Neuroscience Letters,* **122**, 187-191.

Jeffrey, K.J., Abraham, W.C., Dragunow, M. and Mason, S.E. (1990) Induction of fos-like immunoreactivity and the maintenance of long-term potentiation in the dentate gyrus of anaesthetized rats. *Molecular Brain Research,* **8**, 267-274.

Jiang, H., Takeda, K., Lazarovici, P., Katagiri, Y., Yu, Z., -X., Dickens, G., Chabuk, A., Liu, X., -W., Ferrans, V. and Guroff, G. (1999) Nerve growth factor (NGF)-induced calcium influx and intracellular calcium mobilization in 3T3 cells expressing NGF receptors. *Journal of Biological Chemistry*, **274**, 26209-26216.

Jing, S. Tapley, P. and Barbacid, M. (1992) Nerve growth factor mediates signal transduction through the trk homodimer receptors. *Neuron,* **9**, 1067-1079.

Johnson, D., Lanaham, A., Buck, C.R., Sehgal, A., Morgan, C., Merver, E., Bothwell, M and Chao, M. (1986) Expression and structure of the human NGF receptor. *Cell,* **47**, 545-554.

Johnson, E.M., Andres, R.Y., and Bradshaw, R.A., (1978) Characterisation of the retrograde transport of nerve growth facotr (NGF) using high speciif activity [^{125}I] NGF. *Brain Research*, **150**, 319-331.

Johnson, E.M., Gorin, P.D., Brandeis, L.D. and Pearson, J. (1980) Dorsal root ganglion neurons are destroyed by exposure *in utero* to maternal antibody to nerve growth factor. *Science*, **210**, 916-918.

Johnson, J.R., Chu, A.K. and Sato-Bigbee, C (2000) Possible role of CREB in the stimulation of oligodendrocyte precursor cell proliferation by neurotrophin-3. *Journal of Neurochemistry*, **74**, 1409-1417.

Jonanovic, J. N., Benfenati, F., Siow, Y. L., Sihra, T. S., Sanghera, J. S., Pelech, S. L., Greengard, P., Czernik, A. J. (1996) Neurotrophins stimulate phosphorylation of synapsin-1 – actin interactions. *Proceeding of the National Academy of Sciences*, **93**, 3679-3683.

Jones, K.R., Farinas, I., Backus, C., and Reichardt, L.F. (1994) Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell*, **76**, 989-999.

Jungbluth, S., Bailey, K. and Barde, Y.A. (1994) Purification and characterisation of a brain-derived neurotrophic factor/neurotrophin-3 (BDNF/NT-3) heterodimer. *European Journal of Biochemistry*, **221**, 677-685.

Kang, H. and Schuman, E. M. (1995) Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science*, **267**, 1658-1662.

Kang, H. and Schuman, E. M. (1996) A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. *Science*, **273**, 1402-1406.

Kleshchevnikov, A. M. and Routtenberg, A. (2001) PKC activation rescues LTP from NMDA receptor blockade. *Hippocampus,* **11**, 168-175.

Knipper, M., Leung, L.S., Zhao, D. and Rylett, R.J. (1994) Short-term modulation of glutamatergic synapses in the adult rat hippocampus by NGF. *Neuroreport,* **5**, 2433-2436.

Kobayashi, M. and Kawakami, K. (1995) ATF-1CREB heterodimer is involved in constitutive expression of the housekeeping Na,K-ATPase alpha 1 subunit gene. *Nucleic Acids Research,* **23**, 2848-2855.

Kohler, M., Burnashev, N., Sakmann, B. and Seeburg, P.H.(1993) Determinants of Ca^{2+} permeability in both TM1 and TM2 of high affinity kainate receptor channels: diversity by RNA editing. *Neuron,* **10**, 491-500.

Kohr, G. and Seeburg, P. H. (1996) Subtype-specific regulation of recombinant NMDA receptor-channels by protein tyrosine kinases of the src family. *Journal of Physiology,* **492**, 445-452.

Kojima, N., Wang, J., Mansuy, I. M., Grant, S. G., Mayford, M. and Kandel, E. R. (1997). Rescuing impairment of long-term potentiation in fyn-deficient mice by introducing Fyn transgene. *Proceeding of the National Academy of Sciences,* **94**, 4761-4765.

Kokaia, M., Asztely, F., Olofsdotter, K., Sindreu, C.B., Kullman, D.M. and Lindvall, O. (1998) Endogenous neurotrophin-3 regulates short-term plasticity at the lateral perforant path-granule cell synapses. *Journal of Neuroscience*, **18**, 8730-8739.

Kornecki, E., Wieraszko, A., Chan, J., and Ehrlich, Y.H. (1996) Platelet-activating factor in memory formation: role as a retrograde messenger in long-term potentiation. *Journal of Lipid Mediated Cell Signalling*, **14**, 115-126.

Korsching, S. and Thoenen, H. (1983) Quantitative demonstration of retorgrade transport of endogenous nerve growth factor. *Neuroscience Letters,* **39**, 1-4.

Korte, M. Kang, H., Bonhoffer, T. and Schuman, E. (1998) A role for BDNF in the late-phase of hippocampal long-term potentiation. *Neuropharmacology,* **37**, 553-559.

Korte, M., Carroll, P., Wolf, E., Brem, G., Thoenen, H. and Bonhoeffer, T. (1995) Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proceedings of the National Academy of Sciences*, **92**, 8856-8860.

Korte, M., Griesbeck, O., Gravel., C., Carroll, P., Staiger, V., Thoenen, H., Bonhoeffer, T. (1996)Virus-mediated gene transfer into hippocampal CA1 region restores long-term potentiation in brain-derived neurotrophic factor mutant mice. *Proceedings of the National Academy of Sciences*, 93, 12547-12552.

Krug, M., Lossner, B. and Ott, T. (1984) Anisomycin blocks the late phase of long-term potentiation in the dentate gyrus of freely moving rats. *Brain Research Bulletins,* **13**, 39-42.

Krüttgen, A., Möller, J.C., Heymach, J.V. and Shooter, E.M. (1998) Neurotrophins induce release of neurotrophins by the regulated secretory pathway. *Proceedings of the National Academy of Sciences*, **96**, 9614-9619.

Kullmann, D. M. and Nicoll, R. A. (1992) Long-term potentiation is associated with increases in quantal content and quantal amplitude. *Nature,* **357**, 240-244.

Kurino, M., Fukunaga, K., Ushio, Y. and Miyamoto, E. (1995) Activation of mitogen-activated protein kinase in cultured rat hippocampal neurons by stimulation of glutamate receptors. *Journal of Neurochemistry,* **65**, 1282-1289.

Kwok, R.P., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G., Green, M.M. and Goodman, R.H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature*, **370**, 223-226.

Lai, C., Gore, M. and Lemke, G. (1994) Structure, expression and activity of Tyro 3, a neural adhesion-related receptor tyrosine kinase. *Oncogene,* **9**, 2567-2578.

Lai, K.O., Fu, W.Y., Ip, F.C. and Ip, N.Y. (1998) Cloning and expression of a novel neurotrophin, NT-7, from carp. *Molecular and Cellular Neuroscience*, **11**, 64-76.

Lamballe, F., Klein, R. and Barbacid, M. (1991) *trk*C, a new member of the *trk* family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell,* **66**, 967-979.

Lambelle, C. and Leclerc, N. (2000) Exogenous BDNF, NT-3 and NT-4 differentially regulate neurite outgrowth in cultured hippocampal neurons. *Developmental Brain Research,* **123**, 1-11.

Larson, J., Wong, D. and Lynch, G. (1986) Patterned stimulation at the theta frequency is optimal for the induction of hippocampal long-term potentiation. *Brain Research,* **368**, 347-350.

Lau, L-F. and Huganir, R. L. (1995) Differential tyrosine phosphorylation of N-Methyl-D-Aspartate Receptor Subunits. *Journal of Biological Chemistry,* **270**, 20036-20041.

Lauri, S. E., Taira, T. and Rauvala, H. (2000) High-frequency synaptic stimulation induces association of fyn and c-src to distinct phosphorylated components. *Molecular Neuroscience*, **11**, 997-1000.

Lee, K. A., Hai, T. Y., SivaRaman, L., Thimmappaya, B., Hurst, H. C., Jones, N. C. and Green, M. R. (1987) A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. *Proceedings of the National Academy of Sciences*, **84**, 8355-8359.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H. and Barde, Y-A. (1989) Molecular cloning and expression of brain-derived neurotrophic factor, *Nature,* **341**, 149-152.

Leßmann, V. and Heumann, R. (1998) Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brain-derived neurotrophic factor in hippocampal microcultures: presynaptic enhancement depends on pre-established paired-pulse facilitation. *Neuroscience,* **86**, 399-413.

Leßmann, V., Gottmann, K. and Heumann, R. (1994) BDNF and NT-4/5 enhance glutamatergic synaptic tranmission in cultured hippocampal neurons. *Neuroreport,* **6**, 21-25.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B. and Schlessinger, J. (1995) Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature,* **376**, 737-745.

Levi-Montalcini, R. (1987) The nerve growth factor 35 years later. *Science*, **237**, 1154-1162.

Levi-Montalcini, R. and Hamburger, V. (1951) Selective growth stimulating effects of mouse sarcoma on the sensory and symapathetic nervous system of the chick embroyo. *Journal of Experimental Zoology,* **116**, 321-360.

Levine, E.S., Crozier, R.A., Plummer, M.R. and Black, I.B. (1998) Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing *N*-methyl-

D-aspartate receptor activity. *Proceedings of the National Academy of Sciences*, **95**, 10235-10239.

Levine, E.S., Dreyfus, C.F., Black, I.B. and Plummer, M.R. (1995) Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. *Proceedings of the National Academy of Sciences*, **92**, 8074-8077.

Levy, W.B. and Steward, O. (1979) Synapses as associative memory elements in the hippocampal formation. *Brain Research,* **175**, 233-245.

Li, Y., -X., Zhang, Y., Lester, H. A., Schuman, E. M. and Davidson, N. (1998) enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons. *Journal of Neuroscience*, **18**, 10231-10240.

Liao, D., Hessler, N. A. and Malinow, R. (1995) Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature,* **375**, 400-404.

Liao, D., Jones, A. and Malinow, R. (1992) Direct Measurement of quantal changes underlying long-term potentiation in CA1 hippocampus. *Neuron,* **9**, 1089-1097.

Lin-, S. -Y., Wu, K., Levine, E. S., Mount, H. T. J., Suen, P. -C and Black, I. B. (1998) BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. *Molecular Brain Research*, **55**, 20-27.

Linden, D.J., Wong, K.L., Sheu, F.S. and Routtenberg, A. (1988) NMDA receptor blockade prevents the increase in protein kinase C substrate (Protein F1) phosphorylation produced by long-term potentiation. *Brain Research,* **458**, 142-146.

Lindholm, D., Castren, E., Berzaghi, M.P., Blochl, A. and Thoenen, H. (1994) Activity-dependent and hormonal regulation of neurotrophin mRNA levels in the brain - implications for neuronal plasticity. *Journal of Neurobiology,* **25**, 1362-1372.

Linstedt, A.D., Vetter, M.L., Bishop, J.M. and Kelly, R.B. (1992) specific association of the proto-oncogene product pp60c-src with an intracellular organelle, the PC12 synaptic vesicle. *Journal of Biological Chemistry,* **117**, 1077-1084.

Liou, J.C. and Fu, W.M. (1997) Regulation of quantal secretion from developing motoneurons by postsynaptic activity-dependent release of NT-3. *Journal of Neuroscience,* **17**, 2459-2468.

Liu, J., Fukunaga, K., Yamamoto, H., Nishi, K. and Miyamoto, E. (1999) Differential roles of Ca^{2+}/Calmodulin-dependent protein kinase II and mitogen-activated protein kinase activation in hippocampal long-term potentiation. *Journal of Neuroscience,* **19**, 8292-8299.

Llinas, R., Sugimori, M. and Silver, R.B. (1992) Microdomains of high calcium concentration in the presynaptic terminal. *Science,* **256**, 677-679.

Lohof, A. M., Ip, N. Y. and Poo, M. (1993) potentiation of developing neuromuscular synapses by neurotrophins NT-3 and BDNF. *Nature,* **362**, 350-353.

Lovinger, D.M. and Routtenberg, A. (1988) Synapse-specific protein kinase C activation enhances maintenance of long-term potentiation in rat hippocampus. *Journal of Physiology,* **400**, 321-333.

Lovinger, D.M., Wong, K.L., Murakami, K. and Routtenberg, A. (1987) Protein kinase C inhibitors eliminate hippocampal long-term potentiation. *Brain Research,* **436**, 177-183.

Lu, B., Yokoyama, CF., Black, IB. (1991) Depolarising stimuli regulate nerve growth factor gene expression in cultured hippocampal neurons. *Proceedings of the National Academy of Sciences*, **88**, 6268-6292.

Lu, Y. -F., Kojima, N., Tomizawa, K., Moriwaki, A., Matsushita, M., Obata, K. and Matsui, H. (1999a) Enhanced synaptic transmission and reduced threshold for LTP induction in fyn-transgenic mice. *European Journal of Neuroscience,* **11**, 75-82.

Lu, Y., -F., Kandel, E. R. and Hawkins, R. D. (1999b) Nitric oxide signaling contributes to late-phase LTP and CREB phosphorylation in the hippocampus. *Journal of Neuroscience,* **19**, 10250-10261.

Lu, Y.M., Roder, J.C., Davidow, J. and Salter, M.W. (1998) Src activation in the induction of long-term potentiation in CA1 hippocampal neurons. *Science,* **279**, 1363-1367.

Lundblad, J.R., Kwok, R.P., Laurance, M.E., Harter, M.L. and Goodman, R.H. (1995). Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. *Nature,* **374**, 85-88.

Lynch, G., Dunwiddie, T. and Gribkoff, V. (1977) Heterosynaptic depression: a postsynaptic correlate of long-term potentiation. *Nature,* **266**, 737-739.

Lynch, G., Larson, J., Kelso, S., Barrionuevo, G. and Schottler, F. (1983) Intracellular injections of EGTA block induction of hippocampal long-term potentiation. *Nature,* **305**, 719-721.

Lynch, M.A. and Voss, K.L. (1991) Membrane arachiodonic acid concentration correlates with age and induction of long-term potentaion. *European Journal of Neuroscience,* **6**, 1008-1014.

Lynch, M.A., Clements, M.P., Voss, K.L., Bramham, C.R. and Bliss, T.V.P. (1991) Is archidonic acid a retrograde messenger in long-term potentiation? *Biochem Soc Trans.*, **19**, 391-396.

Lynch, M.A., Errington, M.L. and Bliss, T.V.P. (1989) Nordihydroguaiaretic acid blocks the synaptic component of long-term potentiation and the associated increases in release of glutamate and arachidonate: an in vivo study in the dentate gyrus. *Neuroscience,* **30**, 693-701.

Lynch, M.A., Voss, K.L., Rodriguez, J. and Bliss, T.V.P. (1994) Increase in synaptic vesicle proteins accompanies long-term potentiation in the dentate gyrus. *Neuroscience*, **60**, 1-5.

Ma, L., Reis, G., Parada, L. F. and Schuman, E. M. (1999) Neuronal NT-3 is not required for synaptic transmission or long-term potentiation in area CA1 of the adult rat hippocampus. *Learning & Memory,* **6**, 267-275.

MacDermott, A.B., Mayer, M.L., Westbrook, G.L., Smith, S.J. and Barker, J.L. (1986) NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal neurons. *Nature,* **321**, 519-522.

Maguire, C., Casey, M., Kelly, A., Mullany, P.M. and Lynch, M.A. (1999) Activation of tyrosine receptor kinase, *trk,* plays a role in expression of long-term potentiation in the rat dentate gyrus. *Hippocampus,* **9**, 519-526.

Maisonpierre, P.C., Belluscio, L., Squinto, S., IP, N. Y., Furth, M. E., Lindsay, R. M. and Yancopoulos, G. D. (1990) Neurotrophin-3: A neurotrophic factor related to NGF and BDNF. *Science,* **247**, 1446-1451.

Maisonpierre, P.C., Belluscio, L., Ffriedman, B., Alderson, R.F., Wiegand, S.J., Furth, M.S., Lindsay, R.M. and Yancopoulos, G.D. (1990) NT-3, BDNF and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. *Neuron,* **5**, 501-509.

Malenka, R.C., Kauer, J.A., Perkel, D.J., Mauk, M.D., Kelly, P.T., Nicoll, R.A. and Waxham, M.N. (1989) An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature,* **340**, 554-557.

Malenka, R.C., Lancaster, B. and Zucker, R.S. (1992) Temporal limits on the rise in postsynaptic calcium required for the induction of long-term potentiation. *Neuron,* **9**, 121-128.

Malenka, R.C., Madison, D.V. and Nicoll, R.A. (1986) Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature,* **321**, 175-177.

Malinow, R. and Tsien, R.W. (1990) Presynaptic enhancements shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature*. **346**, 177-190.

Malinow, R., Madison, D.V. and Tsien, R.W. (1988) Persistent protein kinase activity underlying long-term potentiation. *Nature,* **335**, 820-824.

Malinow, R., Schulman, H., and Tsien, R.W. (1989) Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science,* **245**, 862-866.

Marsh, N. and Palfrey, C. (1996) Neurotrophin-3 and brain-derived neurotrophic factor activate multiple signal transduction events but are not survival factors for hippocampal pyramidal neurons. *Journal of Neurochemistry*, **67**, 952-963.

Martin, K. C., Michael, D., Rose, J. C., Barad, M., Casadio, A., Zhu, H., and Kandel, E. R. (1997) MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in Aplysia. *Neuron,* **18**, 899-912.

Martínez, A., Alcántara, S., Borrell, V., Del Río, J. A., Blasi, J., Otal, R., Campos, N., Boronat, A., Barbacid, M., Silos-Santiago, I. and Soriano, E. (1998) TrkB and trkC signaling are required for maturation and synaptogenesis of hippocampal connections. *Journal of Neuroscience,* **18**, 7336-7350.

Mason, A.J., Evans, B.A., Cox, D.A., Shine, J., and Richards, R.I. (1983) Structure of mouse kallikrein gene family suggests a role in specific processing of biologically active peptides. *Nature,* **300**, 300-307.

Matsubara, M., Kusubata, M., Ishigurao, K., Uchida, T., Titani, K. and Taniguchi, H. (1996) Site-specific phosphorylation of synapsin I by mitogen-activated protein kinase and Cdk5 and its effects on physiological functions. *Journal of Biological Chemistry,* **271**, 21108-21113.

Matthies, H. and Reymann, K.G. (1993) Protein kinase A inhibitors prevent the maintenance of hippocampal long-term potentiation. *Neuroreport,* **4**, 712-714.

Matthies, H., Schulz, S., Thiemann, W., Seimer, H., Schmidt, H., Krug, M. and Hollt, V. (1997) Design of the multiple interface chamber and application for resolving the temporal pattern of CREB phosphorylation in hippocampal long-term potentiation. *Journal of Neuroscience Methods,* **78**, 173-179.

Matveeva, E. A., Whiteheart, S. W., Vanaman, T. C. and Slevin, J. T. (2001) Phosphorylation of the N-Ethylmaleimide-sensitive factor is associated with depolarization-dependent Neurotransmitter release from synaptosomes. *Journal of Biological Chemistry,* **276**, 12174-12181.

Maycox, P.R., Deckwerth, T., Hell, J.W. and Jahn, R. (1988) Glutamate uptake by brain synaptic vesicles. Energy dependence of transport and functional reconstitution in proteoliposomes. *Journal of Biological Chemistry,* **263**, 15423-15438.

Mayer, M.L., and Westbrook, G.L. (1987) The physiology of excitatory amino acids in the vertebrate central nervous system. Progress in Neurobiology, **28**, 197-276.

McBean, G.J. and Roberts, P.J. (1985) Neurotoxicity of L-glutamate and DL-threo-3-hydroxyaspartate in the rat striatum. *Journal of Neurochemistry,* **44**, 247-254.

McCleskey, E.W. (1994) Calcium channels: cellular roles and molecular mechanisms. *Current Opinion in Neurobiology,* **4**, 304-312.

McCormick, F. (1994) Activators and effectors of ras p21 proteins. *Current Opinion in Genetic Development,* **4**, 71-76.

McGahon, B. and Lynch, M.A. (1994) A study of the synergism between metabotropic glutamate receptor activation and arachidonic acid in the rat hippocampus. *Neuroreport,* **5**, 2353-2357.

McGahon, B. and Lynch, M.A. (1998) Analysis of the interaction between arachidonic acid and metabotropic glutamate receptor activation reveals the phospholipase C acts as a coincidence detector in the expression of long-term potentiation in the rat dentate gyrus. *Hippocampus,* **8**, 1-9.

McGahon, B., Maguire, C., Kelly, A. and Lynch, M. A. (1999) Activation of p42 mitogen-activated protein kinase by arachidonic acid and trans-1-amino-cyclopentyl-1,3-dicarboxylate impacts on long-term-potentiation in the dentate gyrus in the rat: analysis of age-related changes. *Neuroscience,* **90**, 1167-1175.

McGuinness, N., Anwyl, R. and Rowan, M. (1991) t-ACPD enhances long-term potentiation in the hippocampus. *European Journal of Neuroscience,* **197**, 231-232.

McNaughton, B.L. Douglas, R.M. and Goddard, G.V. (1978) Synaptic enhancement in fascia dentata: cooperativity among coafferents. *Brain Research,* **157**, 277-294.

Merlio, J.-P., Ernfors, P., Jaber, M. and Persson, H. (1992) Molecular cloning of rat *trk*C and distribution of cells expressing messenger rnas for members of the *trk* family in the rat central nervous system. *Neuroscience*, **51**, 513-532.

Meshul, C.K. and Hopkins, W.F. (1990) Presynaptic ultrastructural correlates of long-term potentiation in the CA1 subfield of the hippocampus. *Brain Research,* **514**, 310-319.

Messaoudi, E., Bardsen, K., Srebro, B. and Bramham, C.R. (1998) Acute intrahippocampal infusion of BDNF induced lasting potentiation of synaptic transmission in the rat dentate gyrus. *Journal of Neurophysiology,* **79**, 496-499.

Messina, A. and Bell, C.B. (1991) Are genetically hypertensive rats deficient in nerve growth? *Neuroreport,* **2**, 45-48.

Middlemas, D.S., Meisenhelder, J. and Hunter, T. (1994) Identification of trkB autophosphorylation sites and evidence that phospholipase C-gamma 1 is a substrate of the trkB receptor. *Journal of Biological Chemistry,* **269**, 5458-5466.

Miller, S.G. and Kennedy, M.B. (1986) Regulation of brain type II Ca^{2+}/calmodulin-dependnet protein kinase by autophosphorylation: a Ca^{2+}-triggered molecular switch. *Cell,* **44**, 861-870.

Miller, S.G., Patton, B.L. and Kennedy, M.B. (1988) Sequences of autophosphorylation sites in neuronal type II CaM kinase that control Ca^{2+}-independent activity. *Neuron,* **1**, 593-604.

Min, M. Y., Asztely, F., Kokaia, M. and Kullmann, D. M. (1998) Long-term potentiation and dual-component quantal signaling in the dentate gyrus. *Proceeding of the National Academy of Sciences,* **95**, 4702-4707.

Mochida, H., Sato, K., Sasaki, S., Yazawa, I., Kamino, K. and Momose-Sato, Y. (2001) Effects of anisomycin on LTP in the hippocampal CA1: long-term analysis using optical recording. *Neuroreport*, **12**, 987-991.

Montminy, M. R. and Bilezikjian, L. M. (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature,* **328**, 175-178.

Morgan, S.L. and Teyler, T.J. (1999) VDCCs and NMDARs underlie two forms of LTP in the CA1 hippocampus in vivo. *Journal of Neurophysiology*, **82**, 736-740.

Morris, R.G., Anderson, E., Lynch, G.S. and Baudry, M. (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature,* **319**, 774-776.

Mowla, S.J., Pareek, S., Farhadi, H.F., Petrecca, K., Fawcett, J.P., Seidah, N.G., Morris, S.J., Sossin, W.S. and Murphy, R.A. (1999) Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. *Journal of Neuroscience*, **19**, 2069-2080.

Mulkey, R. M. and Zucker, R. S. (1991) Action potentials must admit calcium to evoke transmitter release. *Nature* , **350**, 153-155.

Mullany, P. and Lynch, M.A, (1997) Changes in protein synthesis of the synaptic vesicle protein, synaptophysin, in entorhinal cortex following induction of long-term potentiation in dentate gyrus: an age-related study in the rat. *Neuropharmacology.*, **36**, 973-980.

Mullany, P. and Lynch, M.A. (1998) Evidence for a role for synaptophysin in expression of long-term potentiation in the rat dentate gyrus. *Neuroreport,* **9**, 2489-2494.

Mullany, P., Connolly, S. and Lynch, M.A. (1996) ageing is associated with changes in glutamate release, protein tyrosine kinase and calcium/calmodulin-dependent kinase II in rat hippocampus. *European Journal of Pharmacology,* **309**, 311-315.

Muller, D., Joly, M. and Lynch, G. (1988) Contributions of quisqualate and NMDA receptors to the induction and expression of LTP. *Science,* **242**, 1694-1697.

Muragaki, Y., Timothy, N., Leight, S., Hempstead, B.L., Chao, M.V., Trojanowski, J.Q. and Lee, V.M. (1995) Expression of trk receptors in the developing and adult human central and peripheral nervous system. *Journal of Compative Neurology,* **356**, 387-397.

Murphy, D. D. and Segal, M. (1997) morphological plasticity of dendritic spines in central neurons is mediated by activation of cAMP response element binding protein. *Proceedings of the National Academy of Sciences*, **94**, 1482-1487.

Nadler, J. V., Vaca, K. W., White, W. F., Lynch, G. S. and Cotman, C. W. (1976) Aspartate and glutamate as possible transmitters of excitatory hippocampal afferents. *Nature,* **260**, 538-540.

Nadler, J.V. and Smith, E.M. (1981) Perforant path lesions deplete glutamate content of fascia dentata synaptosomes. *Neuroscience Letters*, **25**, 275-280.

Nakajima, T., Uchida, C., Anderson, S.F., Parvin, J.D. and Montminy, M. (1997) Analysis of a cAMP-responsive activator reveals a two-component mechanism for transcriptional induction via signal-dependent factors. *Genes Developement,* **11**, 738-747.

Nakanishi, S. (1992) Molecular diversity of glutamate receptors and implications for brain function. *Science,* **258**, 597-603.

Obermeier, A., Bradshaw, R. A., Seedorf, K., Choidas, A., Schlessinger, J. and Ullrich, A. (1993) Neuronal differentiation signals are controlled by nerve growth factor receptor/trk binding sites for SHC and PLC. *EMBO Journal,* **13**, 1585-1590.

Ohmichi, M., Decker, S. and Saltiel, A. (1992) Activation of phosphatidylinosytol 3-kinase by nerve growth factor involves indirect coupling of the trk proto-oncogne with src Homology 2 domains. *Neuron,* **9**, 769-777.

Okumura, N., Okada, M. and Nakagawa, H. (1994) Depolarization-induced tyrosine phosphorylation in PC12 cells. *Journal of Biochemistry,* **116**, 346-350.

Omkumar, R.V., Kiely, M.J., Rosenstein, A.J., Min, K.T. and Kennedy, M.B. (1996) Identification of a phosphorylation site for calcium/calmodulin dependent protein kinase II in the NR2B subunit of the N-methyl-D-aspartate receptor. *Journal of Biological Chemistry*, **271**, 31670-31678.

Ordronneau, P., Abullah, L. and Petruse, P. (1991) An efficient enzyme immunoassay for glutamate using glutaraldehyde coupling of the hapten to microtiter plates. *Journal of Immunological Methods,* **142**, 169-176.

Orphanides, G., Lagrange, T. and Reinberg, D. (1996) The general transcription factors of RNA polymerase II. *Genes Development,* **10**, 2657-2683.

Otani, S. and Abraham, W.C. (1989) Inhibition of protein synthesis in the dentate gyrus, but not the entorhinal cortex, blocks maintenance of long-term potentiation in rats. *Neuroscience Letters,* **106**, 175-180.

Otani, S., Roisin-Lallemand, M.-P. and Ben-Ari, Y. (1992) Enhancement of extracellular protein concentrations during long-term potentiation in the rat hippocampal slice. *Neuroscience,* **47**, 265-272.

Pang, D.T., Wang, J.K.T., Valtorta, F., Benifenati, F and Greengard, P. (1988) protein tyrosine phosphorylation in synaptic vesicles. *Proceeding of the National Academy of Sciences,* **85**, 762-766.

Patneau, D.K. and Mayer, M.L. (1990) Structure-activity relationships for amino acid transmitter candidates acting at the N-methyl-D-aspartate and quisqualate receptors. *Journal of Neuroscience,* **10**, 2385-2399.

Patneau, D.K. and Mayer, M.L. (1991) Kinetic analysis of interactions between kainate and AMPA: evidence for activation of a single receptor in mouse hippocampal neurons. *Neuron,* **6**, 785-798.

Patterson, S.L., Abel, T., Deuel, T.A.S. Martin, K.C., Rose, J.C. and Kandel, E.R. (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron,* 1137-1145.

Patterson, S. L., Grover, L. M., Schwartzkroin P. A. and Bothwell, M. (1992) Neurotrophin expression in rat hippocampal slices: a stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT3 mRNAs. *Neuron,* **9**, 1081-1088.

Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Sha, J., Hunt, D. F., Weber, M. J. and Sturgill, T. W. (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO Journal,* **10**, 885-892.

Pevsner, J., Hsu, S-C., Braun, J.E.A., Calakos, N., Ting, A.E., Bennett, M.K. and Scheller, R.H. (1994) Specificity and regulation of a synaptic vesicle docking complex. *Neuron,* **13**, 353-361.

Phillips, H.S., Hains, J.M., Laramee, G.R., Rosenthal, A. and Winslow, J.W. (1990) Widespread expression of BDNF but not NT-3 by target areas of basal forebrain cholinergic neurons. *Science,* **250**, 290-294.

Phillis, J.W., Song, D. and O'Regan, M.H. (1996) Inhibition of tyrosine phosphorylation attenuates amino acid neurotransmitter release from the ischemic/reperfused rat cerebral cortex. *Neuroscience Letters,* **207**, 151-154.

Pin, J-P. and Duvosin, R. (1995) the metabotropic glutamate receptors: structure and function. *Neuropharmacology,* **34**, 1-26.

Posanda, J. and Cooper, J.A. (1992) Requirements for phosphorylation of MAP kinase during meiosis in Xenopus oocytes. *Science,* **255**, 212-215.

Potier, B. and Rovira, C. (1999) Protein tyrosine kinase inhibitors reduce high-voltage activating calcium currents in CA1 pyramidal neurones from rat hippocampal slices. *Brain Research,* **816**, 587-597.

Pozzo-Miller, L., Gottschalk, W., Zhang, L., McDermott, K., Du, J., Gopalakrishnan, R., Oho, C., Sheng, Z-H. and Lu, B. (1999) Impairments in high-frequency transmission, synaptic vesicle docking and synaptic protein distribution in the hippocampus of BDNF knockout mice. *Journal of Neuroscience*, **19**, 4972-4983.

Radeke, M.J., Misko, T.P., Hsu, C., Herzenberg, L.A. and Shooter, E.M. (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature*, **325**, 593-597.

Radpour, S. and Thomson, A. M. (1992) Synaptic enhancement induced by NMDA and Qp receptors and presynaptic activity. *Neuroscience Letters,* **138**, 119-122.

Recesens, M. (1995) Putative molecular mechanisms underlying long-term potentiation (LTP): the key role of excitatory amino acid receptors. *Therapie,* **50**, 19-34.

Raman, I. M., Tong, G. and Jahr, C.E. (1996) Beta-adrenergic regulation of synaptic NMDA receptors by cAMP-dependent protein kinase. Neuron, **16**, 415-421.

Rettig, J., Heinemann, C., Ashery, U., Sheng, Z. H., Yokoyama, C. T., Catterall, W. A. and Neher, E. (1997) Alteration of Ca^{2+} dependence of neurotransmitter release by disruption of Ca^{2+} channel/syntaxin interaction. *Journal of Neuroscience* , **17**, 6647-6656.

Rettig, J., Sheng, Z. H., Kim, D. K., Hodson, C. D., Snutch, T. P. and Catterall, W. A. (1996) Isoform-specific interaction of the Alpha1A subunits of brain Ca^{2+} channels with the presynaptic proteins syntaxin and SNAP-25. *Proceeding of the National Academy of Sciences,* **93**, 7363-7368.

Reymann, K.G. and Matthies, H. (1989) 2-Amino-4-phosphonobutyrate selectively eliminates late phases of long-term potentiation in rat hippocampus. *Neuroscience. Letters,* **98**, 166-171.

Richardson, C.L., Tate, W.P., Mason, S.E., Lawlor, P.A., Dragunow, M. and Abraham, W.C. (1992) Correlation between the induction of an immediate early gene, zif/268, and long-term potentiation in the dentate gyrus. *Brain Research,* **580**, 147-154.

Riedel, G. and Reymann, K. (1993) An antagonist of the metabotropic glutamate receptor prevents LTP in the dentate gyrus of freely moving rats. *Neuropharmacology,* **32**, 929-931.

Ringstedt, T., Lagercrantz, H. and Persson, H. (1993) Expression of members of the trk family in the developing posstnatal rat brain. *Brain Research Development Brain Research,* **72**, 119-131.

Robbins, D. J. and Cobb, M. H. (1992) Extracellular signal-related kinases 2 autophosphorylates on a subset of peptides phosphorylated in intact cells in response to insulin and nerve growth factor: analysis by peptide mapping. *Molecular and Biological. Chemistry,* **3**, 299-308.

Roberson, E. D. and Sweatt, J. D. (1996) Transient activation of cyclic AMP-dependent protein kinase during hippocampal long-term potentiation. *Journal of Biological Chemistry,* **271**, 30436-30441.

Roberson, E.D., English, J.D., Adams, J.P., Selcher, J.C., Kondratick, C. and Sweatt, J.D. (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of the hippocampus. *Journal of Neuroscience,* **19**, 4337-4348.

Robinson, M.B. and Dowd, L.A. (1997) Heterogeneity and functional properties of subtypes of sodium-dependent glutamate transporter in the mammalian central nervous system. *Advanced Pharmacology,* **37**, 69-1515.

Robinson, R.C., Radziejewski, C, Stuart, D.I. and Jones, E.Y. (1995) Structure of the brain-derived neurotrophic factor/neurotrophin-3 heterodimer. *Biochemistry,* **34**, 4139-4146.

Rodriguez-Tebar, A., Dechant, G. and Barde, Y.A. (1990) Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron,* **4**, 487-492.

Rodriguez-Viciana, P., Warne, P., Hand, R.D., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D. and Downward, J. (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature*, **370**, 527-532.

Rose, G.M. and Dunwiddie, T.V. (1986) Induction of hippocampal long-term potentiation using physiologically patterned stimulation. *Neuroscience Letters*, **69**, 244-248.

Rosenblum, K., Schul, R., Meiri, N., Hadari, Y. R., Zick, Y. and Dudai, Y. (1995) Long-term potentiation increases tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit 2B in rat dentate gyrus in vivo. *Proceeding of the National Academy of Sciences, **93**, 10457-10460.*

Rostas, J. A. P., Brent, V. A., Voss, K., Errington, M. L., Bliss, T. V. P. and Gurd, J. W. (1996). Enhanced tyrosine phosphorylation of the 2B subunit of the N-methyl-D-aspartate receptor in long-term potentiation. *Proceeding of the National Academy of Sciences, **93**, 10452-10456.*

Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P.G. (1992) Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature,* **360**, 689-692.

Sandoval, M.E., Horch, P. and Cotman, C.W. (1978) Evaluation of glutamate as a hippocampal transmitter: glutamate uptake and release from synaptosomes. *Brain Research,* **142**, 285-299.

Schiaro, G., Stenbeck, G., Rothman, J.E. and Söllner, T.H. (1997) Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25 can explain docked vesicles as neurotoxin-treated synapses. *Proceedings of the National Academy of Sciences,* **94**, 997-1001.

Schinder, A. F., Berniger, B. and Poo, M-M. (2001) Postsynaptic target specificity of neurotrophin-induced presynaptic potentiation. *Neuron*, **25**, 151-163.

Schlessinger, J. (1994) SH2/SH3 signaling proteins. *Current Opinion in Genetic Development,* **4**, 25-30.

Schoepp, D.D., Tizzano, J.P., Wright, R.A., and Fix, A.S. (1995) Reversible and irreversible neuronal injury induced by intrahippocampal infusion of the mGluR agonist 1S, 3R-ACPD in the rat. *Neurodegeneration,* **4**, 71-80.

Schultz, S., Seimer, H., Krug, M. and Hollt, V. (1999) Direct evidence for biphasic cAMP response element-binding protein phosphorylation during long-term potentiation in the rat dentate gyrus in vivo. *Journal of Neuroscience,* **19**, 5683-5692.

Schuman, E.M. (1997) Synapse specificity and long-term memory storage. *Neuron,* **18**, 339-342.

Schuman, E.M. and Madison, D.V. (1991) A requirement for the intracellular messenger in long-term potentiation. *Science,* **254**, 1503-1506.

Scott, J., Selby, M, Urdea, M., Quiroga, M., Bell, G.I. and Rutter, W.J. (1983) Isolation and nucleotide sequence of cDNA encoding the precursor of mouse nerve growth factor. *Nature,* **302**, 538-540.

Segal, and Greenberg, M.E. (1996) Intracellular signaling pathways activated by neurotrophic factors. *Annual Review of Neuroscience,* **19**, 463-489.

Segal., R.A., Bhattacharyya, A., Rua, L.A., Alberta, J.A., Stephens, R.A., Kaplan, D.R. and Stiles, C.D. (1996) Differential utilization of Trk autophosphorylation sites. *Journal of Biological Chemistry,* **271**, 20175-20181.

Seger, R. and Krebs, E.G. (1995) The MAPK signaling cascade. *FASEB Journal,* **9**, 726-735.

Seidah, N.G., Benjannet, S., Pareek, S., Chretien, M. and Murphy, R.A. (1996a) Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. *FEBS Letters,* **379**, 247-250.

Seidah, N.G., Benjannet, S., Pareek, Savaria, D., Hamelin, J., Goulet, B., Laliberte, J., Lazure, C., Chretien, M. and Murphy, R.A. (1996b) Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. *Biochemical Journal,* **314**, 951-960.

Seidah, N.G., Chretien, M. and Day, R. (1994) The family of subtilisin/kexin-like proprotein and prohormone convertases: divergent or shared functions. *Biochimie,* **76**, 197-209.

Selcher, J., Atkins, C. M., Paylor, R. and Sweatt, J. D. (1999) A necessity for MAP kinase activation in mammalian spatial learning. *Learn and Memory,* **6**, 97-110.

Sequeira, S. M., Malva, J. O., Carvalho, A. P. and Carvalho, C. M. (2001) Presynaptic *N*-methyl-D-aspartate receptor activation inhibits neurotransmitter release through nitric oxide formation in rat hippocampal nerve terminals. *Molecular Brain Research,* **89** 111-118.

Shao, X., Fernandez, I., Sudhof, T. and Rizo, J. (1998) Solution structures of the Ca^{2+}-free and Ca^{2+}-bound C2A domain of synaptotagmin I: does Ca^{2+} induce a conformational change? *Biochemistry,* **37**, 16106-16115.

Shao, N., Wang, H., Zhou, T. and Liu, C. (1993) 7S nerve growth factor has different biological properties from 2.5S nerve growth factor in vitro. Brain Research, **609**,338-340.

Sheng, M., McFadden, G. and Greenberg, M.E. (1990). Membrane depolarisation and calcium induce c-fos transcription via phosphorylation of transcription CREB. *Neuron,* **4**, 571-582.

Shigemoto, R., Kinoshita, A., Wada, E., Nomura, S., Ohishi, H., Takada, M., Flor, P.J., Neki, A., Abe, T., Nakanishi, S. and Mizuno, N. (1997) Differential presynaptic localisation of metabotropic glutamate receptor subtypes in the rat hippocampus. *Journal of Neuroscience,* **17**, 7503-7522.

Siciliano, J.C., Gelman, M. and Girault, J.A. (1994) Depolarization and neurotransmitters increase neuronal protein tyrosine phosphorylation. *Journal of Neurochemistry,* **62**, 950-959.

Silva, A.J., Stevens, C.F., Tonegawa, S. and Wang, Y. (1992) Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. *Science,* **257**, 201-206.

Söllner, T, Bennett, M.K., Whiteheart, S.W., Scheller, R.H. and Rothamn, J.E. (1993) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell,* **75**, 409-418.

Son, H., Hawkins, R.D., Martin, K., Kiebler, M., Huang, Fishman, M.C. and Kandel, E.R. (1996) Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. *Cell,* **87**, 1015-1023.

Springer, J. E., Gwag, B. J. and Sessler, F. M. (1994) Neurotrophic factor mRNA expression in dentate gyrus is increased following in vivo stimulation of the angular bundle. *Molecular Brain Research,* **23**, 135-143.

Squire, L.R. and Zola-Morgan, S. (1991) The medial temporal lobe memory system. *Science,* **253**, 1380-1386.

Stanton, P.K. and Sarvey, J.M. (1984) Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis. *Journal of Neuroscience,* **4**, 3080-3088.

Stephens, R.D., Loeb, D., Copeland, T., Pawson, T., Greene, L. and Kaplan, D. (1994) Trk receptors use redundant signal transduction pathways involving Shc and PLC gamma 1 to mediate NGF responses. *Neuron,* **12**, 691-705.

Stevens, C.F. and Wang, Y. (1993) Reversal of long-term potentiation by inhibitors of haem oxygenase. *Nature*, **364**, 147-149.

Steward, O. and Scoville, S.A. (1976) Cells of origin of the entorhinal cortical afferents to the hippocampus and fascia dentata of the rat. *Journal of Comp Neurology,* **114**, 181-200.

Stoop, R. and Poo, M.M. (1996) Synaptic modulation by neurotrophic factors: differential and synergistic effects of brain-derived neurotrophic factor and ciliary neurotrophic factor. *Journal of Neuroscience,* **16**, 3256-3264.

Storm-Mathisen, J. and Ottersen, O. P. (1987) Tracing of neurons with glutamate or gamma-aminobutyrate as putative transmitters. *Biochem. Soc. Trans.* **15**, 210-213.

Stricker, C., Field, A. C. and Redman, S. J. (1996) Changes in quantal parameters of EPSCs in rat CA1 neurones in vitro after the induction of long-term potentiation. *Journal of Physiology (London)* **490**, 43-54.

Struhl, K. (1998) Histone acetylation and transcriptional regulatory mechanisms. *Genes Development,* **12**, 599-606.

Suen, P-C., Wu, K., Levine, E.S., Mount, H.T.J., Xu, J-L, Lin, S-Y., Black, I.B. (1997) Brain-derived neurotrophic factor rapidly enhances phosphorylation of the postsynaptic *N*-methyl-*D*-aspartate receptor subunit 1, *Proceedings of the National Academy of Sciences*, **94**, 8191-8195.

Sutton, R.B., Ernst, J.A. and Brunger, A.T. (1999) Crystal structure of the cytosolic C2AC2B domains of synaptotagmin III. Implications for Ca^{2+}-independent SNARE complex interaction. *Journal of Cellular Biology,* **147**, 589-598.

Takahashi, M., Arimatsu, Y., Fujita, S., Fujimoto, Y, Kondo, Y., Hama, T. and Miyamoto, E. (1991) Protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II phosphorylate a novel 58-kDa protein in synaptic vesicles. *Brain Research,* **551**, 279-292.

Tan, S.E., Wenthold, R.J. and Soderling, T.R. (1994) Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. *Journal of Neuroscience,* **14**, 1123-1129.

Tancredi, V., D'Arcangelo, G., Mercanti, D. and Calissano, P. (1995) Nerve growth factor inhibits the expression of long-term potentiation in hippocampal slices. *NeuroReport,* **4**, 147-150.

Tessarollo, L., Tsoulfas, P., Martin-Zanka, D., Gilbert, D. J., Jenkins, N. A., Copeland, N.G. and Parada, L.F. (1993) trkC, a receptor for neurotrophin-3, is widely expressed in the developing nervous system and in non-neuronal tissues. *Development,* **118**, 463-475.

Teyler, W.J. and Pozzo-Miller, L. (2001) BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. *Journal of Neuroscience*, **21**, 4249-4258.

Thoenen, H. (1995) Neurotrophins and Neuronal Plasticity. *Science,* **270**, 593-598.

Thomas, K.L., Laroche, S., Errington, M.L., Bliss, T.V.P. and Hunt, S.P. (1994) Spatial and temporal changes in signal transduction pathways during LTP. *Neuron,* **13**, 737-746.

Thomas, L. and Betz, H. (1990) Synaptophysin binds to physophillin, a putative synaptic plasma membrane protein. *Journal of Biological Chemistry,* **111**, 2041-2052.

Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S. and Brugge, J. S. (1992) ras is essential for nerve growth factor and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell*, **68**, 1031-1040.

Tingley, W. G., Roche, K. W., Thompson, A. K. and Huganir, R. L. (1993) Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. *Nature*, **364**, 70-73.

Tokuyama, W., Hashimoto, T., Li, Y. X., Okuno, H. and Miyashita, Y. (1999) Quantification of neurotrophin-3 mRNA in the rat hippocampal subregions using the RT-PCR-based coamplification method. *Brain Research Protocols*, **4**, 407-414.

Toledo-Aral, J.J., Brehm, P., Halegoua, S. and Madel, G. (1995) A single pulse of nerve growth factor triggers long-term neuronal excitability through sodium channel gene induction. *Neuron,* **14**, 607-611.

Toni, N., Buchs, P-A., Nikonenko, I., Bron, C.R. and Muller, D. (1999) LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. *Nature,* **402**, 421-425.

Treisman, R. (1996) Regulation of transcription by MAP kinase cascades. *Current Opinion in Cell Biology,* **8**, 205-215.

Turner, R.W., Baimbridge, K.G. and Miller, J.J. (1982) Calcium-induced long-term potentiation in the hippocampus. *Neuroscience*, 7, 1441-1416.

Urfer, R., Tsoulfas, P., Soppet, D., Escandón, E., Parada, L. F., and Presta, L.G. (1994) The binding epitopes of neurotrophin-3 to its receptors trkC and gp75 and the design of a multifunctional human neurotrophin. *EMBO Journal,* **13**, 5896-5909.

Vanhoutte, P., Barnier, J-V., Guibert, B., Pages, C., Besson, M-J, Hipskind, R.A. and Caboche, J. (1999) Glutamate induces phosphorylation of Elk-1 and CREB, along with c-fos activation, via extracellular signal-regulated kinase-dependent pathway in brain slices. *Molecular and Cellular Biology,* **19**, 136-146.

Varon, S., Nomura, J., and Shooter, E.M. (1967a) Subunit structure of a high-molecular weight form of nerve growth factor from mouse submaxillary gland. *Proceedings of the National Academy of Sciences,* **57**, 1782-1789.

Varon, S., Nomura, J., and Shooter, E.M. (1967b) The isolation of the mouse nerve growth factor protein is a high-molecular weight form. *Biochemistry*, **6**, 2202-2209.

Verhratsky, A. and Toescu, E. C. (1998) Calcium and Neuronal Ageing. *Trends in Neuroscience,* **21**, 2-7.

Vetter, M.L., Martin-Zanca, D., Parada, L.F., Bishop, J.M. and Kaplan, D.R. (1991) Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-gamma 1 by a kinase activity associated with the product of the trk protooncogene. *Proceedings of the National Academy of Sciences,* **88**, 5650-5654.

Vicario-Abejón, C., Collin, C., McKay, R. D. G. and Segal, M. (1998) Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. *Journal of Neuroscience,* **18**, 7256-7271.

Walker, D and De Waard, M. (1998) Subunit interaction sites in voltage-dependent Ca^{2+} channels: role in channel fucntion. *Trends in Biological Sciences,* **21**, 148-154.

Wang, X.H. and Poo, M.M. (1997) Potentiation of developing synapses by postsynaptic release of neurotrophin-4. *Neuron,* **19**, 825-835.

Wang, Y. T. and Salter, M. W. (1994) Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature,* **369**, 233-235.

Wenzel, J., Desmond, N.L. and Levy,W.B., (1993) Somatic ribosomal changes induced by long-term potentiation of the perforant path-hippocampal CA1synapses. *Brain Research,* **619**, 331-333.

Wetmore, C., Ernfors, P., Persson, H. and Olson, L. (1990) Localisation of brain-derived neurotrophic factor mRNA to neurons in the brain by in situ hybridization. *Experimental Neurology,* **109**, 141-152.

Widmer, H. R., Kaplan, D. R., Rabin, S. J., Beck, K. D., Hefti, F. and Knüsel, B. (1993) Rapid phosphorylation of phospholipase cγ1 by brain-derived neurotrophic factor and neurotrophin-3 in cultures of embryonic rat cortical neurons. *Journal of Neurochemistry*, **60**, 2111-2123.

Wieraszko, A., Li, G., Kornecki, E., Hogan, M. V. and Ehrlich, Y. H. (1993) Long-term potentiation in the hippocampus induced by platelet-activating factor. *Neuron,* **10**, 553-557.

Wijetunge, S., Lymn, J.S. and Hughes, A.D. (2000) Effects of protein tyrosine kinase inhibitors on voltage-operated calcium currents in vascular smooth muscle cells and pp60(c-src) kinase activity. *British Journal of Pharmacology,* **129**, 1347-1354.

Williams, J. H. and Bliss, T. V. P. (1989) An in vitro study of the effect of lipoxygenase and cyclo-oxygenase inhibitors of arachidonic acid on the induction and maintenance of long-term potentiation in the hippocampus. *Neuroscience Letters,* **107**, 301-306.

Williams, R., Bäckström, A. and Kullander, K. (1995) Developmentally regulated expression of mRNA for neurotrophin high-affinity (trk) receptors within chick trigeminal sensory neurons. *European Journal of Neuroscience*, 7, 116-128.

Wood, K. W., Sarnecki, C., Roberts, T. M., and Blenis, J. (1992) ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. *Cell*, **68**, 1041-1050.

Woodrow, S., Bissoon, N. and Gurd, J.W. (1992) Depolarization-dependent tyrosine phosphorylation in rat brain synaptosomes. *Journal of Neurochemistry,* **59**, 857-862.

Xia, Z., Dudek, H., Miranti, C. K. and Greenberg, M. E. (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. *Journal of Neuroscience,* **16**, 5425-5436.

Xie, K., Wang, T., Olafsson, P., Mizuno, K. and Lu, B. (1997) Activity-dependent expression of NT-3 in muscle cells in culture: implications in the development of neuromuscular junctions. *Journal of Neuroscience,* **17**, 2947-2958.

Xing, J., Ginty, D. D. and Greenberg, M. E. (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB Kinase. *Science*, **273**, 959-963.

Yan , Q., Radeke, M.J., Matheson, C.R., Talvenheimo, J., Welcher, A.A., and Einstein, S.C. (1997) Immunocytochemical localisation of TrkB in the central nervous system in the adult rat. *Journal of Comparative Neurology,* **378**, 546-471.

Yang, F., He, X., Feng, L., Mizuno, K., Liu, X., Russell, J., Xiong, W. and Lu, B. (2001) PI-3 kinase and IP3 are both necessary and sufficient to mediate NT3-induced synaptic potentiation. *Nature Neuroscience*, **4**, 19-28.

Yazejian, B., Sun, X.P. and Grinnell, A.D. (2000) Tracking presynaptic Ca^{2+} dynamics during neurotransmitter release with Ca^{2+}-activated K^{+} channels. *Nature Neuroscience*, 566-571.

Yoon, S. O., Casaccia-Bonefil, P., Carter, B. and Chao, M. V. (1998) Competitive signalling between TrkA and P75 nerve growth receptors determines cell survival. *Journal of Neuroscience*, **18**, 3273-3281.

Yu, X-M., Askalan, R., Kell, G.J., Salter, M.W. (1997) NMDA channel regulation by channel-associated protein tyrosine kinase src. *Science,* **275**, 674-678.

Young, M., Blanchard, M.H., Sessions, F. and Boyle, M.D. (1998) Subunit structure of high molecular weight mouse nerve gowth factor. Biochemistry, **27**, 6675-6681.

Yuen, E. C. and Mobley, W. C. (1999) Early BDNF, NT-3, and NT-4 signaling events. *Experimental Neurology*, **159**, 297-308.

Zafra, F., Hengerer, B., Leibrock, J., Thoenen, H., and Lindholm, D. (1990) Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. *EMBO Journal*, **9**, 3545-3550.

Zhao, W., Cavallaro, S., Gusev, P. and Alkon, D. L. (2000) Nonreceptor tyrosine protein kinase pp60c-src in spatial learning: synapse-specific changes in its gene expression, tyrosine phosphorylation, and protein-protein interactions. *Proceeding of the National Academy of Sciences,* **97**, 8098-8103.

Zhou, A., Webb, G., Zhu, X. and Steiner, D. F. (1999) Proteolytic processing in the secretory pathway. *Journal of Biological Chemistry*. **274**, 5812-5818.

Zhou, X-F. and Rush, R.A. (1994) Localisation of neurotrophin-3-like immunoreactivity in the rat central nervous system. *Brain Research,* **643**, 162-172.

IX Appendix I Solutions used

Krebs solution

NaCl, 136mM

KCl, 2.54mM

KH_2PO_4, 1.18mM

$MgSO_4.7H_2O$, 1.18mM

$NaHCO_3$, 16mM

Glucose, 10mM

Containing $CaCl_2$

Incubation buffer for $^{45}Ca^{2+}$ experiments

NaCl, 128mM

KCl, 4.8mM

$CaCl_2$, 1.3mM

$MgSO_4$, 1.2mM

$NaHCO_3$, 7.5mM

Glucose, 11mM

Ascorbic acid, 0.1mM

HEPES, 15mM

Disodium EDTA, 0.03mM

Stop buffer for $^{45}Ca^{2+}$ experiments

NaCl, 118mM

KCl, 4.8mM

$CaCl_2$, 1.3mM

$MgSO_4$, 1.2mM

KH_2PO_4, 1.2mM

$NaHCO_3$, 26mM

Glucose, 11mM

Oubain, 10μM

Separating gel

Acrylamide/Bisacrylamide (30%stock) 33% v/v

Tris-HCl, 1.5M, pH 8.8

SDS, 1% W/v

Ammonium persulphate, 0.5% w/v

Distilled water

Temed, 0.1% v/v

Stacking gel

Acrylamide/Bisacrylamide (30%stock) 6.5% v/v

Tris-HCl, 0.5M, pH 6.8

SDS, 1% W/v

Ammonium persulphate, 0.5% w/v

Distilled water

Temed, 0.1% v/v

Electrode running buffer

Tris base, 25mM

Glycine, 200mM

SDS, 17mM

Phosphate-buffered saline (PBS), pH 7.4

Na_2HPO_4, 80mM

NaH_2PO_4, 20mM

NaCl, 100mM

PBS-Tween (PBS-T)

A 0.1% Tween-20 solution in PBS.

Tris-buffered saline (TBS) pH 7.6

TBS-Tween (TBS-T)

A 0.1% Tween-20 solution in TBS.

Transfer buffer, pH 8.3

Tris base, 25mM

Glycine, 192mM

Methanol, 20%, v/v

SDS, 0.05% w/v

Distilled water

Sample buffer

Tris-HCl, 0.5M, pH 6.8

Glycerol, 10% v.v

SDS, 0.05% w/v

ß-mercaptoethanol, 5% v/v

Bromophenol blue, 0.05% w/v

Immunoprecipitation buffer

Triton x-100, 1.25% v/v

NaCl, 1910mM

Tris-HCl, 60mM, pH 7.4

EDTA, 6mM, pH 8.0

Aprotonin, 10U/ml

Preparation of acrylamide

Acrylamide (29.2g) was added to N'N'Bis-methylene-acrylamide (0.8g) and dissolved in 100ml of distilled water. This solution was filtered and stored in the dark at 4°C for a maximum of one month.

Appendix II

Alomome

Alomone Labs,
Shatner Center 3,
P.O. Box 4287,
Jerusalem 91042,
Israel.

Amersham

Amersham International plc.,
Lincoln Place,
Green End,
Aylesbury,
Buckinghamshire,
HP202TP, U.K.

Calbiochem

Calbiochem European Distribution Centre,
Boulevard Industrial Park,
Nottingham,
NG9 1BR, U.K.

Lennox

Lennox Lab Supplies,
John F. Kennedy Drive,
Naas Road,
Dublin 12, Ireland.

Pierce
P.O. Box 117,
Rockford,
IL 61105, USA.

3747, N. Meridian Road,

Promega	Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA.
Roche Molecular Biochemicals	Roche Diagnostic GmbH, Sandhoferstrasse 116, 68305 Mannheim, Germany.
Sartorius	Sartorius, 5 Sussex Street, Dun Laoghaire, Co. Dublin, Ireland.
Sigma	Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, U.K.
Tocris Neuramin	Tocris Neuramin 14 Charlotte Street, Bristol, B51 5PP, U.K.
Whatman	Whatman International Ltd., Maidstone, Kent, U.K.

X Publications

Casey, M., Maguire, C., Kelly, A., Gooney, M.A. and Lynch. M.A. (2001) Analysis of the presynaptic mechanism underlying the inhibition of LTP in the rat dentate gyrus by the tyrosine kinase inhibitor, genistein. *Hippocampus* (in press)

Casey, M and Lynch, M.A. (2000) The role of ERK and Calcium channel phosphorylation in LTP. Society for Neuroscience Abstracts

Analysis of the presynaptic signalling mechanisms underlying the inhibition of LTP in rat dentate gyrus by the tyrosine kinase inhibitor, genistein.

M. Casey, C. Maguire, Á. Kelly, M.A Gooney and M.A. Lynch[+]
Physiology Department,
Trinity College,
Dublin 2.
Ireland

Running title: Tyrosine kinase and LTP in dentate gyrus
No. text pages: 30
No. figures: 5
No. tables: 0

[+] **Corresponding author**
Telephone: (353)-1-608 1770
Fax: (353)-679 3545
Email: lynchma@tcd.ie
Grant Sponsor: Health Research Board (Ireland) and Forbairt (Ireland)
Keywords: glutamate release, calcium influx, ERK, CREB, calcium channel α-subunit

ABSTRACT

A great deal of recent evidence points to a role for tyrosine kinase in expression of LTP and data has been presented which is consistent with the idea that tyrosine phosphorylation of proteins occurs in both the presynaptic and postsynaptic areas. In this study we set out to investigate the role that tyrosine kinase might play presynaptically to modulate release of glutamate in an effort to understand the mechanism underlying the persistent increase in release which accompanies LTP in perforant path- granule cell synapses. We report that LTP was associated with increased calcium influx and glutamate release. LTP was also associated with an increase in phosphorylation of the α-subunit of calcium channels and ERK in synaptosomes prepared from dentate gyrus and these effects were inhibited when LTP was blocked by the tyrosine kinase inhibitor, genistein. LTP was accompanied by increased protein synthesis and increased phosphorylation of CREB in entorhinal cortex, effects which were also blocked by genistein. We conclude that tetanic stimulation leads to enhanced tyrosine phosphorylation of certain presynaptically-located proteins which modulate glutamate release and contribute to expression of LTP.

INTRODUCTION

Among the changes which accompanies long-term potentiation in dentate gyrus is an increase in glutamate release which is inhibited when LTP is blocked, for example by the NMDA receptor inhibitor, AP5 (Errington et al., 1987), the *Trk* inhibitor, tyrphostin AG879 (Maguire et al., 1999), the ERK inhibitor, PD 98059 (McGahon et al., 1999), or when LTP is impaired, for example in aged rats (Murray and Lynch, 1998). Thus there is a tight coupling between LTP and increased glutamate release pointing to presynaptic involvement in LTP in perforant path-granule cell synapses.

Several proteins kinases contribute to expression of LTP (see Bliss and Collingridge, 1993) and there is now convincing evidence that tyrosine kinase also plays a role. Consistent with this is the finding that tyrosine kinase inhibitors block LTP (O'Dell et al., 1991; Abe and Saito, 1993; McGahon and Lynch, 1998; Lu et al., 1998; Maguire et al., 1999; Huang and Hsu, 1999), while LTP is accompanied by increased tyrosine phosphorylation of synaptophysin (Mullany and Lynch, 1998), PLCγ (McGahon and Lynch, 1998) and the 2B subunit of the NMDA receptor (Rosenblum et al., 1996; Rostas et al., 1996). A role for at least two non-receptor tyrosine kinases, *fyn* (Grant et al., 1992; Kojima et al., 1997) and *src* (Lu et al., 1998; Huang and Hsu, 1999) has been documented in LTP, while receptor tyrosine kinases have also been shown to be involved (Maguire et al., 1999; Kang and Schuman, 1995; Messaoudi et al., 1998).

An essential role for protein synthesis in maintenance of the long-lasting components of LTP has been identified by several laboratories (Charriaut-Marlangue et al., 1988; Otani et al., 1989; Fazeli et al., 1993; Mullany and Lynch, 1997), but the trigger leading to the appropriate changes remains to be identified. One transcription factor, cAMP response

element binding protein (CREB), is a candidate trigger since it appears to mediate in the transduction of neuronal stimulation into gene expression (Ginty, 1997). Thus morphological changes in the spine (Murphy and Segal, 1997), late-phase LTP (Nguyen and Kandel., 1996) and BDNF-induced transcription (Finkbeiner et al., 1997), all of which require protein synthesis, appear to rely on increased CREB phosphorylation. CREB phosphorylation also plays a role in LTP, perhaps as a consequence of increased activation of the mitogen-activated protein kinase, ERK (English and Sweatt, 1997; McGahon and Lynch, 1998; Maguire et al., 1999; Davis et al., 2000) as shown directly (e.g. Impey et al., 1996; Davies et al., 2000) and by the observation that LTP is impaired in CREB mutant mice (Bourtchuladze et al., 1994).

In this study we focussed on changes which occur presynaptically after tetanization following treatment with saline or the tyrosine kinase inhibitor, genistein. We report that genistein inhibited expression of LTP in dentate gyrus and also the LTP-associated increases in glutamate release and calcium influx. Our findings indicate that LTP was associated with increased phosphorylation of ERK and the α-subunit of calcium channels in synaptosomes prepared from dentate gyrus, both of which are likely to enhance glutamate release, and increased phosphorylation of CREB and protein synthesis in entorhinal cortex.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (250-350g) were used in these experiments; rats were obtained from the BioResources Unit, Trinity College Dublin, where they were maintained under veterinary supervision at an ambient temperature of between 22 and 23°C, under a 12-hour light schedule.

The minimum number of rats was used compatible with statistical analysis and every effort was made to minimize discomfort. All experimentation was conducted as required by local regulations and conformed to Department of Health and Children (Ireland) and international guidelines on the ethical use of animals.

Induction of LTP in vivo

Rats were anaesthetized by intraperitoneal injection of urethane (1.5g/kg i.p) and LTP was induced as described previously (McGahon and Lynch, 1998). Briefly, a bipolar stimulating electrode was placed in the perforant path (4.4mm lateral to Lambda) and a unipolar recording electrode in the dorsal cell body region of the dentate gyrus (2.5mm lateral and 3.9mm posterior to Bregma). Test shocks were given at 30 sec intervals for 10 min before, and 40 min after, tetanic stimulation (3 trains of stimuli delivered at 30 sec intervals; 250 Hz for 200 msec). In some experiments, genistein (250μM; 5μl; Calbiochem, UK) or saline (5μl) was injected intracerebroventricularly (0.4mm posterior to Bregma; 0.2mm lateral to midline; 3.5mm depth) 30 min before recording commenced and the experiment proceeded as described. In a separate series of experiments, the effect of delivering the same total number of stimuli without the high frequency train was assessed; in this case stimuli were delivered at a rate of 1 stimulus/12sec. At the end of the recording period, rats were killed by cervical dislocation, cross-chopped slices (350μm) were prepared from ipsilateral and contralateral dentate gyri and ipsilateral and contralateral entorhinal cortices. These samples were separately frozen in Krebs solution containing 10% DMSO (Haan and Bowen, 1981). Samples were stored at -80°C and for analysis, slices were thawed rapidly and rinsed in fresh oxygenated Krebs solution before preparation of tissue for analysis.

Release of glutamate

Release was assessed in samples of P_2 prepared from untetanized and tetanized slices which were frozen as described above, or in freshly prepared P_2 according to a method described previously (McGahon and Lynch, 1998). In both cases, samples of synaptosomal tissue were resuspended in ice-cold Krebs solution containing 2mM $CaCl_2$, aliquotted onto Millipore filters (0.45μm) and rinsed under vacuum. Tissue was incubated in 250μl oxygenated Krebs solution at 37°C for 3min and filtrate was collected and stored. Release of transmitter was stimulated by the addition of 40mM KCl to Krebs solution. In some cases, genistein (50μM) or PD98059 (2μM) was added to assess its effect on KCl-stimulated release *in vitro*. In these experiments synaptosomes were preincubated (15 min at 37°C) in Krebs solution containing 2mM $CaCl_2$ with added genistein or PD98059.

Glutamate was analysed as described previously (Ordronneau et al., 1991). Glutaraldehyde (0.5% in 100mM NaH_2PO_4 buffer, pH 4.5; 320μl) was added to 96-well plates, incubated for 60 min at 37°C, and washed with 100mM NaH_2PO_4 buffer. Triplicate samples (50μl) or glutamate standards (50μl; 50nM to 10μM prepared in 100mM Na_2HPO_4 buffer, pH 8.0) were added, incubated for 2 hours at 37°C and washed. Ethanolamine (320μl; 0.1M in 100mM Na_2HPO_4 buffer) was added to bind any unreacted aldehydes and donkey serum (200μl; 3% in phosphate-buffered saline containing Tween-20 (0.5%; PBS-T)) was used to block non-specific binding. Antiglutamate antibody (raised in rabbit; 100μl; 1:5,000 in PBS-T; Sigma, UK) was added, incubated overnight at 4°C and washed with PBS-T. Anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (95μl; 1:10,000 in PBS-T; Amersham, UK) was added, incubated for 60 min at room temperature and washed. 3,3',5,5'-Tetramethylbenzidine liquid

substrate was added as chromogen and incubation continued for exactly 60 min at room temperature. H_2SO_4 (4M; 50µl) was added to stop the reaction and optical densities were determined at 450nm. Values were calculated with reference to the standard curve, corrected for protein (Bradford, 1976) and expressed as µmol glutamate/mg protein.

Assessment of ^{45}Ca influx

^{45}Ca influx was assessed in samples of P_2 prepared from untetanized and tetanized slices of dentate gyrus obtained from saline- and genistein-treated rats by a method described previously (Kelly and Lynch, 2000). P_2 was resuspended in oxygenated ice-cold incubation buffer (composition in mM: NaCl, 128; KCl 4.8; KH_2PO_4, 1.2; $MgSO_4.7H_2O$, 1.2; $NaHCO_3$, 7.5; $CaCl_2$, 1.3; glucose 11; ascorbic acid, 0.1; HEPES, 15; disodium EDTA, 0.3) and incubated for 5 sec at 37°C in buffer containing ^{45}Ca (final concentration 1µCi/ml; Specific activity, 2.1 mCi/ml; Amersham, UK) ± KCl). In some experiments, genistein (50µM) was added during incubation. Reactions were stopped by addition of 1 ml ice-cold 'stop' buffer (composition in mM: NaCl, 118; KCl 4.8; KH_2PO_4, 1.2; $MgSO_4.7H_2O$, 1.2; $NaHCO_3$, 26; $CaCl_2$, 1.3; glucose, 11; ouabain, 10mM). Samples were rinsed in a filtration manifold and filters added to scintillation fluid for assessment of radioactivity. Data were expressed as nmol $^{45}Ca^{2+}$/mg protein.

Analysis of tyrosine kinase activity

Tyrosine kinase activity was assessed in P_2 prepared from untetanized and tetanized tissue obtained from saline-and genistein-treated rats by ELISA using a non-radioactive tyrosine kinase kit (Roche Molecular Biochemicals, UK). Briefly P_2 was resuspended in Krebs solution containing 2mM $CaCl_2$ and the tyrosine phosphatase inhibitor, sodium orthovanadate (10mM) and aliquots were added to the assay solution to start the reaction. The assay solution consisted

of peptide substrate (10µl; 5µM reconstituted in PBS-BSA (1mg/ml), assay buffer (10µl; Tris HCl, 50mM; pH 7.5; $C_4H_6O_4Mg{\cdot}4H_2O$, 20mM; NaF, 5mM; EDTA, 0.2mM; EGTA, 0.8mM; dithiothreitol, 1mM; sodium orthovanadate, 30µM; ATP, 1mM), ATP-Mg^{2+} solution (10µl; ATP, 5mM; $MgCl_2$, 50mM, pH 7) and distilled water (10µl). Samples were incubated for 1h at room temperature. The reaction was stopped by addition of the tyrosine kinase inhibitor, piceatannol (10µl; 3mM in 10% DMSO). Samples were placed on ice to reduce the temperature to 4°C and centrifuged at 10,000g for 1min, aliquots (50µl) were added to streptavidin-coated 96-well plates, incubated for 20 min at 37°C and washed. Peroxidase-labelled antiphosphotyrosine antibody (75µl; 1:100 in PBS/BSA 1mg/ml) was added, samples were incubated for 1h at 37°C and washed. The manufacturer's substrate was added as chromogen and incubation continued for 45 min at room temperature to allow maximum colour development and optical densities were determined at 405nm. Values were calculated with reference to a standard curve, corrected for protein (Bradford, 1976) and expressed as picomoles tyrosine kinase/mg protein.

Analysis of calcium channel α-subunit phosphorylation

P_2 was prepared from untetanized and tetanized sides of dentate gyri obtained from saline-treated and genistein-treated rats. Samples were equalized for protein, immunecomplexes were prepared by incubating tissue for immunobuffer (155µl; TritonX-100, 1.25%; NaCl, 1.9mM; Tris-HCl, 60mM, pH 7.4; EDTA, 6µM, pH 8; aprotonin, 10U/ml) for 1h at 37°C, in the presence of an antibody raised against the α1-subunit of voltage-gated calcium channels (anti-pan α1-subunit, Alomone Labs, Israel; 1:25). Immune complexes were separated on 7.5% SDS gels and transferred onto nitrocellulose strips

(225mA for 2h) and reacted with antiphosphotyrosine (1:160 in 2% Tris-buffered saline-Tween-20 (0.1% Tween; TBS-T) Affiniti, UK) overnight at 4°C. Nitrocellulose strips were washed and incubated for 2h at room temperature with secondary antibody (HRP-linked anti-rabbit antibody; 1:2,000 dilution; Amersham, UK) and protein complexes were visualized by ECL detection (Amersham, UK). Immunoblots were exposed to film overnight and processed using a Fuji X-ray processor. Protein bands were quantitated by densitometric analysis.

Analysis of ERK phosphorylation

P_2 was prepared from untetanized and tetanized sides of dentate gyri obtained from saline-treated and genistein-treated rats. by a method described previously (McGahon and Lynch, 1998). Samples were analysed for protein, diluted to equalize for protein concentration and aliquots (10µl, 1mg/ml) were added to 10µl sample buffer (Tris-HCl, 0.5mM, pH 6.8; glycerol 10%; SDS, 10%; β-mercaptoethanol, 5%; bromophenol blue, 0.05% w/v), boiled for 2 min and loaded onto gels (10% SDS). Proteins were separated by application of 30mA constant current for 25-30 min, transferred onto nitrocellulose strips (225mA for 75 min) and immunoblotted with anti-active ERK (Promega, USA; 1.5:1,000 in TBS-T) containing 2% non-fat dried milk) overnight at 4°C. Nitrocellulose strips were washed and incubated for 60 min at room temperature with secondary antibody (HRP-linked anti-rabbit antibody; 1:2,000; Amersham, UK) and protein complexes were visualized by ECL detection (Amersham, UK). Immunoblots were exposed to film overnight and processed using a Fuji X-ray processor. Protein bands were quantitated by densitometric analysis.

Assessment of protein synthesis: [^{35}S]-Methionine labelling

Slices of entorhinal cortex were thawed rapidly (1.5-2 min) by agitation at 37°C and rinsed 4 times in excess fresh Krebs solution. The method used for analysis of protein synthesis has been

described previously (Mullany and Lynch, 1997). Briefly, slices were preincubated at 37°C for 10 min in 500µl oxygenated Krebs solution containing $CaCl_2$ (2mM), resuspended in oxygenated Krebs solution containing $CaCl_2$ (2mM), ATP (3.5mM) and [^{35}S]-methionine (specific activity, 37TBq/mmol; 0.2µl/ml) and incubated for 60 min at 37°C. At the end of the incubation period, samples were placed on ice, added to TCA (50µl; final concentration, 10%), homogenized and added to Millipore filters (pore size 0.45µm) and filtered under vacuum. Samples were washed at least 10 times by addition of ice-cold 5%TCA in H_2O (200µl). Filter papers were added to scintillant and counted for 1.5 min. Values were expressed as cpm/mg protein.

Analysis of CREB phosphorylation

Slices prepared from untetanized and tetanized sides of entorhinal cortex of saline-treated and genistein-treated rats were homogenized in Krebs solution containing 2mM CaCl2. Samples were analysed for protein, diluted to equalize for protein concentration and aliquots (10µl, 1mg/ml) were added to 10µl sample buffer (Tris-HCl, 0.5mM, pH 6.8; glycerol 10%; SDS, 10%; β-mercaptoethanol, 5%; bromophenol blue, 0.05% w/v), boiled for 10 min and loaded onto gels (14% SDS). Proteins were separated by application of 36mA constant current for 25-30 min, transferred onto nitrocellulose strips (225mA for 75 min) and immunoblotted with anti-phospho-CREB (New England BioLabs, UK; 1:1,000 in 5%BSA in Tris buffered saline-Tween (0.1% Tween-20) containing 5% BSA) overnight at 4°C. Nitrocellulose strips were washed and incubated for 60 min at room temperature with secondary antibody (HRP-linked anti-rabbit antibody; 1:2,500; Amersham, UK) and protein complexes were visualized by ECL detection (Amersham, UK). Immunoblots were exposed to film overnight and processed using a Fuji X-ray processor. Protein bands were quantitated by densitometric analysis.

Statistical analysis

A one-way analysis of variance (ANOVA) was performed to determine whether there were significant differences between conditions. When this analysis indicated significance (at the 0.05 level), the post hoc student Newmann-Keuls test analysis was used to determine which conditions were significantly different from each other. In some cases the Student's t-test for was used to establish statistical significance.

RESULTS

As a first step in elucidating the role of tyrosine kinase in modulating transmitter release, we assessed the effect of a depolarizing pulse of KCl (40mM) on $^{45}Ca^{2+}$ influx and glutamate release (Figure 1). KCl significantly enhanced $^{45}Ca^{2+}$ influx (Figure 1A) and glutamate release (Figure 1B; $p< 0.05$ in each case; student's t-test for paired values), but both KCl-induced responses were inhibited by incubation in the presence of genistein (50μM).

Synaptosomes were prepared from untetanized dentate gyrus and dentate gyrus which had sustained LTP following tetanic stimulation (Figure 2A). The data indicate that addition of 40mM KCl to the incubation medium enhanced both $^{45}Ca^{2+}$ influx (Figure 2B) and glutamate release (Figure 2C) to a more marked degree in synaptosomes which had been prepared from tetanized dentate gyrus ($p < 0.01$; student's t-test for paired values), compared with untetanized tissue ($p< 0.05$ in each case; student's t-test for paired values). Comparison of the data indicated that KCl-stimulated glutamate release was significantly increased in tetanized, compared with untetanized, tissue ($p < 0.05$; student's t-test for unpaired means), but unstimulated glutamate release was not altered by tetanic stimulation.

Comparison of KCl-stimulated $^{45}Ca^{2+}$ influx in tetanized and untetanized tissue revealed no statistical significance, although an LTP-associated decrease in unstimulated $^{45}Ca^{2+}$ influx was observed ($p < 0.05$; student's t-test for unpaired means). We observed that addition of KCl (40mM) to the incubation medium increased glutamate release to a similar extent in synaptosomes prepared from dentate gyrus of rats which received low frequency stimulation and synaptosomes prepared from unstimulated tissue (Figure 3B). In these rats, the mean percentage change in the epsp slope in the last 5 min of the experiment, compared with the mean value in the time period which corresponded to the pre-tetanus period in the group of rats which received high frequency stimulation, was 97.5 (± 3.43, SEM; Figure 3A).

The mean population epsp slope in the 10 min prior to tetanic stimulation was 1.25mV/msec (± 0.13) in saline-treated rats; intracerebroventricular injection of genistein resulted in a decrease in mean epsp slope to 0.85mV/msec (± 0.26). The data also show that intracerebroventricular injection of genistein (250μM) inhibited expression of LTP in dentate gyrus (Figure 4A); the mean percentage increases in population epsp slope in the 2 min immediately following tetanic stimulation, compared with the mean value in the 5 min immediately prior to tetanic stimulation were 143.73 (± 5.76, SEM) and 115.40 (± 2.74) in saline- and genistein-pretreated rats respectively. The corresponding mean values in the last 5 min of the experiment were 130.08 (± 1.58) and 107.87 (± 2.11). A total of 12 saline-treated and 12 genistein-treated rats were analysed. At the end of the recording period, untetanized and tetanized dentate gyri were retained for subsequent preparation of synaptosomes for analysis; tissue from at least 6 rats was used in each assay.

Figure 4B indicates that incubation of tissue in the presence of KCl increased tyrosine kinase activity in untetanized and tetanized tissue; this effect was statistically significant in

synaptosomes prepared from dentate gyrus which had sustained LTP ($p < 0.05$; student's t-test for paired values) but, although the increase was of a similar order in synaptosomes prepared from untetanized dentate gyrus, the increase was not statistically significant due to the very large inter-sample variability. Pretreatment with genistein prevented the KCl-stimulated release in tetanized samples and Figure 4C demonstrates that while KCl significantly increased glutamate release in synaptosomes prepared from untetanized dentate gyrus of saline-pretreated rats ($p < 0.05$; ANOVA), release was enhanced to a greater extent in synaptosomes prepared from tetanized dentate gyrus ($p < 0.01$; ANOVA). Both unstimulated and KCl-stimulated glutamate release were decreased in synaptosomes prepared from dentate gyrus of genistein-pretreated rats, and genistein pretreatment also inhibited the stimulatory effect of KCl on glutamate release; similar data were obtained in both untetanized and tetanized tissue.

Figure 5A shows one sample immunoblot which indicates that tyrosine phosphorylation of the calcium channel α-subunit was increased in tetanized tissue prepared from saline-treated, but not genistein-treated, rats. Densitometric analysis of the individual experiments allowed assessment of the mean changes and showed that tyrosine phosphorylation of the calcium channel α-subunit was significantly enhanced in synaptosomes prepared from tetanized, compared with untetanized, dentate gyrus of saline-treated rats ($p < 0.05$; ANOVA). This LTP-associated increase in phosphorylation was inhibited by genistein pretreatment (Figure 5A).

While ERK phosphorylation was not affected by low frequency stimulation as shown by the sample immunoblot and by the mean data obtained following densitometric analysis (Figure 3C), Figure 5B shows one sample immunoblot which demonstrates that ERK

entorhinal cortex were affected by low frequency stimulation to the perforant path (Figure 3D and 3E).

DISCUSSION

We set out to investigate the possibility that tyrosine kinase activity exerted an impact on LTP in perforant path-granule cell synapses by modulating glutamate release. We report that inhibition of tyrosine kinase by intracerebroventricular injection of genistein, blocked expression of LTP and the LTP-associated increases in glutamate release and calcium influx. The data indicate that LTP was accompanied by increases in phosphorylation of the α1-subunit of voltage-sensitive calcium channels and ERK in presynaptic terminals and increased phosphorylation of CREB in the entorhinal cortex.

As a first step in analysis of the effect of tyrosine phosphorylation on synaptic function in hippocampus, we assessed the effect of genistein on endogenous glutamate release and calcium influx in synaptosomes prepared from hippocampal synaptosomes and observed that depolarization-induced increases in both measures were inhibited by genistein. This suggests that tyrosine phosphorylation of one or more proteins is required to permit a depolarizing pulse of KCl to stimulate calcium entry and to release glutamate. The inhibitory effect of genistein on endogenous glutamate release is consistent with earlier findings (Mullany et al., 1996; Phillis et al., 1996).

The present data also argue for a role for tyrosine kinase in LTP in dentate gyrus (McGahon and Lynch, 1998) and CA1 in which activation of *fyn* (Grant et al., 1992; Kojima et al., 1997; Lu et al., 1999) and *src* (Lu et al., 1998; Huang and Hsu, 1999) were coupled with LTP. The present data do not assist in identification of the family of tyrosine

kinases which are activated following tetanic stimulation, but we have previously shown that LTP in dentate gyrus is accompanied by increased tyrosine phosphorylation of the NGF receptor, *TrkA*, suggesting a role for activation of cascades triggered by stimulation of receptor tyrosine kinases (Maguire et al., 1999).

The question of identification of the protein substrates targetted by the LTP-associated increase in tyrosine kinases arises. Since LTP in dentate gyrus is accompanied by increased glutamate release as described here and in several previous reports (Errington et al., 1987; Canevari et al., 1994; McGahon et al., 1999; Maguire et al., 1999; Kelly and Lynch, 2000), it is appropriate to consider proteins which might modulate transmitter release. We investigated the possibility that the α1-subunit of calcium channels might be a substrate for tyrosine kinase (a) because the *in vitro* data presented suggested that calcium influx was sensitive to tyrosine kinase activity, (b) because calcium channel activity modulates calcium influx, which in turn modulates transmitter release and (c) because several reports have indicated that calcium channel activity is altered by tyrosine phosphorylation (Arnoult et al., 1997; Potier and Rovira, 1999; Wijetunge et al., 2000; Strauss et al., 2000). Our findings reveal that tyrosine phosphorylation of the α1-subunit of calcium channels was markedly increased in synaptosomes prepared from tetanized dentate gyrus and this effect was inhibited by genistein. One consequence of a phosphorylation-induced increase in calcium influx is likely to be an increase in glutamate release; the present data which show parallel changes in tyrosine phosphorylation of α1-subunit of calcium channels, calcium influx and release following induction of LTP, and the parallel inhibitory effects of genistein, suggest a possible causal relationship between these synaptic events. In the context of these findings, it is significant that an NMDA-independent form of LTP, described in CA1 both *in vitro* and

in vivo, is dependent on activation of voltage-sensitive calcium channels (Grover and Teyler, 1994; Morgan and Teyler, 1999). Interestingly, the tyrosine kinase inhibitors, genistein and lavendustin A blocked this form of LTP but not the NMDA-dependent form (Çavus and Teyler, 1996).

The mitogen-activated protein kinases, of which ERK is a member, are activated by dual phosphorylation on threonine and tyrosine residues. Several regulators of ERK have been identified, including growth factors and neurotransmitters, but activation by calcium and tyrosine kinase have also been reported (see Derkinderen et al., 1999). In this study, we observed an LTP-coupled increase in ERK activation, coincident with increased tyrosine kinase activity and increased calcium influx and parallel inhibition of these functions in tissue prepared from genistein-treated rats. The coupling of these events permits us to suggest that a causal relationship exists between them, and that the activation of ERK described here might be stimulated by tyrosine kinase acting directly, or indirectly as a consequence of increased calcium influx. However we have previously found that LTP is associated with increased release of both NGF (Kelly et al., 2000) and BDNF (Gooney et al., 2000) from slices of dentate gyrus, and that they act on presynaptic *TrkA* and *TrkB* respectively; it is possible that these neurotrophins contribute to activation of ERK observed here. Other modulators of ERK include PI3-kinase (eg Lopez-Ilasaca et al., 1997), phosphorylation of which is increased with LTP (Kelly and Lynch, 2000), and the small GTP-binding protein, *ras* (acting through the *ras/raf*/MEK cascade) which may be activated by tyrosine kinase-induced stimulation of the adaptor protein, SHC (see Graves and Krebs, 1999). We propose that one consequence of increased ERK phosphorylation is upregulation of glutamate release since the data presented provides direct evidence that inhibition of ERK

blocked stimulus-dependent glutamate release. Therefore one mechanism by which tyrosine kinase inhibition might contribute to the downregulation of LTP is by attenuating ERK activation, which in turn attenuates glutamate release.

One downstream consequence of an increase in ERK activity is an increase in activation of the transcription factor, CREB (Finkbeiner, 2000), which is also activated by protein kinase A (Nguyen and Kandel., 1996) and Ca^{2+}/calmodulin-dependent kinase (Finkbeiner et al., 1997). Recent data have indicated that phosphorylation of CREB may be a necessary step in inducing morphological changes stimulated by oestradiol in hippocampal neurons (Murphy and Segal, 1997). In support of such a role for CREB, a coupling between increased CREB phosphorylation and protein synthesis are considered to be vital cell responses in the stabilization of long-term facilitation in Aplysia (Casadio et al., 1999), while CREB phosphorylation also seems to mediate BDNF-induced transcription (Finkbeiner et al., 1997). The data presented here provide direct evidence that tetanically-induced LTP in perforant path-granule cell synapses is coupled with increased CREB phosphorylation in entorhinal cortex and that this change is accompanied by increased protein synthesis. While these observations reflect presynaptic changes, increased CREB phosphorylation has been reported postsynaptically following induction of LTP in dentate gyrus (Schultz et al., 1999; Davis et al., 2000) and CA1 (Matthies et al., 1997), in which an increase in phospho-CREB immunofluorescence was also observed following stimulation of the Schaffer collaterals (Lu et al., 1999). Despite some experimental differences, it is significant that the timing of the response in the present study was similar to that observed elsewhere (Schultz et al., 1999).

The fact that increased protein synthesis accompanied increased CREB phosphorylation following induction of LTP, and that both effects were inhibited when LTP was blocked, is consistent with the view that they are causally related. It is tempting to propose that phosphorylation of CREB is the first of a number of changes which stimulate synthesis of specific proteins, which in turn might orchestrate the morphological changes which are required for the more persistent aspects of LTP (Lisman and Harris, 1993; Edwards, 1995). In the context of the present study it is significant that the cAMP-induced synaptic plasticity in Drosophila neuromuscular junction is a consequence of a CREB-mediated increase in transmitter release (Davis et al., 1996). On the basis of this latter observation, we might speculate that CREB phosphorylation might contribute to the LTP-associated increase in glutamate release.

The focus of the present study was to assess changes that occur presynaptically following induction of LTP in dentate gyrus which might play a role in modulating glutamate release. We did not address changes in the postsynaptic cell. However it must be acknowledged that the data correlate LTP with biochemical changes, for example increases in release and protein synthesis; the fact that these changes are inhibited when LTP is blocked as shown here, or by the NMDA receptor antagonist, AP5 (Mullany and Lynch, 1997; 1998), provides strong evidence of a causal relationship between the measures. The data presented here support the view that activation of tyrosine kinase, one consequence of tetanic stimulation, stimulates the increase in glutamate release which accompanies LTP. It is likely that this is achieved by phosphorylating a number of proteins which include the α1-subunit of voltage-sensitive calcium channels, phosphorylation of which leads to enhanced calcium influx. It seems likely that activation of ERK, which is inhibited when tyrosine kinase

activity is inhibited, contributes to the enhancement of release and to phosphorylation of CREB, which in turn may trigger synthesis of specific proteins which are likely to underpin the more persistent components of LTP.

REFERENCES

Abe K, Saito H Tyrosine kinase inhibitors, herbimycin A and lavendustin A, block formation of long-term potentiation in the dentate gyrus in vivo. Brain Res 1993; 621: 167-170.

Arnoult C, Lemos JR, Florman HM Voltage-dependent modulation of T-type calcium channels by protein tyrosine phosphorylation. EMBO J 1997; 16: 1593-1599.

Bliss TVP, Collingridge GL A synaptic model of memory: Long-term potentiation in the hippocampus. Nature 1993; 361: 31-39.

Bourtchuladze R, Frenfuelli B, Blendy J, Coiffi D, Schutz G, Silva AJ Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell 1994; 79: 59-68.

Bradford MM A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. Anal Biochem 1976; 72: 248-254.

Canevari L, Richter-Levin G, Bliss TVP LTP in the dentate gyrus is associated with a persistent NMDA receptor-dependent enhancement of synaptosomal glutamate release. Brain Res 1994; 667: 115-117.

Casadio A, Martin KC, Giustetto M, Zhu H, Chen M, Bartsch D, Bailey CH, Kandel ER A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. Cell 1999; 99: 221-237.

Cavus I, Teyler T (1996) Two forms of long-term potentiation in area CA1 activate different signal transduction cascades. J Neurophysiol 1996; 76: 3038-3047.

Charriaut-Marlangue C, Aniksztejn L, Roisin MO, Ben-Ari Y. Release of proteins during long-term potentiation in the hippocampus of the anaesthetized rats. Neurosci Letts 1991; 91: 308-314.

Davis S, Vanhoutte P, Pages C, Caboche J, Laroche S (2000) The MAPK/ERK cascade targets both Elk-1 and cAMP response element binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. J Neurosci 2000; 20: 4563-4572.

Derkinderen P, Enslen H, Girault J-A The ERK/MAP-kinases cascade in the nervous system. NeuroReport 1999; 10: R24-R34.

Edwards F LTP- a structural model to explain the inconsistencies. TINS 1995; 18: 250-255.

English JD, Sweatt JD A requirement for the mitogen-activated protein kinase cascade in hippocampal long-term potentiation. J Biol Chem 1997; 272: 19103-19106.

Errington ML, Lynch MA, Bliss TVP (1987) Long-term potentiation in the dentate gyrus: induction and increased glutamate release are blocked by D(-)amino-phosphonovalerate. Neuroscience 1987; 20: 279-284.

Fazeli MS, Corbet J, Dunn MJ, Dolphin AC, Bliss TVP Changes in protein synthesis accompanying long-term potentiation in vivo. J Neurosci 1993; 13: 1346-1353.

Finkbeiner S, Tavazoie SF, Malorarsky A, Jacobs KM, Harris KM Greenberg ME. (1997) CREB: A major mediator of neuronal neurotrophin responses. Neuron 1997; 19: 1031-1047.

Finkbeiner S. CREB couples neurotrophin signals to survival messages. Neuron 2000; 25: 11-14.

Ginty DD. Calcium regulation of gene expression: isn't that spatial? Neuron 1997; 18: 183-186.

Gooney M, O'Mara S, Lynch MA. Long-term potentiation induces brain-derived neurotrophic factor release and TrkB phosphorylation. Soc Neurosci Abstr 2000; 26: 360.

Grant SGN, O'Dell TJ, Karl KA, Stein PL, Soriano P, Kandel ER. Impaired long-term potentiation, spatial learning and hippocampal development in fyn mutant mice. Science 1992; 258: 1903-1910.

Graves JD, Krebs EG. (1999) Protein phosphorylation and signal transduction. Pharmacol Ther 1999; 82: 111-121.

Grover LM, Teyler TJ. Two components of long-term potentiation induced by different patterns of afferent activation. Nature 1990; 347: 477-479.

Haan EA, Bowen DM. Protection of neocortical prisms from freeze-thaw injury by dimethylsulphoxide. J Neurochem 1981; 37: 243-246

Huang C-C, Hsu KS. Protein tyrosine kinase is required for the indiction of long-term potentiation in the rat hippocampus. J Physiol 1999; 520.3: 783-796.

Impey S, Mark M, Villacres EC, Poser S, Chavkin C, Storm DT. Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron 1996; 16: 973-982.

Kang H, Schuman EM. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 1995; 267: 1658-1662.

Kelly Á, Maguire C, Lynch MA. Deficits in NGF release and trk phosphorylation are associated with age-related impairment in LTP in dentate gyrus. Neuroscience 2000; 95: 359-365.

Kelly Á, Lynch MA Long-term potentiation in dentate gyrus of the rat is inhibited by the phosphoinositide 3-kinase inhibitor, wortmannin. Neuropharmacology 2000; 39: 643-651.

Kojima N, Wang J, Mansuy IM, Grant SGN., Mayford M, Kandel ER. Rescuing impairment of long-term potentiation in fyn-deficient mice by introducing Fyn transgene. Proc Natl Acad Sci 1997; 94: 4761-4765.

Lisman JE, Harris KM. Quantal analysis and synaptic anatomy - integrating two views of the hippocampal plasticity. TINS 1993; 16: 141-146.

Lopez-Ilasaca M, Crespo P, Pellici PG, Gutkind JS, Wetzker R. Linkage of G-protein-coupled receptors to the MAPK signalling pathway through PI-1 kinase gamma. Science 1997; 275: 384-397.

Lu YM, Roder JC, Davidow J, Salter MW Src activation in the induction of long-term potentiation in CA1 hippocampal neurons. Science 1998; 279: 1363-1367.

Lu YF, Kojima N, Tomizawa K, Moriwaki A, Matsushita M, Obata K, Matsui H. Enhanced synaptic transmission and reduced threshold for LTP induction in fyn-transgenic mice. Eur J Neurosci 1999; 11: 75-82.

Maguire C, Casey M, Kelly A, Mullany PM, Lynch MA. Activation of tyrosine receptor kinase, trk, plays a role in expression of long-term potentiation in the rat dentate gyrus. Hippocampus 1999; 9: 519-526.

Matthies H, Schulz S, Thiemann W, Seimer H, Schmidt H, Krug M, Hollt V. Design of a multiple interface chamber and application for resolving the temporal pattern of CREB phosphorylation in hippocampal long-term potentiation. J Neurosci Meths 1997; 78: 173-179.

McGahon B, Lynch MA Analysis of the interaction between arachidonic acid and metabotropic glutamate receptor activation reveals that phospholipase C acts as a coincidence detector in the expression of long-term potentiation in the rat dentate gyrus. Hippocampus 1998; 8: 1-9.

McGahon B, Maguire C, Kelly A, Lynch MA. Activation of p42 mitogen-activated protein kinase by arachidonic acid and ACPD impacts on long-term potentiation in dentate gyrus in the rat: Analysis of age-related changes. Neuroscience 1999; 90: 1167-1175.

Messaoudi E, Barsden K, Srebro B, Bramham CR. Acute intrahippocampal infucion of BDNF induces long-lasting synaptic potentiation of synaptic transmission in the rat dentate gyrus. J Neurophysiol 1998; 79: 496-499

Morgan SL, Teyler TJ. VDCCs and NMDARs underlie two forms of LTP in CA1 hippocampus in vivo. J Neurophysiol 1999; 82: 736-740.

Mullany P, Connolly S, Lynch MA. Ageing is associated with changes in glutamate release, protein tyrosine kinase and calcium/calmodulin-dependent protein kinase II in rat hippocampus. Eur J Pharmacol 1996; 309: 311-315.

Mullany PM, Lynch MA. Changes in protein synthesis and synthesis of the synaptic vesicle protein, synaptophysin, in entorhinal cortex following induction of long-term potentiation in dentate gyrus: an age-related study in the rat. Neuropharmacology 1997; 36: 973-980.

Mullany PM, Lynch MA Evidence for a role for synaptophysin in expression of long-term potentiation in rat dentate gyrus. NeuroReport 1998; 9: 2489-2494.

Murphy DD, Segal M. Morphological plastivity of dendritic spines in central neurons is mediated by activation of cAMP response element binding protein. Proc Natl Acad Sci 1997; 94: 1482-1487.

Murray C, Lynch MA. Evidence that increased hippocampal expression of the cytokine, IL-1β, is a common trigger for age and stress-induced impairments in long-term potentiation. J Neurosci 1998; 18: 2974-2981.

Murray C, Lynch MA. Analysis of the mechanism by which dietary supplementation with vitamin E and vitamin C restores ability of aged animals to sustain long-term potentiation in dentate gyrus. J Biol Chem 1998; 273: 12161-12168.

Nguyen PV, Kandel ER. A macromolecular synthesis-dependent late phase of long-term potentiation requiring cAMP in the medial perforant pathway of rat hippocampal slices. J Neurosci 1996; 16: 3189-3198.

O'Dell TJ, Kandel ER, Grant SGN. Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. Nature 1991; 353: 558-560.

Ordronneau P, Abdullah L, Petruse P. An efficient enzyme immunoassay for glutamate using glutaraldehyde coupling of the hapten to microtiter plates. J Immunol Meth 1991; 142: 169-176.

Otani S, Marshall CJ, Tate WP, Goddard GV, Abraham WC. Maintenance of long-term potentiation in rat dentate gyrus requires protein synthesis but not messenger RNA synthesis immediately post-tetanization. Neuroscience 1989; 28: 519-526.

Phillis JW, Song D, O'Regan MH Inhibition of tyrosine phosphorylation attenuates amino acid neurotransmitter release from the ischemic/reperfused rat cerebral cortex. Neurosci Letts 1996; 207: 151-154.

Potier B, Rovira C. Protein tyrosine kinase inhibitors reduce high-voltage activating calcium currents in CA1 pyramidal neurones from rat hippocampal slices. Brain Res 1999; 816: 587-597.

Rosenblum K, Dudai Y, Richter-Levin G. Long-term potentiation increases tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit 2B in rat dentate gyrus in vivo. Proc Natl Acad Sci 1996; 93: 10457-10460.

Rostas JAP, Brent VA, Voss K, Errington ML, Bliss TVP, Gurd JW Enhanced tyrosine phosphorylation of the 2B subunit of the N-methyl-D-aspartate receptor in long-term potentiation. Proc Natl Acad Sci 1996; 93: 10452-10456.

Schultz S, Seimer H, Krug M, Hollt V. Direct evidence for biphasic cAMP response element-binding protein phosphorylation during long-term potentiation in the rat dentate gyrus in vivo. J Neurosci 1999; 19: 5683-5692.

Strauss O, Buss F, Rosenthal R, Fischer D, Mergler S, Stumpff F., Thieme H. Activation of neuroendocrine L-type channels (alpha 1D subunits) in retinal pigment epithelial cells and brain neurons by pp60 (c-src). Biochem Biophys Res Commun 2000; 270: 806-810.

Wijetunge S, Lymn JS, Hughes AD. Effects of protein tyrosine kinase inhibitors on voltage-operated calcium channel currents in vascular smooth muscle cells and pp60(c-src) kinase activity. Brit J Pharmacol 2000; 129: 1347-1354.

FIGURE LEGENDS

FIGURE 1.

Genistein inhibits KCl stimulated [^{45}Ca] influx and glutamate release.

(A) Addition of KCl (40mM) significantly increased ^{45}Ca influx into hippocampal synaptosomes (*$p < 0.05$; student's t-test for paired samples); this effect was inhibited in tissue which was incubated in the presence of genistein. Values are the means of 12 observations and expressed as nmol/mg. (B) Addition of KCl (40mM) significantly increased glutamate release in hippocampal synaptosomes (*$p < 0.05$; student's t-test for paired samples); this effect was inhibited by preincubation in genistein (50μM); incubation of synaptosomes in the presence, compared with in the absence, of genistein significantly decreased both unstimulated and KCl-stimulated glutamate release (+$p < 0.01$). Values are the means of 7 observations and expressed as μmol/mg.

FIGURE 2

LTP is associated with increased ^{45}Ca influx and glutamate release.

Synaptosomes were prepared from ispilateral and contralateral dentate gyrus 40 min after induction of LTP unilaterally (A). (B) Addition of KCl (40mM) significantly increased [^{45}Ca] influx into synaptosomes prepared from untetanized dentate gyrus (*$p < 0.05$; student's t-test for paired samples); influx was further increased in synaptosomes prepared from tetanized dentate gyrus (**$p < 0.01$). Values are the means of 10 observations and expressed as nmol/mg. (C) Addition of KCl (40mM) significantly increased glutamate release in synaptosomes prepared from untetanized dentate gyrus (*$p < 0.05$; student's t-test for paired samples); release was further increased in synaptosomes prepared from

tetanized dentate gyrus (**$p < 0.01$). Values are the means of 7 observations and expressed as μmol/mg.

FIGURE 3

Low frequency stimulation failed to increase glutamate release, ERK activation, CREB activation or protein synthesis.

(A) Mean epsp slope was unchanged by stimulation at a rate of 1 shock per 12 secs for a 50 min recording period. (B) Addition of KCl significantly increased glutamate release in synaptosomes prepared from both sides of dentate gyrus of rats (both sides; $p < 0.05$; ANOVA); the effect of KCl was similar in the unstimulated dentate gyrus and in the dentate gyrus which received low frequency stimulation. (C-E) Activation of ERK (C), and CREB (D) and protein synthesis (E) was similar in both sides all preparations. Values are means (± SEM) of 4 –6 separate experiments.

FIGURE 4

Genistein pretreatment inhibits LTP and the LTP-associated increases in tyrosine kinase and glutamate release.

Intracerebroventricular injection of genistein (250μM) 40 min before tetanic stimulation markedly attenuated LTP in dentate gyrus (A). (B) Addition of KCl (40mM) increased tyrosine kinase activity in synaptosomes prepared from untetanized dentate gyrus of saline-treated rats, but this did not reach statistical significance due to the large variability; a significant increase was observed in tetanized tissue (*$p < 0.05$; student's t-test for paired samples). Unstimulated tyrosine kinase activity in synaptosomes prepared from tetanized tissue was significantly increased compared with untetanized tissue (+$p < 0.05$; student's t-test for paired samples). KCl failed to increase kinase activity in untetanized or tetanized

tissue prepared from genistein-treated rats. Values are the means of 6 observations and expressed as nmol/mg. (C) Addition of KCl (40mM) increased glutamate release in synaptosomes prepared from untetanized dentate gyrus of saline-treated rats (*$p < 0.05$; student's t-test for paired samples); KCl-stimulated release was more marked in synaptosomes prepared from tetanized dentate gyrus (**$p < 0.01$). Unstimulated release was significantly reduced in synaptosomes prepared from both tetanized and untetanized tissue of genistein-treated rats (+$p < 0.01$; student's t-test for unpaired samples) and KCl failed to increase release in these preparations. Values are the means of 6 observations and expressed as μmol/mg.

FIGURE 5

Genistein inhibits the LTP-associated increases in phosphorylation of the α1-subunit of voltage sensitive calcium channels and ERK.

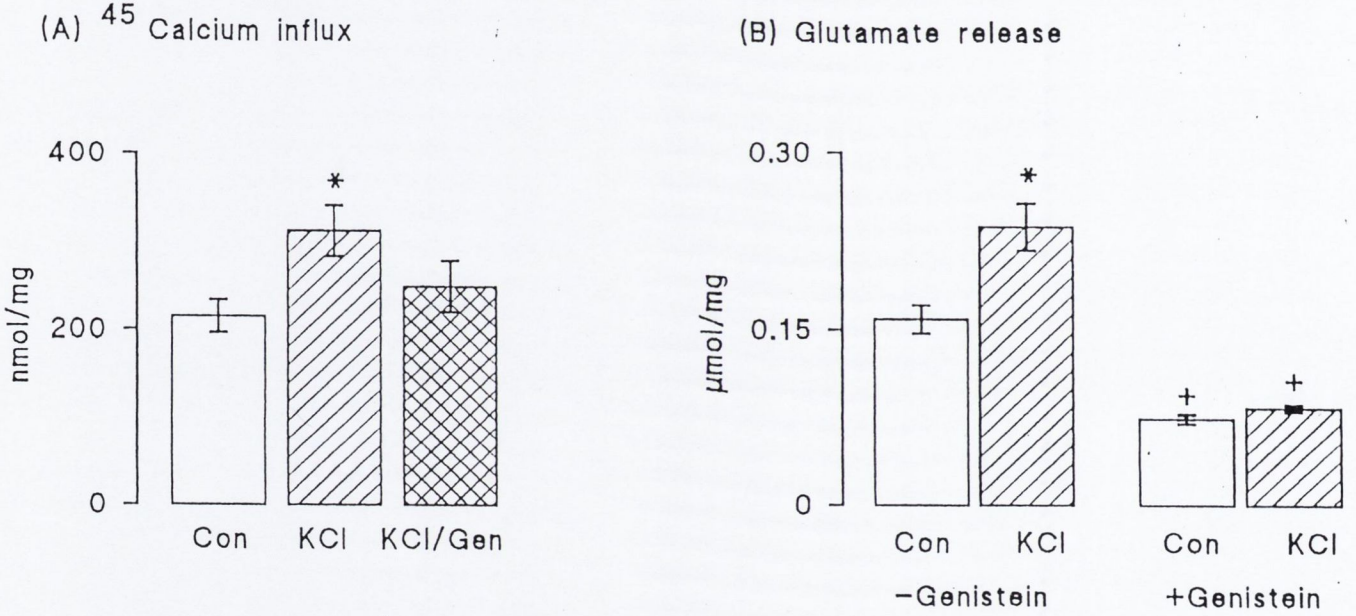
(A) The sample immunoblot indicates that phosphorylation of the α1-subunit of voltage sensitive calcium channels was increased in synaptosomes prepared from tetanized (lane 2), compared with untetanized (lane 1) dentate gyrus of saline-treated rats. Densitometric analysis of data from 6 separate experiments, given in arbitrary units, indicate that the difference was statistically significant (*$p < 0.05$; student's t-test for paired samples). This effect was inhibited in tissue prepared from genistein-pretreated rats as shown on the sample immunoblot (compare lanes 3 and 4) and by analysis of the mean values obtained by densitometric analysis. (B) The sample immunoblot indicates that phosphorylation of ERK was increased in synaptosomes prepared from tetanized (lane 2), compared with untetanized (lane 1), dentate gyrus of saline-treated rats. Densitometric analysis of data from 6 separate experiments, given in arbitrary units, indicate that the difference was statistically significant

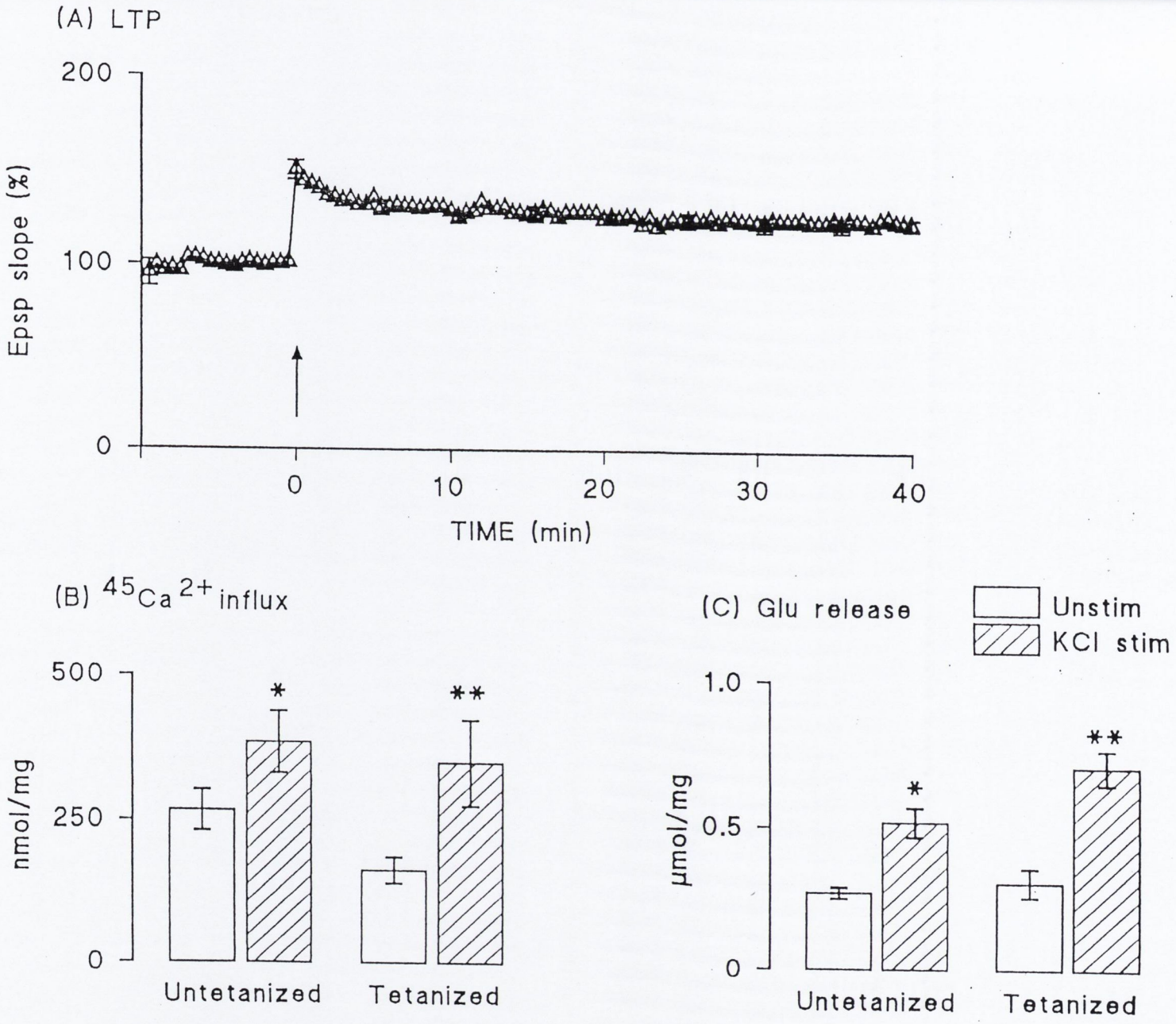
(*$p < 0.05$; student's t-test for paired samples). This effect was inhibited in tissue prepared from genistein-pretreated rats as shown on the sample immunoblot (compare lanes 3 and 4) and by analysis of the mean values obtained by densitometric analysis. (C) Addition of KCl (40mM) to the incubation medium significantly increased glutamate release in synaptosomes prepared from dentate gyrus (**$p < 0.01$; student's t-test for paired samples); this effect was attenuated when PD98059 (2μM) was included in the incubation. Values are means of 6 experiments and expressed as μmol glutamate/mg protein.

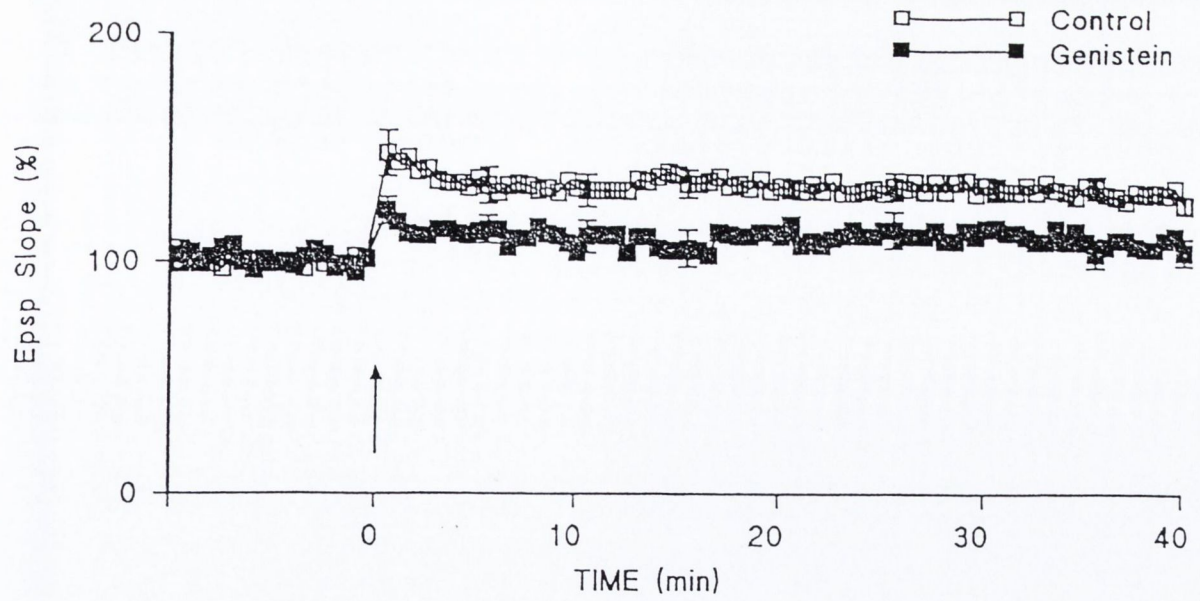
FIGURE 6.

Genistein inhibited the LTP-associated increases in CREB phosphorylation and protein synthesis in entorhinal cortex.

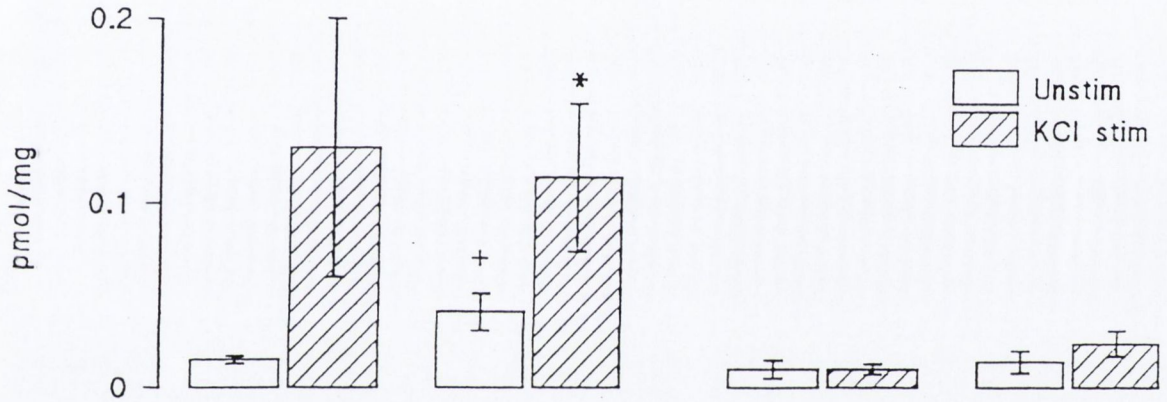
(A) The sample immunoblot indicates that phosphorylation of CREB was increased in homogenate prepared from entorhinal cortex obtained from the tetanized side of the brain of saline-treated rats (lane 2), compared with the untetanized side (lane 1). Densitometric analysis of data from 6 separate experiments, given in arbitrary units, indicate that the difference was statistically significant (*$p < 0.05$; student's t-test for paired samples). This effect was inhibited in tissue prepared from genistein-pretreated rats as shown on the sample immunoblot (compare lanes 3 and 4) and by analysis of the mean values obtained by densitometric analysis. (B) ^{35}S-Methionine labelling of proteins was significantly increased in slices of entorhinal cortex prepared from the tetanized side of the brain of saline-treated rats, compared with the untetanized side (*$p < 0.05$; student's t-test for paired samples). This effect was inhibited in tissue prepared from genistein-pretreated rats.


(A) 45 Calcium influx
400
200
0
nmol/mg
*
Con
KCl
KCl/Gen
(B) Glutamate release
0.30
0.15
0
µmol/mg
*
+
+
Con
KCl
Con
KCl
−Genistein
+Genistein

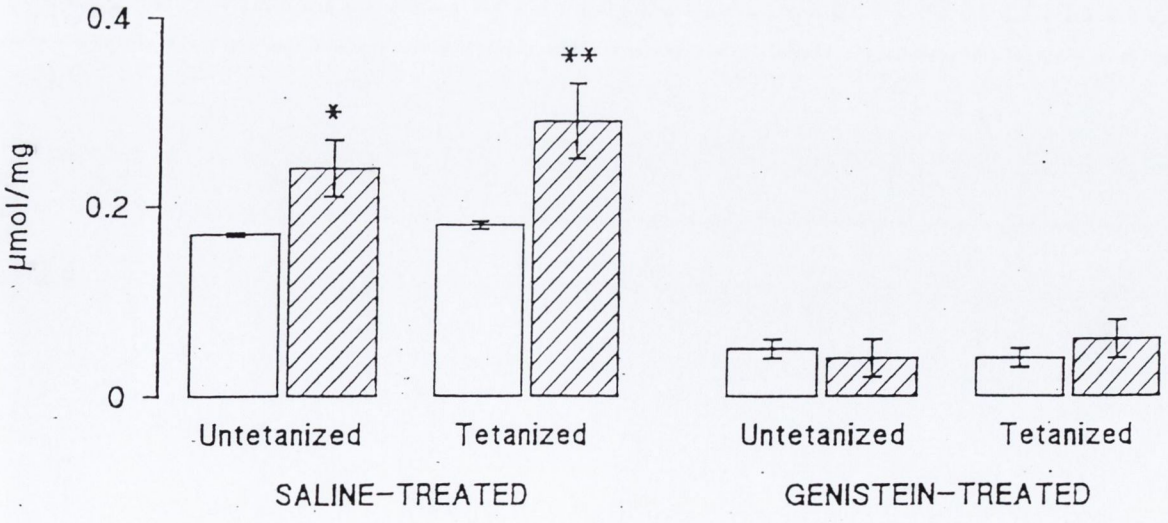

(A) LTP
200
100
0
Epsp slope (%)
0
10
20
30
40
TIME (min)
(B) $^{45}Ca^{2+}$ influx
500
250
0
nmol/mg
*
**
Untetanized
Tetanized
(C) Glu release
Unstim
KCl stim
1.0
0.5
0
µmol/mg
*
**
Untetanized
Tetanized

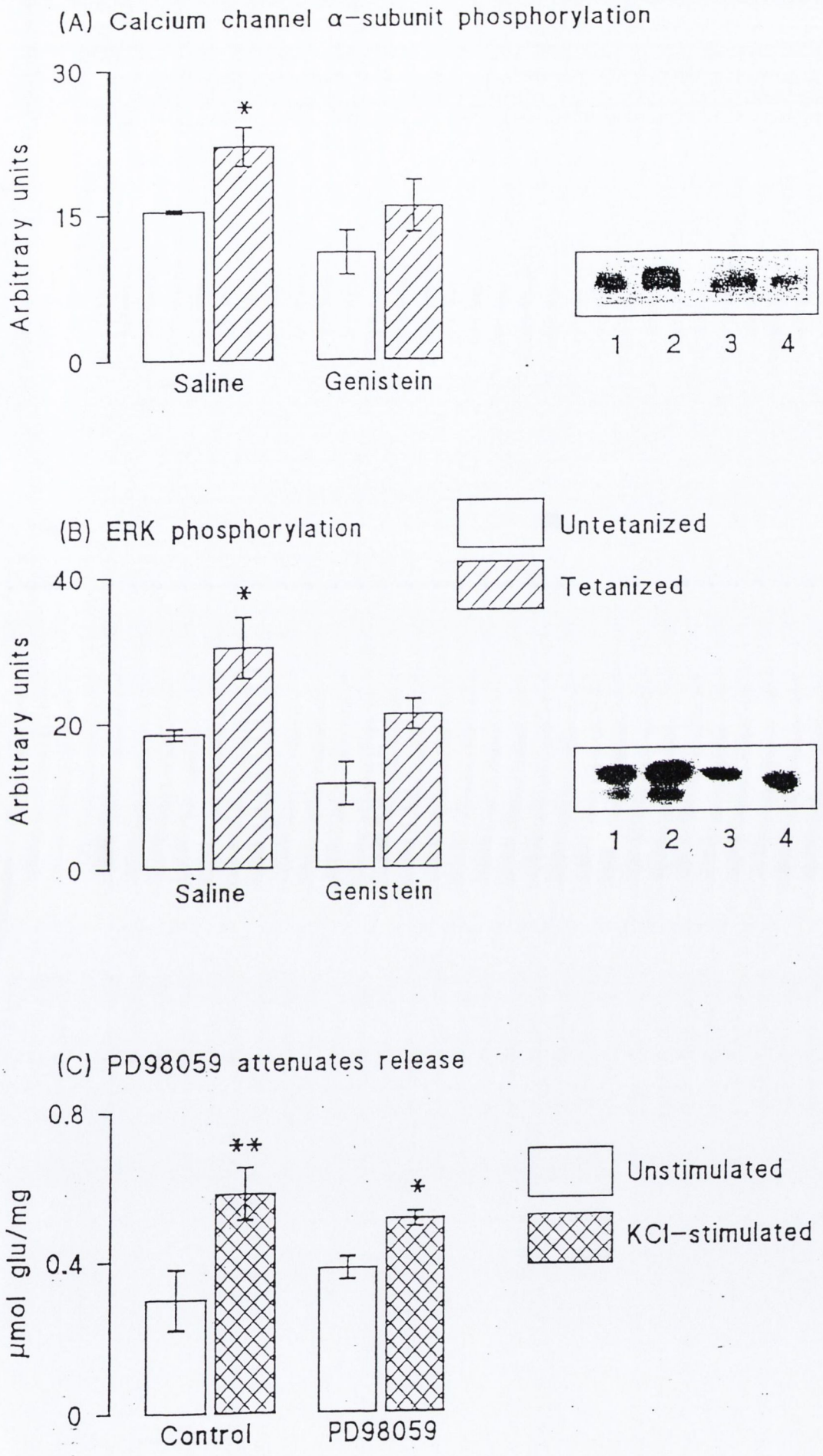


(B) Tyrosine kinase activity

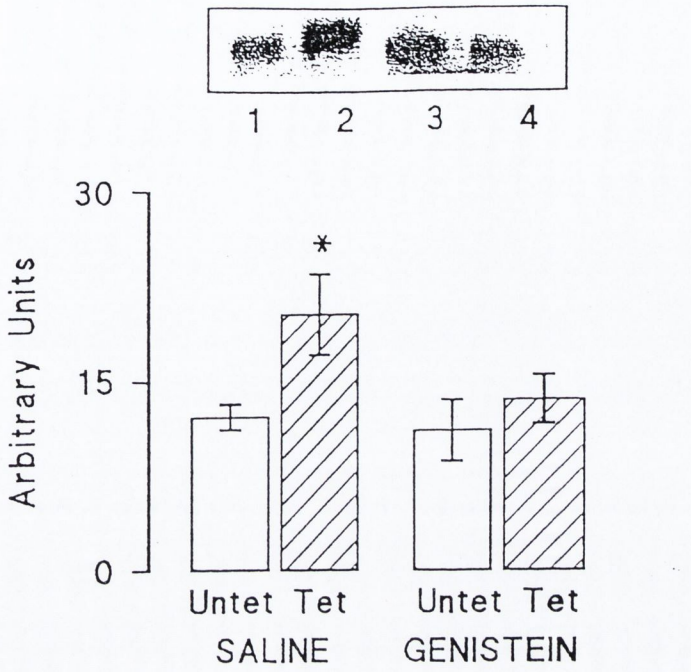


(C) Glutamate release

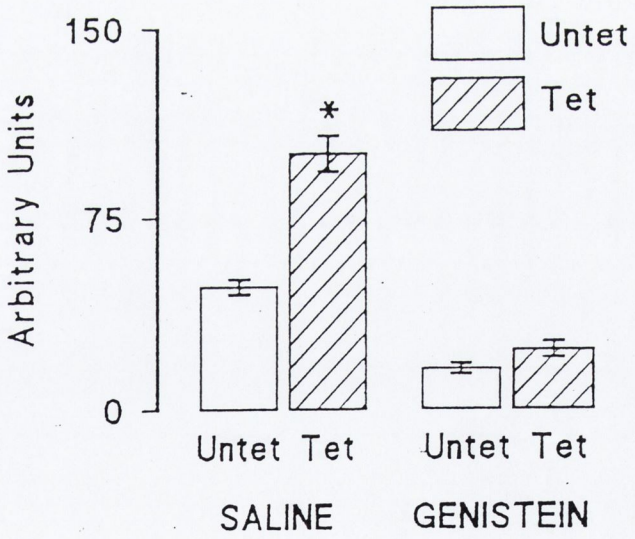


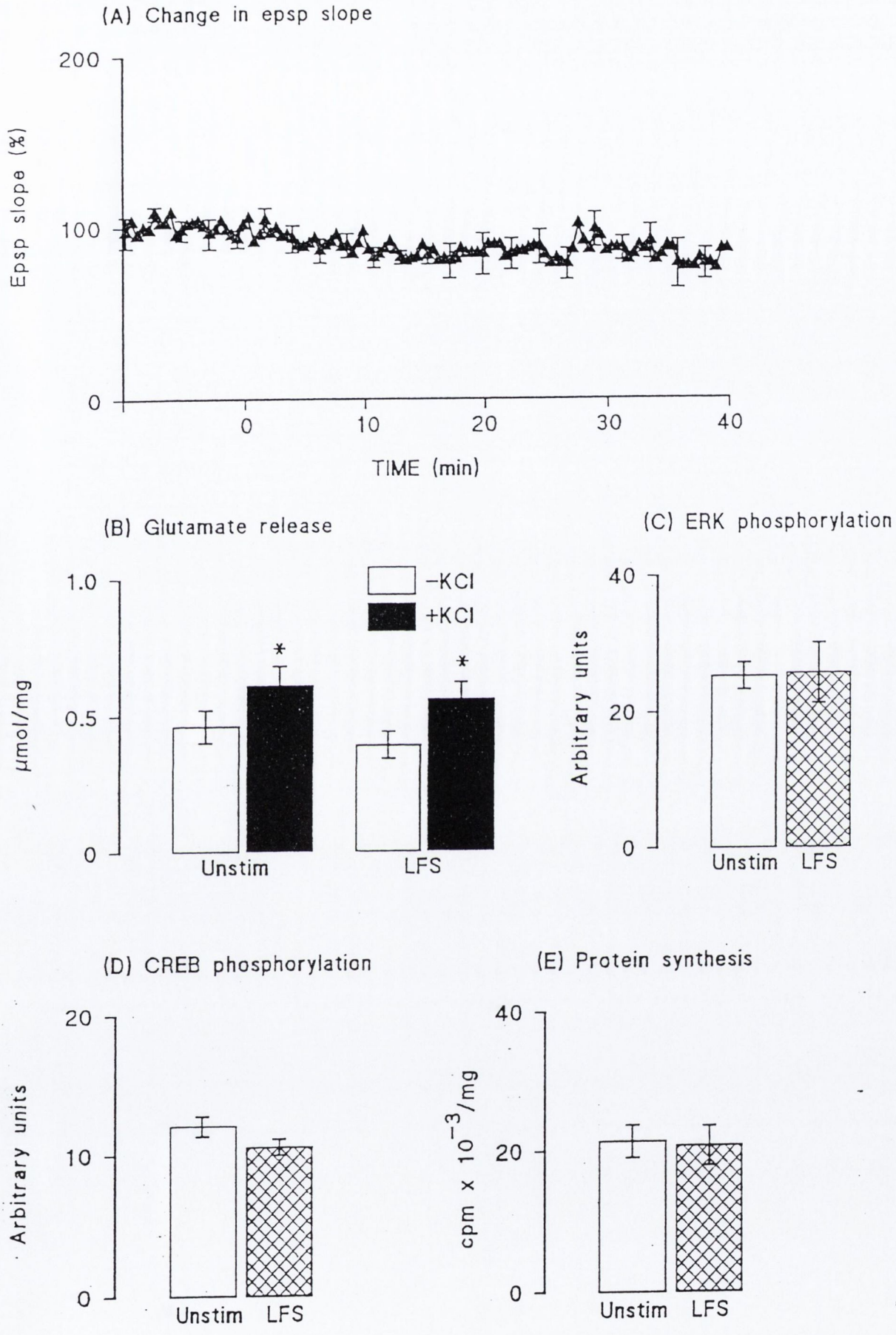

(A) Calcium channel α–subunit phosphorylation
30
15
0
Arbitrary units
*
Saline
Genistein
1 2 3 4
(B) ERK phosphorylation
Untetanized
Tetanized
40
20
0
Arbitrary units
*
Saline
Genistein
1 2 3 4
(C) PD98059 attenuates release
0.8
0.4
0
µmol glu/mg
**
*
Control
PD98059
Unstimulated
KCl–stimulated

(A) CREB phosphorylation



(B) Protein synthesis




(A) Change in epsp slope
Epsp slope (%)
200
100
0
0
10
20
30
40
TIME (min)
(B) Glutamate release
μmol/mg
1.0
0.5
0
−KCl
+KCl
*
*
Unstim
LFS
(C) ERK phosphorylation
Arbitrary units
40
20
0
Unstim
LFS
(D) CREB phosphorylation
Arbitrary units
20
10
0
Unstim
LFS
(E) Protein synthesis
cpm x 10^{-3}/mg
40
20
0
Unstim
LFS