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The Effect of Neuronal Insult on Neurotrophin Receptor Expression and Signalling

Amy Hennigan

Thesis submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College, Dublin 2, Ireland

October 2006

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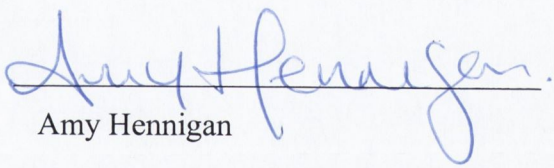

Amy Hennigan

II Abstract

The neurotrophins were initially identified as a group of target-derived neuronal factors but have since been shown to mediate a wide range of biological functions including cell survival, maintenance, differentiation and apoptosis. These diverse functions are mediated via two types of receptor: the tropomyosin-related kinase (Trk) receptor tyrosine kinases which are traditionally known as the high-affinity neurotrophin receptors, and the low-affinity p75 neurotrophin receptor (p75NTR). The Trk receptors transmit positive signals such as enhanced growth and survival, while the p75NTR is somewhat of a biological paradox in that it has been shown to transmit both positive and negative signals. Increased expression of the p75NTR has been observed post injury and in neurodegenerative conditions and its expression has been associated with increased cell death. The aim of this study is primarily to assess the role of the p75NTR following neuronal insult *in vivo* and to establish reliable models by which expression of the p75NTR can be successfully upregulated.

We found that administration of LPS or kainic acid upregulated p75NTR expression. This increase in p75NTR expression was associated with increased apoptotic signalling as evidenced by increased activation of JNK. Concomitant with increased p75NTR was an impairment in long-term potentiation (LTP). LTP was induced in dentate gyrus following high-frequency stimulation of the perforant path. Both LPS (300µl, 100µg/Kg i.p.) and kainic acid (5µl of 100µM i.c.v) administration blocked expression of LTP; mean % change in EPSP slope in the last 5 mins of recording compared with the 5 min immediately prior to tetanic stimulation was for LPS 100.72 ± 3.49% (n=6, mean ± SEM) compared with saline (0.9% w/v) treated controls (118.21± 2.91%); kainic acid 83.74 ± 0.7314% (n=4, mean ± SEM) compared with saline (5µl, 0.9% w/v) 117.3± 0.2977%.

Evidence suggests that the fate of a cell is dependent on the ratio of expression of Trk receptors to p75NTR. When co-expressed the p75NTR enhances TrkA survival signalling but when the ratio of p75NTR expression is greater than TrkA expression apoptotic signalling occurs. To isolate p75NTR signalling *in vivo* genetically hypertensive (GH) rats which are deficient in NGF and TrkA were

treated with LPS. LPS administration increased p75NTR expression in normotensive (N) rats and was associated with a functional impairment as assessed by the failure to successfully complete a novel object preference task. There was no increase in p75NTR in GH rats treated with LPS. This experiment failed to isolate p75NTR signalling *in vivo* but once again demonstrated that p75NTR expression was associated with functional impairment.

To fully elucidate the role of increased p75NTR expression in the functional impairments observed a p75NTR functional inhibitor was co-administered with kainic acid. Kainic acid administration impaired LTP in the rat hippocampus. This kainic acid induced impairment was associated with increased expression of p75NTR. Administration of the p75NTR inhibitor REX failed to reverse the kainic acid induced impairment in LTP indicating that the p75NTR is not intrinsic to the functional impairment observed. However, administration of the p75NTR inhibitor REX enhanced LTP in control animals suggesting that the p75NTR may be tonically supressing LTP.

These data demonstrate that neuronal insult increases p75NTR expression and this increase in expression is concomitant with increased JNK activation and impairment in synaptic function. p75NTR blockade does not reverse the impairment in function caused by neuronal insult. However a role for the p75NTR in synaptic plasticity has been elucidated.

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V Acknowledgements

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Last but by no means least I would like to thank my family; Mom, Dad, Paul and Sarah for all their patience, encouragement and support over the last few years. This thesis is dedicated to them.

VI List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ANOVA	Analysis of variance
AP5	D-amino phosphonovalerate
APS	Ammonium persulfate
ARMS	Ankyrin repeat-rich membrane spanning adaptors
ATP	Adenosine 5'-triphosphate
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CaMKII	Calcium-calmodulin-proteinkinase-II
CARD	Caspase recruitment domain
CNS	Central nervous system
CREB	cAMP-responsive element binding protein
DAB	Diaminobenzidine
DAG	Diacylglycerol
DMSO	Dimethyl sulphoxide
dNTP	Deoxynucleotidetriphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethylene glycol bis (β-aminoethylether) *N,N* 'tetraacetic acid
EPSP	Excitatory postsynaptic potential
ERK	Extracellular regulated kinase
FITC	Flourescein isothiocyanate
GFAP	Glial fibrillary acidic protein
GH	Genetically hypertensive
G-protein	GTP-binding protein
GTP	Guanosine 5'- triphosphate
HFS	High-frequency stimulation
HRP	Horseradish peroxidase conjugate
IgG	Immunoglobulin G
IP_3	Inositol 1,4,5-trisphosphate
JNK	c-jun N-terminal kinase
KCL	Potassium chloride

kDa	Kilo Dalton
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
Mg	Milligram
$MgCl_2$	Magnesium Chloride
MMP	matrix metalloproteinase
mRNA	Messenger ribonucleic acid
N	Normotensive
NFκB	Nuclear factor kappa-B
NGF	Nerve growth factor
NMDA	*N*-methyl-D-aspartate
NogoR	Nogo receptor
NRIF	Neurotrophin receptor interacting factor
NT	Neurotrophin
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
NT-6	Neurotrophin-6
NT-7	Neurotrophin-7
OMgP	Oligodendrocyte myelin glycoprotein
p75NTR	p75 neurotrophin receptor
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol-3-OH kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
RIP2	Receptor interacting protein 2
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-ployacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SNARE	Soluble NSF attachment receptor

SOS	Son of sevenless
TRAF	TNF-Receptor-Associated factor
TBS	Tris-buffered saline
TBS-T	TBS-tween
TdT	Terminal deoynucleotidyl transferase
Thr	Threonine
TNF	Tumour necrosis factor
Trk	Tropomyosin-related kinase
TUNEL	TdT-mediated-UTP-end nick labelling
Tyr	Tyrosine

Chapter 1

General Introduction

1.1 Introduction

The neurotrophins were initially identified as a group of target-derived neuronal factors but have since been shown to mediate a wide range of biological functions including cell survival, maintenance, differentiation and apoptosis. These diverse functions are mediated via two types of receptor; the tropomyosin-related kinase (Trk) receptor tyrosine kinases which are traditionally known as the high affinity neurotrophin receptors, and the low affinity p75 neurotrophin receptor (p75NTR). The Trk receptors transmit positive signals such as enhanced growth and survival, while the p75NTR is somewhat of a biological paradox in that it has been shown to transmit both positive and negative signals. The ambiguity surrounding the role of the p75NTR still persists despite its discovery over 30 years ago. The aim of this study is primarily to assess the role of the p75NTR following neuronal insult *in vivo* and to establish reliable models by which expression of the p75NTR can be successfully upregulated.

1.2The Neurotrophins

In the early 1950's Rita Levi-Montalcini and Viktor Hamburger discovered that a mouse sarcoma tumour implanted close to the spinal cord of a developing chicken *in ovo* secreted a soluble factor that induced hypertrophy and outgrowth of dorsal root ganglion suggesting that targets contacted by neurons produce trophic substances that support neuronal survival. This soluble factor was later termed nerve growth factor (NGF). In 1982, Barde *et al.* succeeded in isolating a neuronal survival factor from pig brain which was named brain-derived neurotrophic factor (BDNF). Over twenty years later the neurotrophin family has grown to include neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) which is found only in fish. All of the neurotrophins are initially produced as pre-proneurotrophins precursors that are approximately 240-260 amino acids in length. The pre-mRNA sequence directs the synthesis of the protein to the endoplasmic reticulum (ER) attached ribosomes, leading to the sequestration of the newly formed polypeptide chain into the ER where the signal peptide is immediately cleaved. This cleavage leads to the formation of the pro-neurotrophins which are transported to the Golgi in vesicles where they are either cleaved by furin in the trans Golgi or by other pro-convertases in vesicles. It is important to note that it has been recently determined that the pro-neurotrophins are

biologically active. Looking at survival and neurite outgrowth assays, Fahnestock *et al.* (2004) found that proNGF exhibits neurotrophic activity similar to mature NGF but it is five times less active. Several groups have reported a more ominous role for the pro-neurotrophins and this will be discussed in further detail in section 1.2.1.2. The mature neurotrophin is a homodimer that is formed by identical peptide chains of roughly 120 amino acids each. The monomers cohere by non-covalent chemical bonds and are stabilized by an inherent highly conserved cysteine knot motif. The uniqueness of each of the neurotrophins is attributed to a specific pattern of charged basic or acidic residues exposed on the surface. Peptide loops protruding from the core β-sheets of the molecule also clarify the differences between each of the neurotrophins.

1.2.1 Neurotrophin receptors

As mentioned in section 1.1 the biological functions of the neurotrophins are mediated via two types of receptor: the Trk receptors and the p75NTR.

1.2.1.1The Trk receptors

TrkA was originally characterized as a transforming onconogene in which tropomyosin was fused to an unknown tyrosine kinase (Martin-Zanca *et al.*, 1989). The corresponding protoonconogene was shown to be a transmembrane protein whose structure suggested that it was a receptor tyrosine kinase. The two other members of the Trk family, TrkB and TrkC, were quickly isolated and all of the genes were shown to be expressed in discrete neuronal populations. It later emerged that these receptors could be activated by a specific neurotrophin, with NGF preferring TrkA, BDNF and NT-4/5 preferring TrkB and NT-3 binding to TrkC. Though initially described in human colon carcinoma, these receptors are expressed in a wide variety of neuronal and non-neuronal cell types, such as the hippocampus, cerebellum, neurons of the peripheral nervous system, arteries, tooth buds and the submaxillary gland (See Roux and Barker, 2002)

Structure of the Trk receptors

All trk receptors are Type I transmembrane proteins that are members of the receptor tyrosine kinase superfamily. The extracellular domains of the Trk receptors contain two cysteine rich regions neighbouring a leucine rich repeat, followed by two immunoglobulin like domains in the juxtamembrane region (Figure 1). Binding studies and deletion work on each of the Trk receptors have shown that the receptors interact with their ligands via the second immunoglobulin like domain with the second leucine rich domain playing an ancillary role in ligand interaction. Interaction of the Trk receptors with the core β-sheets of the neurotrophins provides the majority of the binding energy. The specificity of neurotrophin binding is derived from interactions with the ligands amino-termini.

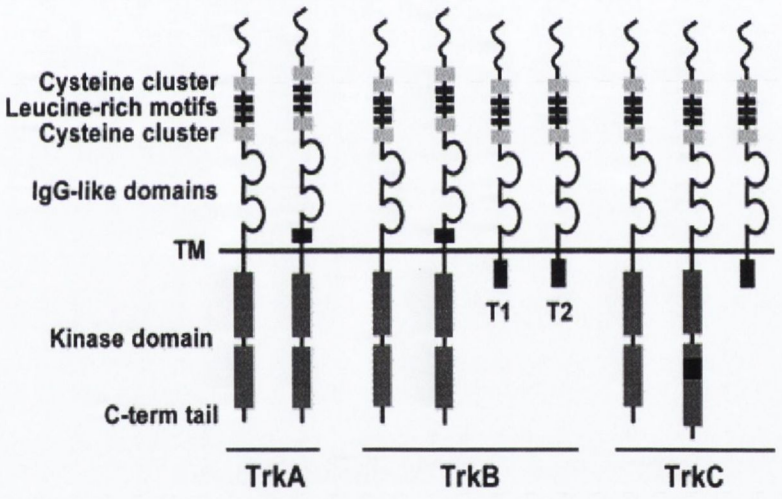


Figure 1.1 Structure of the Trk Receptors: Image taken from Roux and Barker (2002).

Signalling through the Trk receptors

Numerous studies have shown that the signalling via the Trk receptors promotes survival, differentiation and outgrowth of many neuronal populations. Upon ligand binding Trk receptors dimerize and become catalytically active which results in autophosphorylation. There are 10 conserved tyrosines in the cytoplasmic region of each of the Trk receptors, three of which are present in the autoregulatory

loop of the kinase domain. Phosphorylation of these residues leads to further activation of the kinase. Phosphorylation of the other residues leads to the creation of docking sites for adaptor proteins that couple the receptors to intracellular signalling cascades, including the Ras/extracellular regulated kinase (ERK) protein kinase pathway, the phosphatidylinositol-3-OH kinase (PI3K)/Akt kinase pathway and phospholipase C-γ1 (PLC- γ1), which culminate in the activation of transcription factors. The various signalling pathways of the Trk receptors are represented in Figure 1.2.

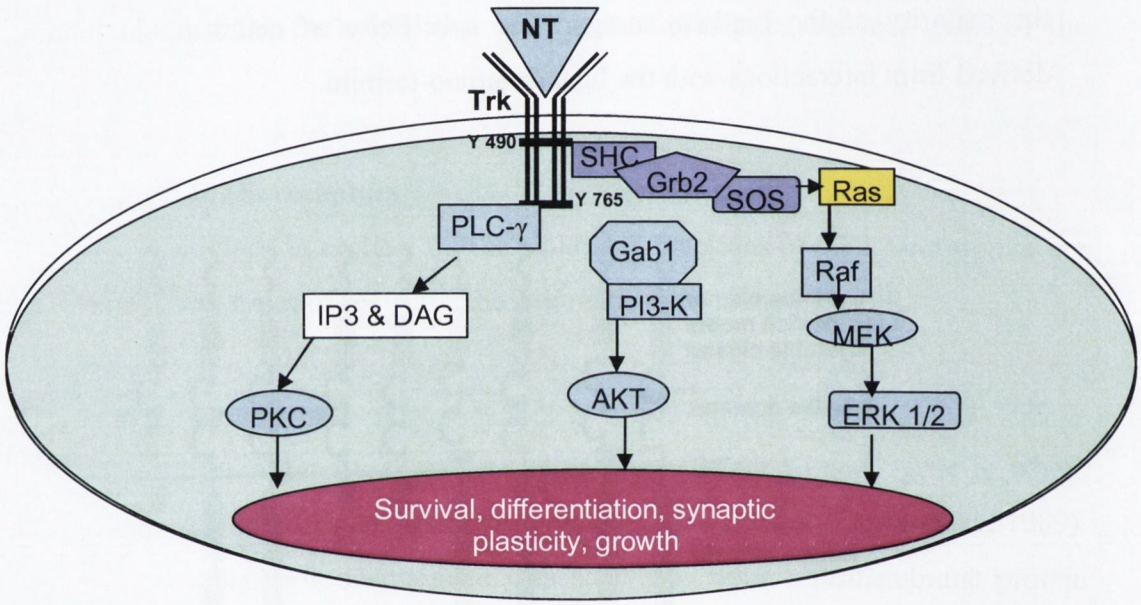


Figure 1.2 Trk signalling pathways

Signalling through Ras

Activation of the Ras signalling cascade is essential for neuronal survival and differentiation through activation of the mitogen-activated protein kinases (MAPK)/ERK pathway. Numerous pathways lead from Trk receptors to the activation of Ras the majority of which appear to involve the phosphorylation of the Y490 tyrosine residue. Upon phosphorylation Y490 creates a recruitment site for binding of the adaptor protein, Shc. When phosphorylated, Shc recruits the adaptor protein, Grb2 and the son of sevenless (SOS) at the membrane which results in Ras activation. The presence of Ras activation leads to the activation of downstream signalling pathways that include PI3-kinase, Raf and p38MAP-kinase. Activation of

Erk1 and Erk2 is preceded by the sequential phosphorylation by Raf of MEK-1 and/or MEK-2 and their phosphorylation of ERK 1 and ERK 2. This Trk receptor mediated stimulation of Ras through Shc and Grb-2/SOS results in transient and not prolonged activation of Erk signalling. Prolonged signalling through ERK appears to be mediated by another signalling pathway involving the adaptor protein Crk, the G protein Rap-1 the protein tyrosine phosphatase Shp2 and the serine threonine kinas B-Raf. The activation of this pathway depends on the recruitment of Frs2 by phosphorylated Y490 (See Roux and Barker, 2002; See Patapoutian and Reichardt, 2001).

Signalling through PI3-K

The PI3-K pathway is another important pathway in Trk-dependent survival. The pathway can be activated through both Ras dependent and Ras independent pathways. The Ras independent pathway involves the activation of the Shc/Grb2 adaptor protein complex. Binding to phosphorylated Y490 of Shc results in recruitment of Grb2. Phosphorylated Grb2 provides a docking site for Gab1, which in turn is bound by PI3-K. PI3-K can activate a number of signalling proteins. Akt is just one of the signalling molecules activated by PI3-K and is known to be involved in NGF mediated survival signalling (See Roux and Barker, 2002; See Patapoutian and Reichardt, 2001).

Signalling through PLC-γ

Phosphorylated Y785 on TrkA and similarly placed tyrosine residues on other Trk receptors recruit PLC-γ. The Trk kinase then phosphorylates and activates PLC-γ1, which acts to hydrolyse phosphatidylinositides to generate diaceylglycerol (DAG) and inositol 1, 4, 5 trisphosphate (IP_3). IP3 promotes the release of calcium ions (Ca^{2+}) from internal stores. This increase in intracellular Ca^{2+} results in the activation of enzymes such as Ca^{2+}- regulated isoforms of protein kinase C and Ca^{2+}-calmodulin-regulated protein kinases (See Roux and Barker, 2002; See Patapoutian and Reichardt, 2001).

1.2.1.2 p75NTR

During the late 1970's binding studies of NGF to chick sensory neurons identified that there were two NGF receptors. One of the receptors identified

displayed a fast rate of dissociation from NGF (kd 10^{-9} M) and was termed the fast NGF receptor. In contrast the other displayed a slow dissociation rate, with a dissociation constant of 10^{-11} M, and was subsequently named the slow NGF receptor. The molecular weights were ascertained by cross linking experiments and were found to be 75kDa and 140kDa respectively. The lower affinity receptor was cloned from rat and human melanoma cells which were found to be highly homologous. Resultantly, the receptor became known as the p75 NGF receptor (p75NGFR). The receptor has since been shown to bind all of the neurotrophins with approximately equal affinity and is now known as the p75 neurotrophin receptor (p75NTR).

Despite its discovery almost 30 years ago the biological functions of the p75NTR are still somewhat ambiguous. Initially, the receptor was believed to play an ancillary role to the Trk receptors and to be biologically redundant on its own. It is now emerging that the p75NTR is important in its own right.

p75NTR: Ligands

The p75NTR has been shown to bind each of the neurotrophins (NGF, BDNF, NT-3 and NT-4) with approximately equal affinity (Sutter *et al.*, 1979; Rodriguez-Tebar *et al.*, 1990; Ernfors *et al.*, 1990). The p75NTR has been shown to bind the pro form of each of the neurotrophins with a higher affinity than that of the mature forms (Hempstead *et al.*, 1991). A number of studies have shown that the p75NTR is capable of binding ligands other than the neurotrophins. Tuffereau *et al.* (1998) revealed that the p75NTR is capable of binding the rabies virus. The receptor has also been found to bind β-amyloid (Yaar *et al.*, 1997) as well as prion peptides (Della- Bianca *et al.*, 2001).

p75NTR Structure

The p75NTR was the first member of what is now the tumour necrosis factor (TNF) superfamily of receptors. Like the Trk receptors it too is a type I transmembrane protein (Figure 1.3). The extracellular domain of the receptor consists of four cysteine rich loops, with six cysteines per loop. It is within these cysteine loops that the neurotrophin binding domains are contained (Yan and Chao, 1991). The cytoplasmic domain features a death domain which consists of six α

helices and possesses a palmitoylation site at Cys 250 or 279. A number of studies have indicated that this region does not have the capability of inducing apoptosis on its own and requires the interaction of adaptor proteins. Contrastingly, it has been suggested that the p75NTR death domain has a prosurvival function (Khursigara *et al.*, 2001). In 2000, Coulson *et al.* identified a cytoplasmic juxtamembrane region of the p75NTR, which they named Chopper, to be essential for the initiation of neural death.

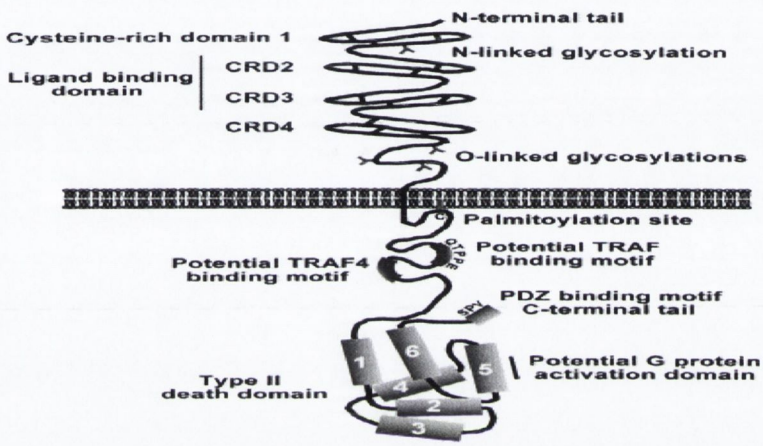


Figure 1.3 Structure of p75NTR: Image taken from Roux and Barker (2002).

p75NTR Expression

Central Nervous System

The p75NTR is expressed in a wide variety of cells in the central nervous system. Its expression appears in several neuronal populations as they differentiate, including spinal motor neurons and brain stem motor nuclei, lateral geniculate nucleus, medial terminal nucleus of the accessory optic tract, ventral and dorsal cochlear nucleus, thalamic nucleus, amygdala, cortical subplate neurons and deep nuclei of the cerebellum. Postnatally, expression of the p75NTR is significantly reduced in most tissue and is confined to the basal cholinergic neurons, motor neurons and the cerebellar Purkinje neurons (See Roux and Barker, 2002).

Peripheral Nervous system

In the peripheral nervous system, there is p75NTR expression in the developing autonomic and sensory nervous system from the earliest stage of development. In general there is a downregulation of p75NTR expression in adulthood (See Roux and Barker, 2002).

Non-neuronal expression of p75NTR

Expression of the p75NTR has been reported in a number of non-neuronal cell types. Its expression has been observed on endothelial cells (Moser *et al.*, 2004), glial cells (Dowling *et al.*, 1999), B cells of the immune system (Torcia *et al.*, 1996) and hair follicles (Botchkarev *et al.*, 2003).

Expression under pathological conditions

An increasing number of studies have found that there is a significant increase in p75NTR expression in certain pathological conditions including mechanical damage, focal ischemia, stroke and epilepsy. Post mortem examination of the brains of Alzheimer's disease sufferers have shown that there is increased p75NTR expression in the cerebral cortex of these patients. A study using a rat epilepsy model found that increased expression of p75NTR in the hippocampus showed a strong positive correlation with a large number of apoptotic neurons (Roux *et al.*, 1999). The results of these studies have lead researchers to question the function of the re-expression of the p75NTR under certain pathological conditions and whether or not it may be responsible for cell death.

Functions of the p75NTR

The functions of the p75NTR have proved somewhat difficult to elucidate, despite the fact that it was discovered over 20 years ago. It was believed for a number of years that the receptor was biologically redundant however this has been shown to be untrue. It is now known that the p75NTR has a number of functions. Firstly, it modulates Trk receptor signalling (Mamidipudi and Wooten, 2002; Bamji *et al.*, 1998; Yoon *et al.*, 1998). Secondly, the receptor can initiate autonomous signalling cascades that can regulate both cell survival and apoptosis earning its title as a biological paradox. Thirdly, it can interact with a number of co-receptors

including sortilin and Nogo resulting in apoptotic signalling (Nykjaer *et al.*, 2004; Wang *et al.*, 2002). Lastly, it has been shown to promote Schwann cell migration (Anton *et al.*, 1994).

p75NTR: Interaction with Trk receptors

Studies in the early 1990's showed that co-expression of p75NTR with Trk receptors produced high affinity binding sites for NGF and induced enhanced activation of TrkA by NGF. Dissociation studies revealed that expression of p75NTR, in the absence of TrkA, has a rapid rate of ligand association and dissociation. TrkA in a similar study was shown to have slow ligand association and dissociation. However, when both receptors are co-expressed the rate at which NGF can associate with TrkA increases 25 fold (Mahadeo *et al.*, 1994), thus producing high affinity binding sites. Co-expression of the receptors also means that the TrkA receptor can be activated by relatively low concentrations of NGF. Hantzopoulos *et al.* (1994) found that a truncated variant of p75NTR enhanced the ability of all the neurotrophins to bind their specific Trk receptor (i.e. NGF to TrkA, BDNF to TrkB, NT-3 to TrkC). Interestingly, the co-expression of p75NTR with Trk receptors attenuates the activation of the receptors by the other neurotrophins. In the presence of p75NTR NGF activates TrkA but its activation by NT-3 and NT-4/5 is greatly attenuated. Likewise, BDNF mediated TrkB activation is not affected by p75NTR expression but its activation by NT-3 and NT4/5 is reduced. The mechanism by which p75NTR expression has these ligand specific effects is not greatly understood but a number of hypotheses have been put forward. It has been suggested that the attenuation in activity is due to a physical interaction between the receptors via several adaptor proteins. Those most likely to be involved are ARMS (ankyrin repeat-rich membrane spanning adaptors) (Kong *et al.*, 2001) and Caveolin (Bilderback *et al.*, 1997) both of which have been demonstrated to interact with both types of receptor. It has also been proposed by Roux and Barker (2002) that the suppression of activity of specific ligands is due to receptor transmodulation in which phosphorylation of specific serine and threonine residues within the cytoplasmic domain of tyrosine kinase receptors results in inhibition of ligand mediated receptor activation. This theory has been supported the work of MacPhee and Barker (1997) who have shown an increase in serine phosphorylation of TrkA following p75NTR activation.

p75NTR can also influence signalling pathways initiated by Trk. Epa *et al.* (2004) demonstrated that p75NTR could bind Shc, an important adaptor protein intrinsically involved in Trk signalling, stimulate its phosphorylation and thereby augment Trk signalling and subsequently enhancing survival signalling.

Trk receptors are also capable of affecting p75NTR signalling and expression. Rankin *et al.* (2005) reported that TrkA expression plays a role in the modulation of p75NTR expression. They found that the expression of p75NTR was upregulated in the presence of NGF in PC12 cells and a mutated PC12 derivative expressing mutated TrkA receptors. Using four mutated cell lines, they identified a role for the individual tyrosine autophosphorylation site Y490 in regulating p75NTR expression. The activation of Trk receptors have been shown to inhibit p75NTR apoptotic signalling. Activation of Trk receptors suppresses the activation by p75NTR of sphingomyelinase. The p75NTR mediated activation of sphingomyelinase leads to the production of ceramide whose accumulation is known to promote apoptosis and mitogenesis in different cell types through control of many signalling pathways (Brann *et al.*, 2002). TrkA activation has also been shown to suppress p75NTR initiated JNK signalling, which in the absence of Trk promotes apoptosis.

p75NTR: Interaction with other receptors

As mentioned previously, the p75NTR is capable of binding the unprocessed forms of the neurotrophins. Upon binding proNGF the p75NTR was demonstrated to induce apoptosis but the mechanism as to how it was binding and mediating the effects were unknown. In 2004, Nykjaer and colleagues reported that the interaction between the p75NTR and the neurotensin receptor sortilin was essential for proNGF-induced neuronal cell death. p75NTR has also been shown to interact with the Nogo receptor (NogoR) and through this receptor complex activate RhoA (Schweigreiter *et al.*, 2004; Wang *et al.*, 2002). RhoA activation mediated the effects of CNS-derived myelin-based growth inhibitors that include Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgP) thereby inhibiting neuronal outgrowth.

p75NTR and Survival

In some circumstances the p75NTR is capable of mediating cell survival. Kume *et al.* (2000) reported that NGF protects cultured cortical neurons that express p75NTR but not TrkA against glutamate cytotoxicity. p75NTR has been shown to protect cortical and hippocampal neurons from calcium mediated hypoglycaemic damage (Cheng *et al.*, 1991). In the presence of NGF, p75NTR can rescue cells from apoptosis by activating NFκB (Gentry *et al.*, 2000). It has been reported that the survival of Schwann cells was mediated via the death domain of the p75NTR and its interaction with the adaptor protein receptor interacting protein 2 (RIP2) (Khursigara *et al.*, 2001). p75NTR signalling through PI3K/Akt has been shown promote survival (Roux *et al.*, 2001). More recently, small nonpeptide, monomeric compounds were identified that interact with the p75NTR (Massa *et al.*, 2006). These peptides promoted survival signalling through p75NTR and inhibited proNGF-induced death.

p75NTR and Apoptosis

The ability of the p75NTR to induce apoptosis was first reported by Rabizadeh *et al.* (1993). Two cell lines, CSM 14.1 and R2 both of which had undetectable levels of TrkA expression, were transfected with high levels of p75NTR expression. They demonstrated that neurotrophin withdrawal induced p75NTR mediated apoptosis. The level of apoptosis was somewhat reduced by the addition of NGF and the application of a p75NTR antibody. Barrett and Bartlett (1994) employed the use of antisense oligonucleotides to down regulate the expression of the p75NTR in sensory neurons isolated from dorsal root ganglia. Interestingly they observed that a downregulation in p75NTR expression prevented NGF mediated survival of sensory neurons from embryonic day 12 and 25 but caused a significant increase in the number of neurons that survived from embryonic day 19 and postnatal day 2. They postulated that the differential expression of the NGF receptor TrkA was responsible for the different outcomes of p75NTR. Frade *et al.* (1996) demonstrated that developmental cell death in the avian retina could be reduced by the application of an antibody directed against the extracellular domain of the p75NTR. In contrast to the work of Rabizadeh, Frade's group found that the application of an antibody directed towards NGF also reduced developmental death suggesting that the p75NTR-induced apoptosis in this instance

was ligand-dependent. Subsequent studies by Frade and colleagues (1996) found that mice lacking the NGF allele or with a p75NTR^{exonIII} deletion had reduced apoptosis in the developing spinal cord and retina when compared to controls. Mice with the same p75NTR^{exonIII} deletion were subsequently found to have supernumerary sympathetic neurons during the neonatal period (Bamji *et al.*, 1998). Using neonatal hippocampal cell cultures, Friedman (2000) demonstrated that the p75NTR-induced apoptosis upon binding of each of the neurotrophins. Neurotrophin binding resulted in a 30-40% loss of neurons when compared to controls. Work carried out by Bono *et al.* (1999) suggests that NGF/p75NTR-induced apoptosis is cell cycle dependent. Using neuroblastoma cells they reported that NGF was pro-apoptotic on growing cells preferentially expressing p75NTR and was anti-apoptotic in quiescent cells, when TrkA expression was prevalent. Similar to Barretts and Bartletts proposal this suggests that the pro-apoptotic signalling of the p75NTR depends on a ratio of expression between it and Trk. As mentioned in section 1.2.1.2, p75NTR expression is greatly reduced in all cell types in adulthood and its expression can be induced by injury. A number of studies have reported that this injury-induced expression is tightly correlated with the degenerative effects observed following the trauma. p75NTR mRNA and protein expression are increased in rat motor neurons following sciatic nerve lesion (Ernfors *et al.,* 1989). The expression of p75NTR was increased in the hippocampus following pilocarpine-induced seizures and was tightly correlated with apoptosis (Roux *et al.*, 1999). Casha *et al.* (2001) reported that oligodendrocyte apoptosis is associated with p75NTR expression following spinal cord injury. p75NTR expression was also associated with neuronal degeneration following experimentally-induced ischemia (Greferath *et al.*, 2001). The binding of β-amyloid to the p75NTR has been shown to induce apoptosis and has been suggested as a possible mechanism for Alzheimer's disease (Yaar *et al.*, 1997; Perini *et al.*, 2002).

p75NTR and the proneurotrophins

Like many growth factors the neurotrophins are synthesized as immature precursors that a proteolytically cleaved by furin, matrix metalloproteinases (MMP's) and other proconvertases (Lee *et al.*, 2001). It had been proposed that the functions of these prodomains were simply to promote protein folding (Suter *et al.*, 1991) and regulate neurotrophin secretion (Rattenholl *et al.*, 2001). Sequencing of

the proneurotrophins revealed that regions of the pro-domain were highly conserved across species (Heinrich and Lum, 2000). These data lead to the speculation that the proneurotrophins had a more complex biological function than originally thought. As outlined the p75NTR has conflicting biological functions in that it can induce pro-survival and pro-apoptotic signalling pathways. It was observed that apoptotic signalling via the p75NTR required high concentrations of neurotrophins to be present (Friedman, 2000). This finding suggested that perhaps there were other more specific ligands for the p75NTR that had yet to be discovered. In 2001, Lee *et al.* reported that both proNGF and proBDNF are secreted and cleaved extracellularly by plasmin and specific MMP's. They also demonstrated that a furin resistant proNGF was a high affinity ligand for the p75NTR and was ten times more potent than mature NGF in inducing apoptosis in a vascular smooth muscle cell line that expressed p75NTR but not Trk receptors. However, not all p75NTR expressing cells respond to proNGF (Nykjaer *et al.*, 2004), suggesting that the presence of other membrane proteins were required for the induction of cell death. Nykjaer and co-workers reported that proNGF creates a signalling complex by binding to both p75NTR and the neurotensin receptor, sortilin and that this signalling complex induces neuronal cell death. ProBDNF was also shown to elicit neuronal apoptosis in sympathetic neurons via the activation of a p75NTR/sortilin receptor complex (Teng *et al.*, 2005).

Increased levels of proNGF have been observed in certain pathological conditions and it has been proposed that the neurodegeneration observed in these conditions may be as a result of proneurotrophins binding to p75NTR. Increased levels of proNGF in the brain have been reported in Alzheimer's disease (Fahnestock *et al.*, 2001). This Alzheimer's disease related increase in proNGF has been correlated with loss of cognitive function (Peng *et al.*, 2004). When isolated from the human brain affected by Alzheimer's disease and introduced to neurons in culture, proNGF-induced apoptosis was mediated by the p75NTR (Pedraza *et al.*, 2005). Both proNGF and p75NTR expression are induced following spinal cord injury and proNGF present in spinal cord lysates induced apoptosis *in vitro* (Beattie *et al.*, 2002). ProNGF is secreted following lesion of corticospinal neurons and induces apoptosis via activation of the p75NTR. This effect was ameliorated by inhibition of proNGF binding to p75NTR *in vivo* (Harrington *et al.*, 2004). Kainic

acid–induced seizures elicited production of proNGF by basal forebrain astrocytes resulting inducing p75NTR activation and caspase activity (Volosin *et al.*, 2006). These data indicate that proneurotrophins are produced in the brain under pathological conditions and can elicit apoptosis via activation of the p75NTR.

It was reported in 2005 that the p75NTR played an integral role in synaptic plasticity in the form of long-term depression (LTD) (Rösch *et al.*, 2005). Later that year it was detailed that the modulation of LTD by the p75NTR involved proBDNF binding to the receptor (Woo *et al.*, 2005). It would appear that the full functional consequences of the proneurotrophins binding to the p75NTR receptor have yet to be fully elucidated.

p75NTR Signalling

The pleiotrophic functions of the p75NTR means that there are several distinct signalling pathways that are activated downstream of the receptor (Figure1.4). Therefore p75NTR will be discussed in relation to each of its functions.

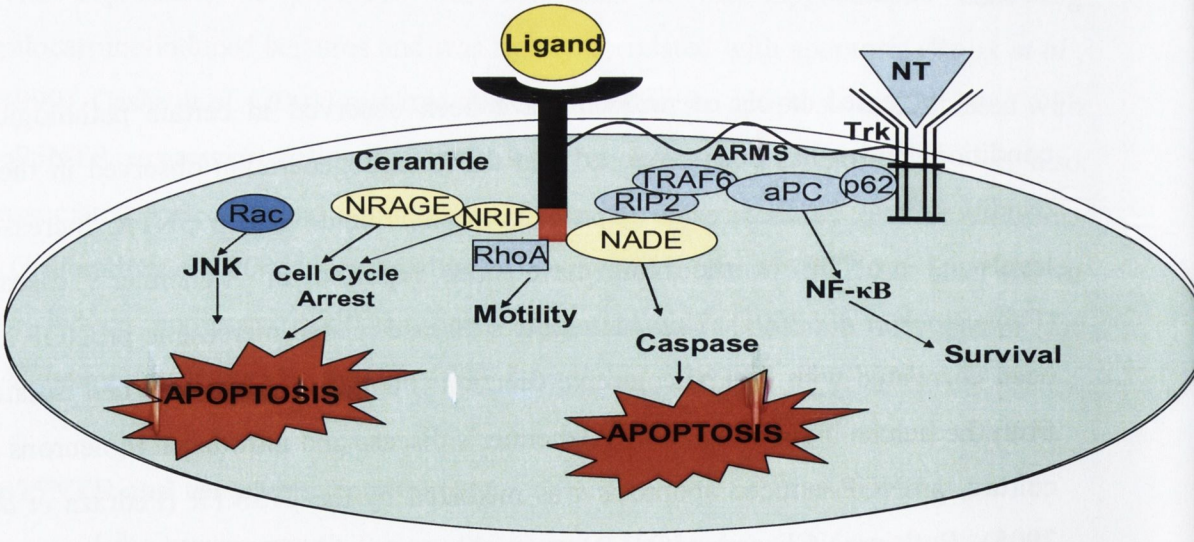


Figure 1.4 p75NTR signalling pathways

p75NTR mediated survival signalling

p75NTR mediated survival appears to involve the activation of nuclear factor kappa-B (NFκB). Activation of NFκB in p75NTR signalling was first reported by Carter *et al.* (1996). They reported that in cultures of Schwann cells, NGF binding to p75NTR resulted in an increase in NFκB DNA binding activity. This p75NTR mediated increase in NFκB activity has been shown in a number of cell types all of which report a promotion of survival following its activation (Hamanoue *et al.*, 1999; Gentry *et al.*, 2000; Khursigara *et al.*, 2001). Khursigara and colleagues identified a role for the p75NTR death domain in survival signalling. They identified an interaction between the death domain of the p75NTR and RIP2. RIP2 is an adaptor protein with a serine threonine kinase and a caspase recruitment domain (CARD). It is the CARD domain of RIP2 that binds to the p75NTR in a ligand-dependent manner. They found that the interaction of RIP2 with p75NTR enhanced NFκB activity and blocked the apoptosis in Schwann cells that was induced by NGF.

p75NTR has also been shown to activate AKT (Protein kinase-B (PKB)) via a PI3-K/AKT dependent pathway (Roux *et al.*, 2001). This pathway is an important survival signalling pathway that is frequently activated downstream of the Trk receptors. Its activation by p75NTR was shown to be independent of Trk receptor activation.

p75NTR and Apoptotic signalling

As with the majority of things concerning the p75NTR, its apoptotic signalling pathway cannot be confined to one particular pathway and a number of pathways and molecules including c-jun N-terminal kinase (JNK) and ceramide have been implicated in its cell death signalling.

The p75NTR activation of JNK has been shown by a number of groups. Friedman (2000) reported that the ligand-dependent p75NTR activation resulted in the death of hippocampal neurons was mediated via JNK signalling. Similarly, Yoon *et al.* (1998) demonstrated that NGF-induced apoptosis could be blocked by an alkaloid that inhibited the JNK pathway. In oligodendrocytes, the mechanism of JNK activation by p75NTR is dependent on the activation of Rac GTPase

(Harrington *et al.*, 2002). NGF, but not BDNF or NT-3, was shown to prolong the activation of Rac and this prolonged activation was positively correlated with the ability of NGF to induce apoptosis. Rac does not directly interact with the p75NTR but there are a number of adaptor proteins that do. The TNF-Receptor-Associated factor (TRAF) 6 has been shown to interact with the p75NTR via the neurotrophin receptor interacting factor (NRIF) (Gentry *et al.*, 2004; Casademunt *et al.*, 1999). It has been reported that NRIF itself is involved in p75NTR mediated apoptosis since NRIF knockout mice are resistant to p75NTR-induced apoptosis (Linggi *et al.*, 2005). p53 is known to be activated downstream of JNK. Aloyz *et al.* (1998) demonstrated that p53 is activated downstream of p75NTR activation and that when its levels are reduced or absent that p75NTR-induced death of sympathetic neurons is inhibited.

Ceramide-induced apoptosis has also been associated with p75NTR. Ceramide can be generated by the condensation of palmitoyl CoA and serine or by the hydrolysis of sphingomyelin by either acid or neutral sphingomyelinase. The involvement of ceramide in p75NTR signalling was first identified by Dobrowsky *et al.* in 1994. The addition of NGF to T9 glioma cells that expressed p75NTR and not TrkA resulted in an increase in ceramide levels. Further studies found that this elevation in ceramide was responsible for the death of oligodendrocytes (Casaccia-Bonnefil *et al.*, 1996). In 2002, Brann *et al.* reported that the NGF-induced death of cultured hippocampal neurons was mediated via ceramide. The neutral sphingomyelinase inhibitor, sycphostatin, inhibited NGF-induced ceramide generation and neuronal death. Sycphostatin was also shown to inhibit NGF-induced JNK phosphorylation suggesting a role for ceramide generated by neutral sphingomyelinase in the diverse neuronal responses induced by p75NTR.

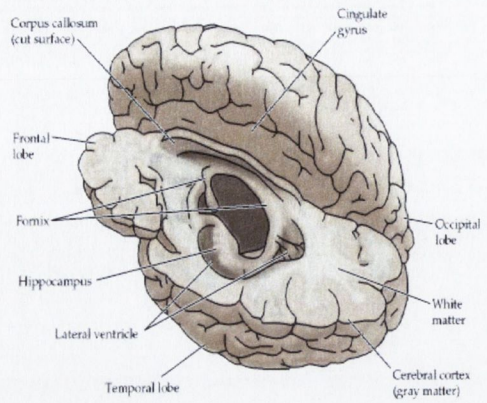
p75NTR mediated neuronal death has also been associated with the activation of caspases. Troy *et al.* (2002) used pilocarpine to induce seizures in order to upregulate the expression of the p75NTR. They elucidated a role for caspase-3 in the neurotrophin induced p75NTR mediated neuronal death that was observed following seizure. The cytoplasmic juxtamembrane region of the p75NTR or "Chopper" as it is known was shown to require the activation of caspase-2, -3 and -8 for execution of cell death (Coulson *et al.*, 2000).

1.3 The Hippocampus

The hippocampus is a structure found in the subcortical brain and forms part of the limbic system. The anatomist Giulio Cesare Anzari first used the term hippocampus having noted that its curved structure resembled that of a seahorse. Structurally, the hippocampus consists of four regions within two c-shaped interlocking cell body layers. These regions are the dentate gyrus, the hippocampus proper which consists of areas CA1, CA2 and CA3, the subicular complex and the entorhinal cortex. Within the hippocampus are three major excitatory pathways. The first is the perforant path, which consists of axons from the entorhinal cortex that synapse with granule cells of the dentate gyrus. Secondly, there are a group of axons which have been designated the mossy fibres of the dentate granule cells which synapse with pyramidal cells of the CA3 region. Finally, the CA3 pyramidal cells synapse with the CA1 neurons to form the Schaffer collateral pathway.

At the beginning of the 1900's Vladimir Bekhterev associated the hippocampus with a role in memory having observed a patient with a profound memory defect. However it was generally considered that like the rest of the limbic system the hippocampus was involved in emotion. It wasn't until the work of Scoville and Milner that the function of the hippocampus was determined after observing patient H.M.. H.M. started suffering from seizures at the age of 10. As he grew older the seizures became progressively worse and it was at the age of 27 that he underwent surgery to correct his epilepsy. A bilateral medial temporal lobe resection was carried out in which the amygdala, uncus, hippocampal gyrus, and the anterior two thirds of the hippocampus were removed. Post-operative evaluation revealed that H.M. had a profound short term memory loss but that he could easily recall his pre-operative memories. Numerous studies have since shown that lesions and damage to the hippocampus inhibits the formation of new memories.

A



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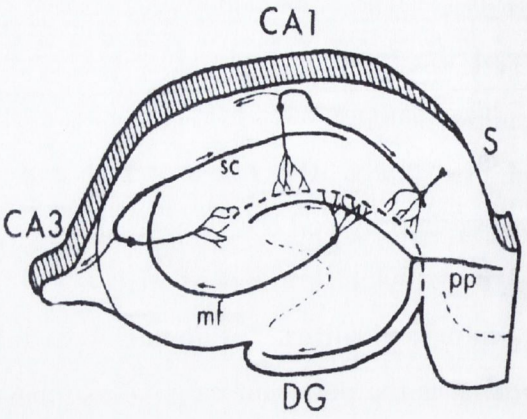


Figure 1.5 The hippocampus

A. **Location of the hippocampus in the human brain**

The hippocampus is located in the medial section of the temporal lobe (Image taken from Neuroscience, Purves et al., 2001)

B. **Pathways in the hippocampus**

The perforant path projects from the entorhinal cortex and forms excitatory connections with the granule cells of the dentate gyrus. Mossy fibre axons of the dentate granule cells synapse with pyramidal cells of the CA3 region. The CA3 pyramidal cells synapse with the CA1 neurons to form the Schaffer collateral pathway.

1.4 Long -Term Potentiation

Alterations and interplay among static and dynamic synaptic connections are required for learning and memory. In 1894, Santiago Ramon y Cajal first proposed this concept of synaptic plasticity. He proposed that new memories might be formed by strengthening the connection between existing neurons. This theory was supported by Donald Hebb in 1949 who went on to propose that neurons may grow new connections in order to enhance synaptic transmission. Long-term potentiation (LTP) is the most intensively studied form of synaptic plasticity and is widely believed to provide an important key to understanding the cellular and molecular mechanisms underlying by which memories are formed and stored. LTP was first described by Timothy Bliss and colleagues in Mill Hill, England in the early 1970's (Bliss and Lømo, 1973). They demonstrated that high-frequency stimulation of the

perforant path fibres of the rabbit hippocampus resulted in a long lasting increase in the synaptic responses of the dendrites of the granule cells of the dentate gyrus and coined this phenonomen LTP. This prolonged enhancement of synaptic transmission was observed to last for hours and even days. This experimental evidence supported the theories proposed by Cajal and Hebb and has provided a model for scientists to employ in order to gain an understanding of the mechanisms underlying memory.

1.4.1 Properties of LTP

LTP has been shown to exhibit certain properties. LTP has been shown to be input specific. This means that when generated at one set of synapses by repetitive activation the increase in synaptic strength does not normally occur in other synapses on the same cell. LTP has also been shown to be state-dependent meaning that the degree of depolarization of the postsynaptic cell determines whether or not LTP occurs. This property of LTP echoes the postulate proposed by Hebb in 1949 which stated that the coordinated activity of the presynaptic terminal and the postsynaptic neuron strengthens the synaptic connections between them. The final property exhibited by LTP is associativity. Weak stimulation of a pathway will not by itself trigger the induction of LTP. However, if one pathway is weakly stimulated at the same time a neighbouring pathway onto the same cell is strongly activated then both synaptic pathways will undergo LTP.

It is these well described properties that lend weight to the argument that LTP is a plausible model that can be constructively used to explain a neural mechanism for long lasting changes in the brain.

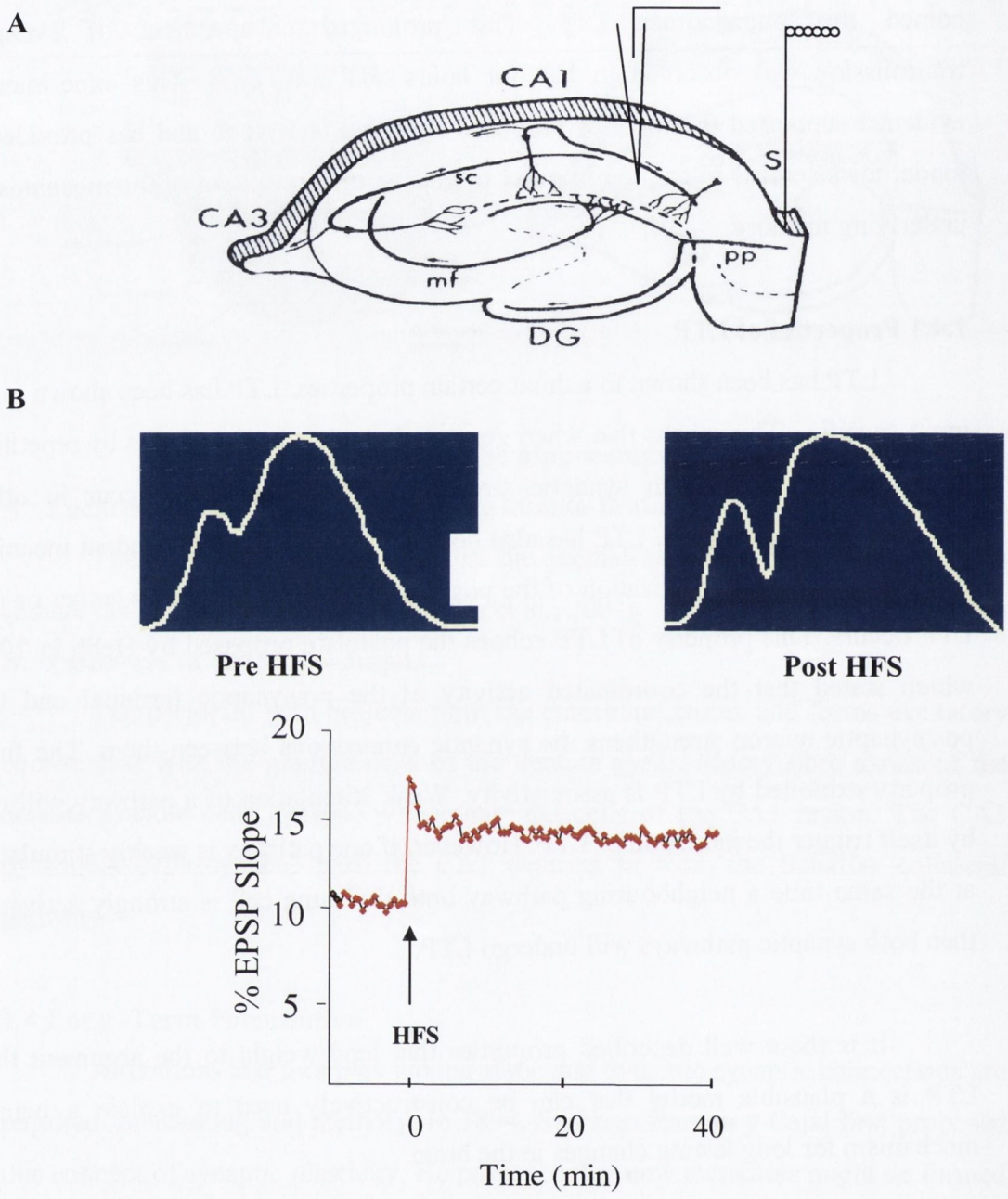


Figure 1.6 Long-term potentiation in the dentate gyrus *in vivo*

A. Stimulating electrodes are placed in the perforant path (pp) of the dentate gyrus a recording electrode was placed in the dorsal cell body of the region. High-frequency stimulation (HFS; 3 trains of stimuli of 200Hz for 200msec at 20sec intervals) was delivered to the perforant path. Recording at test shock frequency resumed for 40min following tetanus.

B. The excitatory postsynaptic potential (EPSP) significantly increases following hfs. This increase is maintained until the end of recording

1.4.2 Induction of LTP

1.4.2.1 Role of the NMDA receptor

Following Bliss and Lomo's initial characterisation of LTP a surge of research was focused on identifying the mechanisms and proteins that attributed to the specific properties of LTP. It was clear that the properties of LTP, particularly associativity relied on some form of communication between synapses. Naturally researchers began to focus on the activation of postsynaptic receptors and as glutamate is the major excitatory amino acid in the central nervous system much of the attention was focussed on the four subtypes of glutamate receptor; *N*-methyl-D-aspartate (NMDA) receptor, α-amino-3 hydroxy-5 methyl4 isoazale proprionic acid (AMPA) receptor, kainate receptor and the metabotropic glutamate receptor (mGluR). It was in the 1980's that the first major breakthroughs emerged providing an insight to the mechanisms behind LTP. In 1983, Collingridge and co-workers observed that application of the NMDA receptor antagonist D-amino phosphonovalerate (AP5) had no effect on basal synaptic activity but blocked LTP in the CA1 region of the hippocampus *in vitro*. This AP5-induced inhibition of LTP was also observed *in vivo* (Errington *et al.*, 1987). These data indicated that the NMDA receptor was playing a pivotal role in the induction of LTP but the mechanism had not yet been elucidated. It was established by Evans and colleagues (1977) that NMDA receptors are blocked by physiological concentrations of magnesium (Mg^{2+}). In 1985, Coan and Collingridge reported that superfusion of hippocampal slices with Mg^{2+}-free medium resulted in an increase in synaptic transmission and that this increase in synaptic excitability was reversed by the application of the NMDA receptor antagonist AP5. This suggested that the NMDA receptors could be activated but that the extracellular concentrations of Mg^{2+} were preventing their activation. Further work ensued and it soon became apparent that the Mg^{2+} block of NMDA receptors was voltage dependent (Nowak *et al.*, 1984). It had been established that AMPA receptors present in the hippocampus were mediators of the synaptic response (Monaghan *et al.*, 1983). Unlike NMDA receptors, AMPA receptors had no physiological activation restrictions. Subsequently the idea that the AMPA receptors would sufficiently depolarize the membrane removing the Mg^{2+} blockade of NMDA receptors and enhanced synaptic transmission began to evolve. The question still remained as to why at low frequency stimulation there was no activation of the NMDA receptors? Receptor

kinetics and pharmacology were to provide the answers. Studies revealed that the NMDA receptor excitatory post synaptic current (EPSC) was slow rising and decaying (Coan and Collingridge, 1987; Collingridge *et al.*, 1988) and it was elucidated that γ-aminobutyric acid (GABA) was inhibiting activation of the NMDA receptor by hyperpolarizing the postsynaptic cell (Herron *et al.*, 1985). Further work established the existence of $GABA_B$ autoreceptors which acting presynaptically inhibited the activity GABAergic inhibitory neurons facilitating the induction of LTP (Davies *et al.*, 1991). These unique properties of the NMDA receptor echoes those of LTP in many respects; NMDA receptors are input specific in that they cannot be activated by depolarization alone but require the presence of glutamate. The properties of associativity and cooperativity are illustrated by the fact that the NMDA receptor requires a significant number of inputs in order to provide sufficient depolarization to facilitate the removal of the Mg^{2+} block of NMDA receptors. While the majority of LTP is dependent on the activation of NMDA receptors it is important to note that not all types of LTP are NMDA dependent. It has been demonstrated that LTP in the CA1 region of the hippocampus can occur in the absence of NMDA receptor activation as a result of a rise in postsynaptic intracellular calcium levels induced by tetanus (Grover *et al.*, 1990).

1.4.2.2 Calcium

The activation of the NMDA receptor alone is not sufficient to induce LTP. It was apparent from the early 1980's that calcium was playing an integral role in the induction of LTP. Intracellular injection of the Ca^{2+} chelator EGTA (Lynch *et al.*, 1983) blocked the induction of LTP. Furthermore, directly raising the concentration of postsynaptic Ca^{2+} by photolysis of stored Ca^{2+} can mimic LTP (Malenka *et al.*, 1988). A fundamental breakthrough in understanding the mechanisms behind the induction of LTP came in 1986 when it was discovered that the activation of the NMDA receptor resulted in an increase in intracellular calcium and that this increase was occurring as a result of calcium entering the cell through NMDA receptor channels (MacDermott *et al.*, 1986). This work has since been confirmed (Regehr and Tank, 1990) and it is now widely accepted that it is the coordination of NMDA receptor activation and the increase in intracellular calcium that leads to the induction of LTP.

1.4.3 Signalling and LTP

Several signalling pathways have been implicated as critical for LTP. For many of the pathways the evidence is conflicting. Those pathways for which the evidence is most convincing are discussed below.

1.4.3.1 Calcium-calmodulin-proteinkinase-II (CaMKII)

Calcium-calmodulin-proteinkinase-II (CaMKII) is one of the molecules for which an overwhelming amount of evidence has been gathered indicating a pivotal role for the molecule in LTP signalling. CaMKII is found in high concentrations in the postsynaptic density and it was established early on that the postsynaptic entry of Ca^{2+} resulted in its activation. The injection of CaMKII inhibitors postsynaptically has been shown to inhibit LTP in the CA1 region of the hippocampus as has the genetic deletion of the CaMKII alpha subunit (Malenka *et al.*, 1989; Malinow *et al.*, 1989; Frankland *et al.*, 2001). Further evidence for CaMKII involvement in LTP came from Lledo and colleagues (1995) who demonstrated that the application of constitutively active CaMKII to a recording pipette lead to a gradual increase in the size of excitatory postsynaptic currents. They also performed occlusion experiments and found that the enhancing action of CaMKII was diminished by prior induction of LTP, indicating that the enhancement in synaptic strength induced by CaMKII shared the same mechanism as the enhancement observed in LTP. The activation of CaMKII by Ca^{2+} initiates autophosphorylation on its threonine 286 residue, rendering it independent of Ca^{2+} and active (De Koninck & Schulman, 1998). This autophosphorylation of CaMKII means that even when Ca^{2+} concentrations return to basal levels the enzyme can remain active. Giese and co-workers in 1998 used a mutant mouse strain in which there was a threonine to alanine substitution at position 286 of α-CaMKII thus preventing autophosphorylation. They observed that these mutants were unable to induce LTP demonstrating that CaMKII autophosphorylation is required for LTP.

1.4.3.2 Mitogen-activated protein kinase (MAPK)

The activation of the mitogen activated protein kinase (MAPK) cascade that leads to the activation of ERK-1 and ERK-2 (also known as the p44 and p42 isoforms respectively) has also been implicated as an important signalling cascade

in the induction and maintenance of LTP. ERK activation comes as a result of an extracellular stimulus which results in the activation of the small G-protein Ras. Activated Ras triggers the activation of the protein kinase Raf which subsequently phosphorylates the MAPK/ERK kinase (MEK) which phosphorylates ERK-1/ERK-2. Upon activation, ERK can interact with transcription factors such as ELK-1, cytoskeletal proteins and other kinases. As mentioned in section 1.2.1.1, ERK activation occurs downstream of Trk receptor activation. However, ERK can also be activated as a result of membrane depolarization as described by Rosen *et al.* in 1994. They found that in PC12 cells, membrane depolarization lead to a calcium influx resulting in the activation of MEK which as previously mentioned phosphorylates MAPK. They also noted that within 30 seconds the calcium influx lead to the activation of Ras which they found to be critical to calcium mediated MAPK signalling. This evidence provided a plausible link between the activation of the MAPK signalling cascade and LTP. The first evidence demonstrating an actual relationship between the two was provided by English and Sweatt in 1997. They employed the use of MEK inhibitor PD 098059 to block the activation of the MAPK signalling cascade during the delivery of LTP-inducing stimuli to the CA1 region of the hippocampus. They reported that the compound PD 0980959 blocked ERK-2 activation and that this inhibition of ERK-2 activity was associated with an impairment in the induction but not the expression of LTP. Since this a number of groups have reported that the inhibition of MAPK signalling results in impairment of LTP not only in the CA1 region of the hippocampus but in also in the dentate gyrus (Coogan *et al.*, 1999).

1.4.3 LTP expression

LTP takes place in two distinct stages: the first is the induction phase or early phase-LTP (E-LTP) which lasts for 2-3 hours and is initiated by the activation of NMDA receptors and an influx of Ca^{2+}. The second phase is known as the maintenance phase or late phase-LTP (L-LTP). L-LTP has been recorded *in vitro* for several hours (Nguyen *et al.*, 1994) and *in vivo* for several months (Abraham *et al.*, 2002) and its expression requires *de novo* protein synthesis.

1.4.3.1 AMPA receptors

For many years after LTP was first described it was debated as to whether the increase in synaptic strength observed in LTP was due to presynaptic or postsynaptic modifications. Several groups have reported that LTP in the dentate gyrus is associated with increased concentrations of extracellular glutamate a finding which was consistent with presynaptic modifications (Dolphin *et al.*, 1982; Bliss *et al.*, 1986). However, these observations have been called into question as other groups have reported no enhancement in glutamate release following LTP (Aniksztejn *et al.*, 1989). More sophisticated electrophysiological experiments have been conducted in order to elucidate the locus of expression. Kauer *et al.* (1988) determined that LTP increases the AMPA receptor-mediated component of the excitatory post-synaptic potential (EPSP) to a greater extent than the NMDA receptor mediated EPSP. This suggested to them that the enhancement of synaptic transmission during LTP is caused by an increased sensitivity of the postsynaptic neuron to synaptically released glutamate. Similarly, a study by Muller and Lynch (1988) determined that the LTP has different effects on the components of the postsynaptic response mediated by the NMDA and AMPA receptors and that it was not a simple case of enhanced glutamate release. Facilitated release of glutamate would have been expected to effect both the NMDA receptor and AMPA receptor component of the field EPSP equally. This was observed using paired pulse facilitation (PPF), a form of presynaptic plasticity, but not LTP. These data lent weight to the argument that the expression of LTP was postsynaptic. It was universally agreed that minimal stimulation could produce failures in synaptic transmission and that the number of failures decreased after the induction of LTP. It was thought that these failures in synaptic transmission were due to failures in neurotransmitter release but the concept of a functionally silent synapse expressing NMDA receptors and not AMPA receptors began to emerge. It was proposed that the induction of LTP at these silent synapses expressing NMDA receptors only resulted in the insertion of AMPA receptors to the postsynaptic membrane rendering them functional and consequently reducing the number of synaptic transmission failures (Liao *et al.*, 1992). Evidence supporting this hypothesis was provided by Isaac *et al.* (1995). They determined that a proportion of excitatory synapses in the CA1 expressed NMDA receptors, had no detectable AMPA receptors, and that these synapses were functionally silent. Furthermore, they observed that when these

synapses were subjected to LTP induction protocol in which the NMDA receptors were repeatedly activated, AMPA receptor EPSC's appeared and remained for the duration of the experiment. It has since been shown that the expression of AMPA receptors on the postsynaptic membrane is modulated by NMDA receptor activation and that this process is accompanied by the mobilization of calcium/calmodulin (CaM) kinase II to the synapse and an increase in AMPA receptor phosphorylation (Liao *et al.*, 2001; Lu *et al.*, 2001). Lu *et al.* demonstrated that AMPA receptor insertion was blocked by inhibiting NMDA receptor activation using AP5, chelating Ca2+ using BAPTA and using intracellular tetanus toxin (TeTx) to determine if the enhanced insertion of AMPA receptors was mediated by a membrane fusion–dependent exocytotic process. Similarly, Luscher *et al.* (1999) reported that inhibition of exocytosis resulted in a decrease in AMPA receptor EPSC. This provided evidence that AMPA receptors are inserted into excitatory synapses via a soluble NSF attachment receptor (SNARE)-dependent exocytosis during LTP. These data supported the findings of several groups who reported an interaction between AMPA receptors and N-ethylmaleimide-sensitive fusion protein (NSF), a protein involved in membrane fusion events (Nishimune *et al.*, 1998; Song *et al.*, 1998). Nishimune and coworkers eloquently demonstrated that interference of NSF binding to the c-terminal of GluR2 in rat hippocampal CA1 pyramidal neurons *in vitro*, results in a progressive decrement of AMPA receptor mediated synaptic transmission. This AMPA/NSF interaction was also observed *in vivo* (Song *et al.*, 1998) and in subsequent experiments performed by the same group a decrease in EPSC amplitude was observed following the disruption of NSF binding to AMPA. These data indicate that insertion of AMPA receptors into the membrane is an integral component of LTP.

1.4.3.2 Metabotropic Glutamate receptors

With the ionotropic glutamate receptors NMDA and AMPA playing a pivotal role in LTP it was not long before investigators turned their attention to the metabotropic glutamate receptors (mGluR's) and raised the question as to whether this group of glutamate receptors were involved in LTP. The first evidence indicating a role for the mGluR's in LTP came in 1991 when it was reported that application of the non-selective mGluR agonist ACPD enhanced LTP in the hippocampus (McGuinness *et al.*, 1991). It would appear that the involvement of

mGluR's in LTP is subtype dependent. There are eight different subtypes of mGluR. Jia *et al.* (1998) described that mice lacking mGluR5 showed a complete loss of NMDA receptor-mediated component of LTP but not the AMPA receptor-mediated component. They also reported that the deficit in LTP could be rescued by the application of 4beta-phorbol-12,13-dibutyrate (PDBu) which stimulates protein kinase C (PKC). This suggests that PKC may couple the postsynaptic mGluR5 to the NMDA-receptor potentiation during LTP.

1.4.3.3Protein synthesis in late-phase LTP

LTP has been recorded for weeks *in vivo* (Abraham *et al.*, 2002). The differential mechanisms between induction and maintenance of LTP have long been the focus of many research groups. It was apparent that a fundamental change occurred resulting in the maintenance of LTP but the specifics of this change was not apparent until the early 1980's. It was reported that LTP lead to an increase in newly synthesized proteins *in vitro* (Duffy *et al.*, 1981). Further evidence supporting the role of protein synthesis in the maintenance of LTP was provided by Krug and co-workers (1984). They reported that intraventricular injection of the protein synthesis inhibitor ansiomycin had no effect on the induction of LTP but 3-4 hours post tetanization a progressive decay in potentiation was observed. Similarly, Otani *et al.* (1992) observed an increase in extracellular protein concentrations which were correlated with the maintenance of LTP. In a subsequent experiment they applied the protein synthesis inhibitor cycloheximide 10–15 min before LTP induction. The drug blocked both the maintenance of LTP and the elevation of extracellular protein concentrations. In a sophisticated study performed by Fazeli and colleagues (1994) gave an insight to the time line of protein synthesis. Induction of LTP unilaterally resulted in the in an increase in protein synthesis 3 hours after the induction of LTP.

1.5 Spatial Memory

"The ability to find objects, recall previous locations and navigate throughout the world is dependent on spatial learning and memory" (Astur *et al.*, 2002). Spatial memory has been the focus of intense research not only because of its fundamental importance to everyday life but also because of its dependence on the integrity of the hippocampus. The importance of the hippocampus to spatial

learning and memory was demonstrated by Morris *et al.* (1982). They employed the use of an open field watermaze in which rats were trained to escape from the water by swimming to a hidden platform. This task was coined the Morris water maze and it relies on the rats use of spatial cues to identify the location of the hidden platform. Morris and co-workers observed that rats with lesions to the hippocampus were impaired in their ability to complete the task. Several other protocols have been developed in order to assess spatial memory and learning. The object displacement task (Poucet *et al.*, 1986; Thinus-Blanc *et al.*, 1987) is commonly used as it relies on an animal's propensity to explore without inducing stress or introducing a reward system. Animals were exposed to an arena in which four objects were placed. Animals were allowed actively explore this environment for a set period of time (e.g. four 3minute sessions with a 3minute rest between each session). Following this exposure to the arena one of the objects was displaced and animals were placed into the arena. Animals with intact hippocampii successfully recognised that one of the objects had been displaced and spent more time actively exploring it (Poucet, 1989).

1.6 Object Recognition Memory

Object recognition memory can be described as the ability to discriminate the familiarity of previously encountered objects. In 1950, Berlyne carried out an experiment that allowed rats to explore three copies of an object in the open field arena. One of the objects was replaced with a novel object and when re-exposed to the arena the rats showed an exploratory preference for the novel object. Ennaceur and Delacour (1988) described the novel object preference protocol that is employed by most groups. A rat is placed in an open field arena and allowed to explore two objects. After a delay the rat is placed back into the arena and with a familiar object and a novel object. Normal rats will spend a greater proportion of their time exploring the novel object as they recognise the familiar object. One benefit of the object-recognition task is that it can be considered as a 'pure' memory test as there are no incentives for the rats to explore, as the usual positive or negative reinforcers (such as food and electric shocks) are absent from the test .

There are a number of studies that implicate that the hippocampus plays a critical role in object recognition learning (Beason-Held *et al.*, 1999; Cave and Squire, 1991)

1.7 The Neurotrophins and Synaptic Plasticity

The neurotrophins are expressed throughout the nervous system but what makes them a particularly interesting group of proteins in relation to synaptic plasticity is that their expression can be activity-dependent. Normal physiological activity that is capable of inducing LTP increases BDNF and NGF mRNA (Castren *et al.*, 1993). Similarly, neuronal depolarization by glutamate also increases the level of BDNF and NGF mRNA (Zafra *et al.*, 1991).

The role of neurotrophins in synaptic plasticity was first demonstrated by Lohof and colleagues in 1993. They reported that applying exogenous BDNF and NT-3 to the neuromuscular junction of *Xenopus* caused an increase in synaptic efficacy. Since the realisation that neurotrophins were not merely involved in the developmental stages of structuring the nervous system several groups began looking at their involvement in synaptic plasticity. Following Lohof's work, it was discovered that neurotrophins could enhance glutamatergic transmission in the CNS (Lessmann *et al.*, 1994; Kang and Schuman, 1995). Since LTP is one of the most investigated forms of synaptic plasticity it wasn't long before a role for the neurotrophins in LTP emerged. The first critical evidence implicating a role for neurotrophins in LTP was provided by Korte *et al.* in 1996. The genetic deletion of BDNF in mice impaired LTP in the CA1 region of the hippocampus. They found that this impairment could be rescued by introducing exogenous BDNF. This work was further supported by groups who found that an impairment in LTP was observed when using a BDNF-chelating TrkB IgG and antibodies directed against BDNF (Chen *et al.*, 1999; Figurov *et al.*, 1996). Interestingly, BDNF has been shown to phosphorylate and activate CaMKII, one of the most important signalling molecules underlying LTP (Blanquet and Lamour, 1997). Using the TrkB inhibitor K252A, Gooney and Lynch (2001) demonstrated that LTP in the dentate gyrus is accompanied by BDNF activation of TrkB. The involvement of TrkB and BDNF has also been demonstrated to be key to the successful completion of a number of hippocampal dependent learning tasks. Both BDNF and TrkB knockout mice failed

to learn the Morris Water Maze (Gorski *et al.*, 2003; Minichiello *et al.*, 1999) and BDNF hippocampal levels have been positively correlated to the ability to successfully complete a spatial task (Francia *et al.*, 2006). Similarly, NGF has been shown to play a role in the induction of LTP. Kelly *et al.* (1998) demonstrated this using an inbred strain of genetically hypertensive (GH) wistar rat in which NGF concentration and Trk expression are decreased. The GH rats were impaired in LTP in the dentate gyrus. Similar to Korte and colleagues, Kelly and co-workers found that the application of exogenous NGF was capable of reversing this deficit. Similar to BDNF and NGF, NT-4 has been shown to play a role in long lasting LTP. Xie *et al.* (2000) found that mice with a targeted deletion of the NT-4 gene were deficient in long-term memory which was assessed by fear conditioning and in long lasting LTP.

There have been few reports on the involvement of the p75NTR and synaptic plasticity. A study by Greferath *et al.* (2000) established a relationship between the expression of the p75NTR and impaired spatial learning in aged rats. Contrastingly, Wright *et al.* (2004) have reported that p75NTR knockout mice perform poorly in the Morris water maze task, which is used to test reference memory. Recently, Rösch *et al.* (2005) investigated the role of the p75NTR in LTP and in another form of synaptic plasticity long-term depression (LTD). LTD occurs when neurons are stimulated at low-frequency (~1Hz) for long periods (10-15 minutes). This depresses the excitatory postsynaptic potential (epsp) for several hours. Like LTP it is specific to the activated synapses. LTD can erase the increase in epsp size due to LTP. Using two strains of mutant mice carrying mutations of the p75NTR gene they reported that LTP was unaffected in the hippocampal slices of both strains of mutant. LTD, however, was impaired in both groups of mutants and interestingly found that the receptor regulated the expression of two of the AMPA receptor subunits. Woo *et al.*, (2005) reported that the facilitation of LTD by the p75NTR is mediated by activation of the receptor by proBDNF. Interestingly, they observed that p75NTR null mice showed a decrease in expression of the NMDA receptor subunit NR2-B and that activation of p75NTR by proBDNF enhanced NR2-B mediated synaptic currents and LTD. Both of these studies, although conflicting in their mechanistic views, show a role for p75NTR in synaptic plasticity.

1.8 Lipopolysaccharide

Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria. It was discovered by Richard Pfeiffer almost 100 years ago when he noted that this heat stable toxin was in fact a part of the bacterial cell and consequently named it an endotoxin. The endotoxin consists of a hydrophilic polysaccharide part and a covalently bound hydrophobic lipid component termed lipid A. LPS is known to induce a profound immunostimulatory and inflammatory response. LPS can cause a variety of pathophysiological reactions including fever, leukopenia, tachycardia, tachypnoe, hypotension, disseminated intravascular coagulation and multiple organ failure.

LPS upon entering the bloodstream binds to lipopolysaccharide binding protein (LBP) (Tobias *et al.*, 1986). This LPS/LBP complex subsequently interacts with CD14, a 55-kDa glycosylphosphatidyl (GPI) anchored protein (Wright *et al.*, 1990).The toll-like receptor 4 (TLR4) was identified as the signalling receptor for LPS (Poltorak *et al.*, 1998) and it was established that a soluble protein MD2 that interacts with TLR4 was essential for LPS-induced activation of the receptor complex (Shimazu *et al.*, 1999). The detrimental impact of LPS is not caused by the toxin itself but comes about as a result of the inflammatory response it induces. LPS activates inflammatory cytokines such as tumour necrosis factor-α (TNF-α), members of the interleukin family (IL-1, IL-6, IL-8, IL-12) and interferon-γ (IFN-γ) and stress activated protein kinases (SAPK's) such as JNK.

1.8.1 Lipopolysaccharide, the neurotrophins and synaptic plasticity

Peripheral injection of LPS has been shown to induce functional impairments in the CNS. In 2000, Vereker *et al.* investigated the effect of intraperitoneal (i.p.) injection of LPS on synaptic function in the rat hippocampus. It was reported that the peripheral injection of LPS impaired LTP in the perforant path granule cell synapses of the dentate gyrus. It was proposed that the injection of LPS lead to an increase in caspase-1 activity and subsequently IL-1β expression in the hippocampus. The increased IL-1β concentration results in degenerative changes consistent with apoptosis and that it is as a consequence of these changes that LTP is impaired. This LPS-induced impairment in LTP has been repeatedly observed (Barry *et al.*, 2005; Shaw *et al.*, 2005; Nolan *et al.*, 2004; Kelly *et al.*, 2003).

Peripheral injection of LPS has also produced profound deficits in spatial learning as evidenced by the poor performance of LPS-treated animals in the Morris water maze (Shaw *et al.*, 2001, 2005). It has also been reported that animals treated with LPS performed poorly in the passive avoidance test with LPS-treated rats requiring a greater number of electric shocks than control animals for acquisition of passive avoidance (Tanaka *et al.*, 2006). Interestingly, this LPS-induced deficit in memory was associated with a decrease in the mRNA of BDNF and its receptor TrkB. This suggests that the impairments in function following LPS administration may be as a result of altered neurotrophin concentrations and receptor expression levels. Guan and Fang (2006) found that i.p. injection of LPS significantly decreased BDNF in the hippocampus, frontal cortex, parietal cortex, temporal cortex and the occipital cortex. NGF was also significantly decreased in the hippocampus and all of the previously mentioned cortical areas, except the occipital cortex. NT-3 concentration was also decreased in the frontal cortex. Similarly, Lapchak *et al.* (1993) found that the systemic injection of IL1-β, an inflammatory cytokine stimulated by LPS, decreased BDNF mRNA in the hippocampus. These data suggest that neurotrophin mediated neuroprotection in the CNS is compromised following LPS administration.

1.9 Kainic acid

Kainic acid was isolated in the 1960's from the seaweed *Digenea simplex.* It was used extensively in post-war Japan to eliminate *ascariasis,* a parasite that infects both humans and animals. It is a conformationally restricted analog of L-glutamic acid and is an agonist of the kainate and AMPA classes of ionotropic glutamate receptors. Kainate receptors are distributed throughout the CNS. Specific subunits contribute to kainate receptors exist (GLU_{K5-7} and GLU_{K1-2}) (Hollmann and Heinemann, 1994). Specific regions of the CNS prominently express different kainate receptor subunits. In situ hybridization studies have revealed that GLU_{K5-7} and GLU_{K2} are distributed throughout the cortex, striatum, cerebellum and hippocampus (See Huettner, 2003). GLU_{K1} subunit expression was observed primarily in the CA3 region of the hippocampus and the dentate gyrus (See Huettner, 2003). Like the other ionotropic glutamate receptors NMDA and AMPA, kainate receptors have the capability postsynaptic current in hippocampal CA3 neurons (Castillo *et al.*, 1997; Vignes and Collingridge, 1997). Activation of kainate

receptors mediates a slow excitatory synaptic current which enhances the excitation of CA3 neurons (Castillo *et al.*, 1997).

Olney *et al.* (1974) reported that kainic acid could in fact be toxic and was capable of producing brain lesions. Kainic acid is frequently used to stimulate temporal lobe epilepsy and induce excitotoxicity in experimental animals (Tremblay and Ben-Ari, 1984; Tasker *et al.*, 2002). Administration of kainic acid, both peripherally and intracereberally, induces the selective degeneration of neurons in the hippocampus with neurons in the CA1, CA3 and dentate hilus regions being particularly susceptible to excitotoxicity (Oh *et al.*, 2000; Shetty *et al.*, 2003; Yi *et al.*, 2003). The excitotoxic effect of kainic acid is thought to be predominantly mediated by an influx of Ca^{2+} (Choi, 1985) but it is probable that the precise mechanisms are more complex and involve a number of proteins. A number of groups have implicated the p75NTR in kainic acid-induced apoptosis. In 2000, Oh and colleagues reported that the administration of kainic acid into the basal forebrain of the rat resulted in increased p75NTR expression and neuronal loss. The level of neuronal loss induced by kainic acid administration was substantially reduced by treatment with an anti-p75NTR antibody indicating that the receptor contributes to excitotoxin-induced neuronal death. The role of p75NTR in kainic acid-induced neuronal death was further supported by Yi and colleagues (2003), who reported that the kainic acid administration co-induced p75NTR expression and p75NTR-associated death executor (NADE) as a result of zinc accumulation in degenerating areas. This co-induction lead to caspase-3 activation and resulted in apoptosis. Likewise, Volosin *et al.* (2006) detailed that kainic acid–induced seizures elicited production of proNGF by basal forebrain astrocytes resulting inducing p75NTR activation and caspase activity which is integral to apoptotic signalling (Volosin *et al.*, 2006).

1.9 Genetically Hypertensive New Zealand Otago Wistar Rats

In 1998, it was reported by Kelly *et al.* that the NGF and its receptor, TrkA, played a role in LTP. This was demonstrated by using an inbred strain of genetically hypertensive (GH) rat which had been previously been shown to be NGF deficient in the peripheral nervous system (Messina and Bell, 1991). Kelly and co-workers ascertained that this NGF deficiency was prevalent in the central nervous system as

shown by decreased levels of NGF in the dentate gyrus of the GH rats. It was also determined that there was a corresponding deficiency in TrkA receptor expression in the dentate gyrus of the GH rats. This deficiency in TrkA and NGF made an ideal model to manipulate the expression of the p75NTR and investigate the effect of increased p75NTR expression.

1.10 Aims

The main aims of this study are:

- To establish reliable models of neurodegeneration in which the expression of the p75NTR is upregulated.
- To investigate the effect of differential neurotrophin receptor expression following neuronal insult.
- To assess the impact of differential neurotrophin receptor expression on synaptic function as assessed by LTP and hippocampal-dependent learning tasks.
- To establish the signalling consequences of upregulated p75NTR expression following neuronal insult.

Chapter 2

Materials and Methods

2. Materials and Methods

Materials

Acrylamide	Sigma
Actin antibody	Santa Cruz
Ammonium persulphate	Sigma
Anti-goat IgG HRP	Vector
Anti-mouse IgG HRP	Sigma
Anti-rabbit IgG (biotinylated)	Vector
BDNF antibody	Chemicon
Bio-Rad dye reagent concentrate	Bio-Rad
Bis-acrylamide	Sigma
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
Calcium chloride	Lennox
DeadEnd™ Colorimetric Apoptosis Detection System	Promega
Digitonin	Sigma
Dimethyl sulphoxide	Sigma
ERK2 antibody	Santa Cruz
Ethanol	Lennox
Glucose	Lennox
Glycerol	Sigma
Glycine	Sigma
Hydrochloric acid	Lennox
Hydrogen peroxide	Sigma
Hyperfilm	Amersham
JNK1 antibody	Santa Cruz
Kainic acid	Sigma
Leupeptin	Sigma
Lipopolysaccharide	Sigma
Magnesium sulphate	Sigma
β-Mercaptoethanol	Sigma
Methanol	Lennox
mNGF 2.5 S	Alomone
NGF antibody	Alomone

Nitrocellulose membrane	Amersham
Normal goat serum	Vector
p75NTR antibody	Sigma/Upstate Signalling/ Gift from Dr. Phil Barker
pERK antibody	Santa Cruz
pJNK antibody	Santa Cruz
Potassium chloride	Sigma
Potassium hydroxide	Sigma
Potassium phosphate	Sigma
Prestained molecular weight standard	Santa Cruz
Prestained molecular weight standard (broad range)	Sigma
Prestained molecular weight standard Kaliedoscope	BioRad
ProBDNF	Alomone
ProNGF antibody	Alomone
ReBlot Plus strong antibody stripping solution	Chemicon
REX (p75NTR inhibitor)	Gift from Dr. Louis Reichardt (USCF)
Sodium carbonate	Sigma
Sodium chloride	Sigma
Sodium dodecylsulphate	Sigma
Sodium hydrogen carbonate	Lennox
Sodium hydroxide	Lennox
Sodium phosphate (monobasic)	Sigma
Sodium phosphate (dibasic)	Sigma
Standard grade No. 3 filter paper	Whatman
SuperSignal	Pierce
Tetramethylbenzidine	R&D Systems
Tris-base	Sigma
Tris-HCl	Sigma
Triton-X 100	Sigma
TrkA antibody	Santa Cruz/ Upstate Signalling

TrkB antibody	Santa Cruz
Tween-20	Lennox
Tyrphostin AG879	Calbiochem
Urethane	Sigma
Vectashield®	Vector Laboratories

2.1 Animals

Three strains of animal were used in these experiments; Wistar rats, normotensive (N) New Zealand Otago Wistar rats and genetically hypertensive (GH) New Zealand Otago Wistar rats.

Male Wistar rats, aged 3-4 months (250-300g), were obtained from the BioResources Unit (BRU), Trinity College Dublin. They were an inbred strain and weighed between 250 and 350g. Animals were housed in groups of 4-6. All rats were maintained under a 12-hour light-dark cycle in the BRU and food and water was available *ad libitum*. Ambient temperature was controlled between 22 and 23°C.

The N and GH rats were obtained from breeding colonies in the BRU (a gift from Professor Bell, Physiology Department, Trinity College Dublin). The GH strain of rat was originally bred in the Department of Medicine in the University of Otago, New Zealand. It was developed as a model of essential hypertension by brother sister matings of successive generations of Wistar rats that displayed high blood pressures (~ 140mmHg systolic blood pressure). The N strain was bred from the ancestral stock from which the GH rats were originally bred and serve as controls for the GH rats. All N and GH rats were male and aged between 3 and 4 months.

2.2 Preparation of tissue

Animals were killed by cervical dislocation and decapitation. The brains were quickly removed and placed on ice and the hippocampi and dentate gyrii were dissected free on ice. The complete procedure took approximately 2 min.

2.3 Preparation of slices for freezing

Tissue slices were stored frozen according to the method of Haan and Bowen (1981). Freshly dissected tissue, either dentate gyrus or hippocampus, was sliced bidirectionally to a thickness of 350µm using a McIlwain tissue chopper and rinsed in ice-cold oxygenated Krebs solution (NaCl, 136mM; KCl, 2.54mM; KH_2PO_4, 1.18mM; $Mg_2SO_4.7H_2O$, 1.18mM; $NaHCO_3$, 16mM; Glucose, 10mM) containing $CaCl_2$ (final concentration: 2mM). The slices were allowed to settle and were rinsed twice more in Krebs $CaCl_2$. Finally, slices were rinsed with ice-cold oxygenated Krebs $CaCl_2$ and DMSO (final concentration: 10%) and then stored in this solution

at -80°C until required for analysis. When required, slices were thawed rapidly at 37°C and washed three times with ice-cold oxygenated Krebs solution containing $CaCl_2$ (2mM).

2.4 Preparation of cryostat sections

For cryostat sectioning the dissected half brain was covered in OCT compound (R.A. Lamb LTD, Sussex, UK), quickly frozen in liquid nitrogen onto cork discs for sectioning and stored at -80°C. Glass slides (76x26mm) were coated with subbing solution (0.5% gelatine (w/v), 0.05% chromalum (w/v) in deionised water and allowed to dry overnight at room temperature, thus providing a surface to which the slices could adhere. On the day of sectioning the half brain was equilibriated to -20°C for 20-30min. Saggital sections were cut (10μm thick) and stained with toludine blue for 1 min and viewed by light microscopy (Nikon Labophot, Nikon Instech co., Ltd, Kanagawa, Japan) for detection of the hippocampus. For each animal 30 sections were cut onto 10 subbed slides (3 sections/slide) and were stored at -20°C until required.

2.5 Protein quantitation using the Bradford assay

Protein concentrations were calculated according to the method of Bradford (1976). Samples, which were analysed in triplicate, were added to 155μl deionised water in a 96-well plate (microtest plate; Sarstedt, Ireland). A standard curve was prepared from a stock solution of 200μg/ml bovine serum albumin (BSA) diluted in deionised water ranging from 3.125μg/ml to 200μg/ml. A blank of deionised water was also included. Bio-Rad reagent (40µl) was added to samples and standards and absorbances were assessed at 630nm using a 96-well plate reader (EIA Mulitwell Reader, Sigma). Regression analysis was used to calculate protein concentrations and values were expressed as mg protein/ml.

2.6.1 Induction of LTP

Rats were anaesthetised by an intraperitoneal injection of urethane (1.5g/kg). The absence of a pedal reflex was used to confirm deep anaesthesia. An initial dose of 1g/kg i.p. was administered and was topped up if necessary. In some experiments rats were subsequently injected with either 300μl of 100μg/ml Escherichia Coli LPS (Sigma) or 300μl of saline (0.9% w/v). Rats were monitored for 3 hours after

injection following which LTP was induced as described below. Fur on the scalp was clipped and the head was positioned in a head holder in a stereotaxic frame. A midline incision was made by a scalpel and the skin pulled back to reveal the skull. The periosteum was scraped clean to reveal the skull plates, allowing identification of lambda and bregma. In some experiments, an injection into the 3rd ventricle (coordinates: 0.5mm lateral and 2.5mm posterior to bregma) of either Kainic acid (5µl, 100µM; Sigma) or saline (5µl, 0.9%) was administered one hour prior to tetanic stimulation. In a subsequent experiment, an injection into the 3rd ventricle (coordinates: 0.5mm lateral and 2.5mm posterior to bregma) of either kainic acid (5µl, 100µM; Sigma) or saline (5µl, 0.9%) was co-administered with either the p75NTR inhibitor REX (5µl, 10mg/ml; generous gift from Dr. Louis Reichardt, University of California, San Francisco) or physiological saline (5µl, 0.9%) one hour prior to tetanic stimulation.

A dental drill was used to remove a window of skull exposing the brain and the dura mater. The dura mater was carefully peeled away to facilitate electrode implantation. The recording chamber consisted of a stereotaxic unit attached to the laboratory bench and surrounded by a Faraday cage to isolate it from interference from the external environment. All instruments in the cage were grounded to eliminate 50Hz cycle noise.

2.6.2 Electrode implantation

Bipolar stimulating electrodes and unipolar recording electrodes (Clark Electromedical, UK) were used in this study. The stimulating electrode was placed on the surface of the brain 4.4mm lateral to lambda and initially lowered to a depth of 2mm. The recording electrode was placed on the surface of the brain 2.5mm lateral and 3.9mm posterior to bregma and was also lowered to a depth of 2mm. The positions of the stimulation and recording electrodes were carefully monitored as they were lowered through the cortical and hippocampal layers to the perforant path and the granule cell layer of the dentate gyrus respectively. This was carried out by generating 0.1msec duration, 2msec delay, 4V pulse through the stimulating electrode at a frequency of 0.1Hz. Evoked responses were picked up by the recording electrode and displayed on an Apple Macintosh computer (Performa 200). The stimulating electrode was lowered in increments into the perforant path. The

recording electrode was lowered into the dentate gyrus until the characteristic perforant path granule cell synapse response was observed. The electrodes were finely adjusted so as to maximise the response. The final depth of the recording electrode was between 2.5 and 3.5mm and for the stimulating electrode was between 2.5 and 3mm. Stimuli were then delivered at 30sec intervals.

2.6.3 EPSP recordings

The population field post-synaptic potential (field EPSP) was used as a measure of excitatory synaptic transmission in the dentate gyrus. EPSPs were achieved by passing a single square wave of current at low frequency (0.033Hz, 0.1sec, 2msec delay) generated by a constant isolation unit (IsoFlex, UK), to the bipolar stimulating electrode. The evoked response was transmitted via a pre-amplifier (DAM 50; Differential Amplifier; gain 75, World Precision Instruments, USA) to an analogue-to-digital converter (Maclab/2e, Analog Digital Instruments). This was a digitised system linked to an Apple Macintosh computer (Performa 200) which interfaced with the converter via a specifically written software package (Scope, Version 3.36). This was customised to control both the generation of the square wave pulses and recording of the evoked potentials. The field EPSP's were displayed on-line and could be analysed at the time of recording or in the case of these studies at a later date.

The slope of the EPSP was taken as the main indicator of excitatory synaptic transmission. Test shocks at 1/30sec were recorded for a 10min control period to establish stable baseline recordings. This was followed by delivery of 3 High-frequency trains of stimuli (250Hz for 200msec) at 30sec intervals. Recording at test shock frequency then resumed for 40min. At the end of the experimental period, animals were killed by cervical dislocation and decapitation.

2.7 Surgical procedure and drug delivery

Rats were anesthetized with ketamine (100µg/kg; Bayer Healthcare, BioResources, Trinity College, Dublin, Ireland) and xylazine (10mg/kg; Rompun®, Bayer Healthcare, BioResources, Trinity College, Dublin, Ireland) and supplemented throughout the surgical procedure as necessary. A single hole was drilled in the skull over the left ventricle (coordinates, bregma, 0.9 mm; midline, 1.3

mm), and a cannula (Plastics 1, Bilaney Consultants Ltd., Kent, UK) was lowered slowly into the ventricle to a depth of 3.6 mm below the brain surface. A guide cannula (Plastics 1, Bilaney Consultants Ltd., Kent, UK) was held in place and fixed to the skull with dental cement (Prestige Dental Products, West Yorkshire, UK). The incision was closed with surgical staples (Promed, Kerry, Ireland). Rats were given 7-10 days to fully recover before being tested. The p75NTR inhibitor REX (5μl, ~10mg/ml) or vehicle (normal rabbit serum; 5μl) were administered via the implanted cannula one hour prior to performing the spatial learning task.

2.8 Object Recognition Task

The apparatus for the object recognition task consisted of a black circular open field (diameter 0.9m, height 0.5m) in a room of dim lighting. In the week prior to performing the task, rats were handled daily and habituated to the experimental apparatus. To ensure that the rats were unable to move the objects they were fixed to the floor. The objects and the open field were cleaned thoroughly between each experiment in order to eliminate olfactory cues.

For the object recognition task, the exploration criteria were based strictly on active exploration. The rats must be seen to have touched to object with at least their nose for it to be deemed as true exploration. The discrimination between familiar and new objects is measured by the time spent exploring and not on the basis of the rat's first choice. The measurement of the time spent exploring each object is recorded and subsequently expressed as a percentage of the total exploration time.

During the one-day experiment, rats were individually placed in the open field. All rats had either been injected with saline or LPS. The rats were allowed to explore two objects for three 5min time periods (Figure 2.1 A). Between each time period the rats were removed from the open field and placed in a holding cage for 5min. The time spent exploring each of the two objects was recorded. The whole experiment lasted for approximately 30min. All rats were killed 5min after their final exposure to the objects.

Day 1 consisted of rats being exposed to two objects for the three 5min time periods as described above. This part of the experiment lasted approximately 30min.

All rats had been injected with either saline or LPS. On day 2, the 12 rats were given a single 5min exposure to one familiar and one novel object to enable object preference to be assessed (Figure 2.1 B). The rats were killed 5min after their day 2 exposure. The time the rats spent exploring each object was recorded on both days.

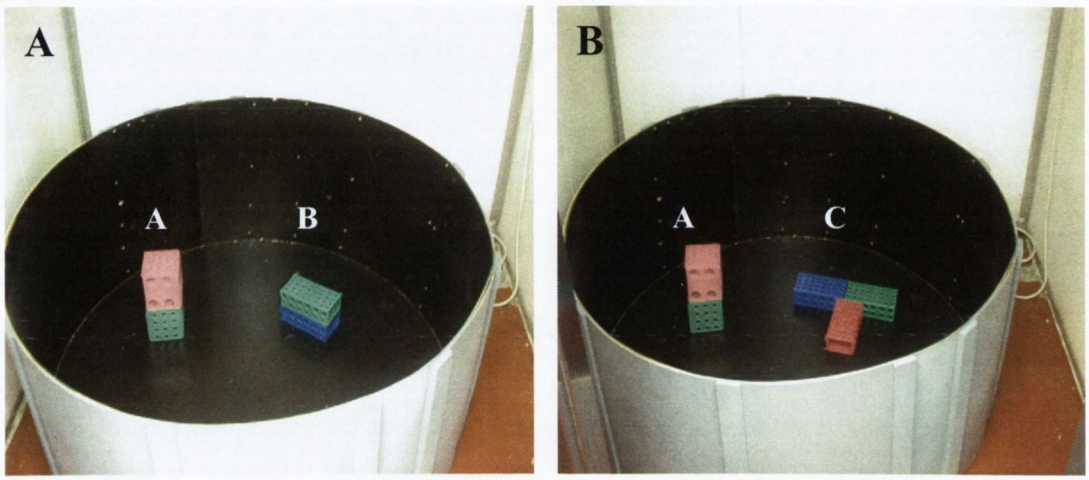


Figure 2.1 Object Recognition Task

2.9 Spatial Learning

Rats were implanted with cannulae as outlined in section 2.6 and allowed to recover for 2 days before being handled daily for 5 days and habituated to an open field (height, 0.5m; diameter, 0.9m). On day 1 the rats were injected with either the p75NTR inhibitor REX (5µl, ~10mg/ml) or saline (5 µl, 0.9% (w/v)) i.c.v. one hour prior to commencement of the spatial learning task. Rats were given three 5-min trials (inter-trial interval 5 min) when they were allowed to explore three novel objects constructed from Lego (Figure 2.2). The time spent actively exploring each of the objects was recorded. On day 2 one of the objects was displaced and rats were placed in the arena for a single 5min trial and allowed to explore. The time spent exploring each of the objects was recorded. For spatial learning assessment, the exploration criteria were based strictly on active exploration. The rats must be seen to have touched the object with at least their nose for it to be deemed as true exploration.

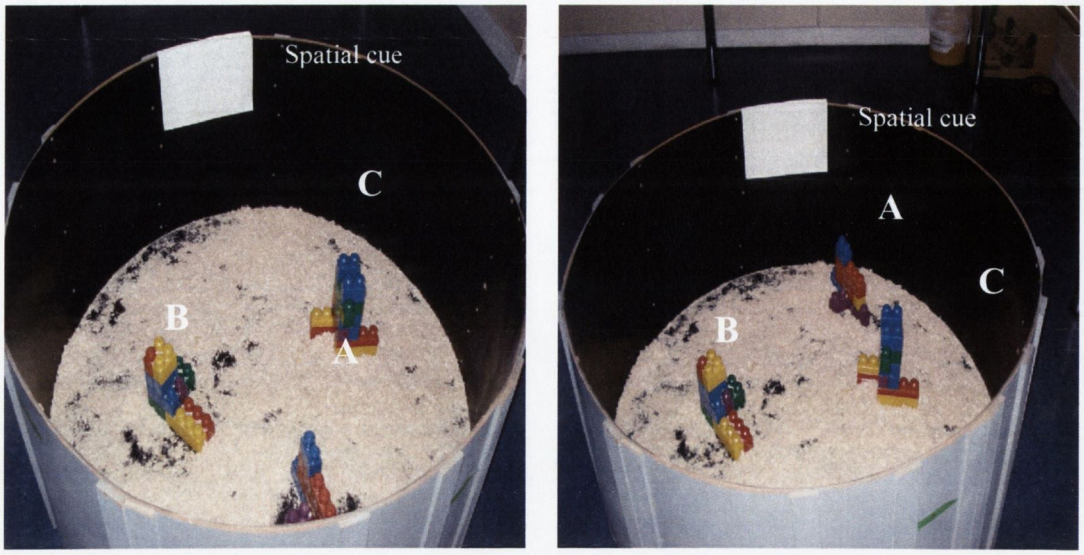


Figure 2.2 Spatial learning task

2.10 *In vitro* incubations

Samples of hippocampus or dentate gyrus were preincubated in oxygenated Krebs solution in the presence or absence of the Trk inhibitor tyrphostin AG879 (1mM) for 15min at 37°C with continuous oxygenation. Following this initial incubation, the samples were further incubated for 25min at 37°C with oxygen in the presence or absence of NGF (100ng/ml) resulting in four treatments, control, NGF alone, Tyrphostin alone and NGF/Tyrphostin. This protocol was repeated using rats that had been pretreated with either saline (0.9%), LPS (300μl of 100μg/ml) or kainic acid (100µM). Following incubation, the samples were equalized for protein concentration according to the method of Bradford (1976). Equal volume of sample buffer was added and the samples were boiled for 2-3min and used for gel electrophoresis and western immunoblotting.

2.11 Preparation of samples for gel electrophoresis

Slices of hippocampus or dentate gyrus were thawed rapidly, washed 3 times in Krebs solution containing 2mM $CaCl_2$ and homogenised (x10 strokes) in Krebs containing 2mM $CaCl_2$ using a 1ml glass homogenizer. Samples of homogenate were equalized for protein concentration (Bradford, 1976) and aliquots of sample (10µl) were added to sample buffer (10µl; composition: Tris-HCL pH 6.8, 0.5M; SDS, 10% w/v; glycerol, 10% v/v; 2-β-mercaptoethanol, 5% v/; bromophenol blue, 0.05% w/v). The samples were boiled for 2-3min.

2.12 Gel electrophoresis

Acrylamide gels (7.5%, 10%, 15% or 20%; see appendix) were cast between 2 glass plates and inserted into the electrophoresis unit (BioRad Mini-PROTEAN 3, BioRad Laboratories, Hertfordshire, England). Electrode running buffer (composition: Tris base, 25mM; glycine, 200mM; SDS 17mM) was added to the inner and outer reservoirs. Prepared samples (10µl) or pre-stained molecular weight markers (5µl; BioRad, Santa Cruz or Sigma) were loaded into the wells and run at 30mA for approximately 40mins.

2.13 Western immunoblotting

The gel slab was washed gently in transfer buffer (composition: Tris base, 25mM; glycine, 200mM; methanol, 20% v/v; SDS pH 8.3, 0.5% w/v). One sheet of nitrocellulose paper (Amersham) and two sheets of filter paper (Whatman No.3), precut to the size of the gel were soaked in transfer buffer for 5min. A layered sandwich was then made in which the nitrocellulose was placed on top of one sheet of filter paper followed by the gel slab and finally the second sheet of filter paper. Air bubbles were removed and the sandwich was placed on the anode of the semi-dry blotter (Apollo Instruments, Alpha Technologies, Dublin, Ireland) which had been pre-moistened with transfer buffer. The lid, containing the cathode, was placed firmly on top and the transfer was carried out at 225mA for 80min. Blots were blocked for non-specific binding overnight at 4°C with a solution of TBS-T (10ml) containing BSA (5%) and probed with an antibody raised against the particular protein in question. This was washed off and a secondary HRP-conjugated antibody was added. Immunoreactive bands were detected with HRP conjugated secondary antibody using super signal West Dura chemiluminescence reagents (Pierce). The membranes were then exposed to photographic (Hyperfilm, Amersham, UK) film and developed using a Fuji Processor.

2.14 Analysis of the activity of protein kinases

In the case of JNK phosphorylation, non-specific binding was blocked by incubation of the nitrocellulose membrane in TBS-T containing 5% BSA overnight at 4°C. The membrane was washed for 10min three times in TBS-Tween and incubated with the primary antibody (mouse anti-phospho JNK monoclonal IgG; Santa Cruz; 1:200 in TBS-T containing 2% BSA) for 2hr at RT. The primary

antibody was a monoclonal antibody raised against a sequence containing phosphorylated Thr-183 and Tyr-185 of JNK of human origin. The membrane was washed three times for 10min and incubated with the secondary antibody (goat anti-mouse IgG-HRP; Amersham; 1:400 in TBS-T containing 2% BSA) for 1hr at RT. The membrane was then washed three times for 10min. Following washing the membranes were incubated with SuperSignal (Pierce) for 5min. The membrane was then exposed to the photographic film for 5-10 secs, depending on the strength of the signal. The film was processed using a Fuji X-ray processor. Following Western immunoblotting for JNK phosphorylation, blots were stripped with an antibody stripping solution (1:10 dilution in dH_2O; Reblot Plus Strong Antibody Stripping Solution; Chemicon) and reprobed for total JNK expression to confirm equal loading of protein. As before, non-specific binding was blocked by incubating the nitrocellulose membrane overnight at 4°C in TBS-T containing 5% BSA. Membranes were washed for 10 min three times in TBS-T. The primary antibody used was a mouse monoclonal IgG antibody raised against non-phosphorylated JNK1 (1:300 dilution in TBS containing 2% BSA; Santa Cruz, California, USA). JNK1 is a mouse monoclonal antibody raised against amino acids 1-384 representing full length JNK1 of human origin. Membranes were incubated for 2 hr at RT in the presence of the antibody and washed for 10 min 3 times in TBS-T. The secondary antibody (1:400 dilution; goat anti-mouse IgG-HRP in TBS containing 2% BSA; Sigma) was added and incubation continued for 1 hr at RT. Membranes were washed three times for 10 min in TBS-T. As before, the membranes were incubated with SuperSignal for 5 min and membranes were exposed to photographic film in the dark followed by film development.

In the case of ERK phosphorylation, non-specific binding was blocked by incubation of the nitrocellulose membrane in TBS-T containing 5% BSA overnight at 4°C. The membrane was then washed for 10min three times in TBS-T and incubated with the primary antibody (mouse anti-phospho ERK monoclonal IgG; Santa Cruz; 1:3000 in TBS-T containing 2% BSA) for 2hr at RT. p-ERK is a mouse monoclonal antibody epitope corresponding to a sequence containing phosphorylated Tyr-204 of ERK of human origin. The membrane was washed three times for 10min and incubated with the secondary antibody (goat anti-mouse IgG-HRP; Sigma; 1:1000 in TBS containing 2% BSA) for 1hr at RT. The membrane

was washed three times for 10min before SuperSignal was added for 5min. The membrane was exposed to the photographic film in the dark and followed by film development. Following Western immunoblotting for ERK phosphorylation, blots were stripped with an antibody stripping solution (1:10 dilution in dH_2O; ReBlot Plus Strong Antibody Stripping Solution; Chemicon) and reprobed for total ERK expression to confirm equal loading of protein. As before, non-specific binding was blocked by incubating the nitrocellulose membrane overnight at 4°C in TBS containing 2% BSA. Membranes were washed for 10 min three times in TBS-T. The primary antibody used was a mouse monoclonal IgG antibody raised against ERK-2 (1:1000 dilution in TBS-T containing 2% BSA; Santa Cruz) with the epitope mapping at the C-terminus of ERK 2 MAP kinase p42 of human origin. Membranes were incubated for 2 hr at RT in the presence of the antibody and washed for 10 min three times in TBS-T. The secondary antibody (1:1000 dilution; goat anti-mouse IgG-HRP in TBS containing 2% BSA; Sigma) was added and incubation continued for 1hr at RT. Membranes were washed for 10min three times in TBS-T. SuperSignal was added for 5 min and membranes were exposed to photographic film in the dark and images were developed.

2.15 p75NTR receptor expression

p75NTR expression was assessed using a protocol similar to above. Non-specific binding was blocked by incubation of the nitrocellulose membrane in TBS-T containing 5% BSA overnight at 4°C. The membrane was washed three times for 10min in TBS-T and incubated with the primary antibody (rabbit polyclonal anti-NGF receptor IgG; Sigma; 1:400 in TBS-T containing 2% BSA. In the case of some studies anti-p75NTR which was received as a generous gift from Dr. Phil Barker was used; 1:1000 in TBS-T containing 2% BSA) overnight at 4°C. The membrane was washed three times for 10min in TBS-T and incubated with the secondary antibody (donkey anti-rabbit IgG-HRP; Sigma; 1:1000 in TBS-T containing 2% BSA) for 1hr at RT. The membrane was washed three times for 10mins in TBS-T prior to incubation with SuperSignal for 5min. The membrane was then exposed to the photographic film images were developed as before. Blots were stripped with an antibody stripping solution (1:10 dilution in dH_2O; ReBlot Plus Strong Antibody Stripping Solution; Chemicon) and reprobed for actin expression to confirm equal loading of protein.

2.16 TrkA receptor expression

TrkA receptor expression was assessed by western immunoblotting. Blots were incubated overnight with 5% BSA containing TBS-T at 4°C to block non-specific binding. The membrane was washed in TBS (3x10mins) and incubated with primary antibody (rabbit anti-TrkA antibody; Upstate Cell Signalling; 1:800 in TBS-T containing 2% BSA) overnight at 4°C. The primary antibody is a bacterially expressed purified protein corresponding to the entire extracellular domain of rat TrkA receptor. The membrane was washed three times for 10min in TBS-T and incubated with the secondary antibody (donkey anti-rabbit IgG-HRP; Sigma; 1:1000 in TBS-T containing 2% BSA) for 1hr at room temperature. The membrane was washed in TBS-T (3x10mins) prior to incubation with SuperSignal for 5min. The membrane was then exposed to the photographic film images were developed as before. Blots were stripped with an antibody stripping solution (1:10 dilution in dH_2O; ReBlot Plus Strong Antibody Stripping Solution; Chemicon) and reprobed for actin expression to confirm equal loading of protein.

2.17 Proneurotrophin expression

ProNGF and proBDNF expression was examined by western immunoblot. Non-specific binding was blocked by incubation of the nitrocellulose membrane in TBS-T containing 5% BSA overnight at 4°C. The membrane was washed three times for 10min in TBS-T and incubated with the primary antibody (rabbit polyclonal anti-proNGF; Alomone; 1:500 in TBS-T containing 2% BSA; rabbit polyclonal anti-proBDNF; Alomone; 1:500 in TBS-T containing 2% BSA) for 2 hours at room temperature. The primary antibody for proNGF was raised in rabbit corresponding to amino acid residues 84-104 of rat NGF (precursor). The primary antibody for proBDNF was raised in rabbit with the epitope corresponding to amino acid residues 72-88 of human BDNF (precursor). The membrane was washed three times for 10min in TBS-T and incubated with the secondary antibody (donkey anti-rabbit IgG-HRP; Sigma; 1:1000 in TBS-T containing 2% BSA) for 1hr at room temperature. The membrane was washed three times for 10mins in TBS-T prior to incubation with SuperSignal for 5min. The membrane was then exposed to the photographic film images were developed as before. Blots were stripped with an antibody stripping solution (1:10 dilution in dH_2O; ReBlot Plus Strong Antibody

Stripping Solution; Chemicon) and reprobed for actin expression to confirm equal loading of protein.

2.18 Mature Neurotrophin expression

NGF and BDNF expression was examined by western immunoblot. Non-specific binding was blocked by incubation of the nitrocellulose membrane in TBS-T containing 5% BSA overnight at 4°C. The membrane was washed three times for 10min in TBS-T and incubated with the primary antibody (rabbit polyclonal anti-NGF; Alomone; 1:500 in TBS-T containing 2% BSA; rabbit polyclonal anti-BDNF; Alomone; 1:500 in TBS-T containing 2% BSA) for 2 hours at room temperature. The primary antibody for NGF was raised in rabbit corresponding to amino acid residues 130-241 of the highly purified 2.5S mouse NGF which is 95.8% homologous with rat.
The primary antibody for BDNF was raised in rabbit with the epitope corresponding amino acid residues 166-178 of human BDNF. The membrane was washed and incubated with the secondary antibody (donkey anti-rabbit IgG-HRP; Sigma; 1:1000 in TBS-T containing 2% BSA) for 1hr at room temperature. The membrane was washed prior to incubation with SuperSignal for 5min. The membrane was then exposed to the photographic film images were developed as before. Blots were stripped with an antibody stripping solution (1:10 dilution in dH_2O; ReBlot Plus Strong Antibody Stripping Solution; Chemicon) and reprobed for actin expression to confirm equal loading of protein.

Protein Target	Antibody Source	Secondary Antibody	Antibody Dilution %BSA in TBS-T	Protein Molecular Weight
Phosphorylated ERK	Mouse	Goat anti-mouse IgG	1° 1:3000 2% BSA 2° 1:1000 2% BSA	ERK1 44kDa ERK2 42kDa
Total ERK expression	Mouse	Goat anti-mouse IgG	1° 1:1000 2% BSA 2° 1:1000 2% BSA	ERK2 42kDa
Phosphorylated JNK	Mouse	Goat anti-mouse IgG	1° 1:200 2% BSA 2° 1:400 2% BSA	JNK1 46kDa
Total JNK expression	Mouse	Goat anti-mouse IgG	1° 1:300 2% BSA 2° 1:400 2% BSA	JNK1 46kDa
p75NTR expression	Rabbit	Donkey anti-rabbit IgG	1° 1:1000 2% BSA 2° 1:2000 2% BSA	75kDa
TrkA expression	Rabbit	Donkey anti-rabbit IgG	1° 1:800 2% BSA 2° 1:1000 2% BSA	140kDa
proBDNF	Rabbit	Donkey anti-rabbit IgG	1° 1:500 2% BSA 2° 1:1000 2% BSA	34kDa
proNGF	Rabbit	Donkey anti-rabbit IgG	1° 1:500 2% BSA 2° 1:1000 2% BSA	28kDa
BDNF	Rabbit	Donkey anti-rabbit IgG	1° 1:500 2% BSA 2° 1:1000 2% BSA	15kDa
NGF	Rabbit	Donkey anti-rabbit IgG	1° 1:500 2% BSA 2° 1:1000 2% BSA	14kDa
Actin	Mouse	Goat anti-mouse IgG	1° 1:1000 2% BSA 2° 1:1000 2% BSA	43kDa

Table 2.1 Antibodies used for Western immunoblotting

2.19 Densitometric Analysis

All protein bands were quantitated by densitometric analysis using the ZERO-DScan Image Analysis System (Scanalytics, Fairfax, USA) or the Gel Doc It Imaging System (UVP, Medical Supply Company, Ireland) in conjuction with LabWorks (Lablogics Inc, Mission Viejo, California, USA).

2.20 Terminal deoxinucleotidyltransferase-mediated biotinylated UTP nick end labelling (TUNEL)

Slides were washed in Tris Buffered Saline (TBS; Tris-HCl 20mM, NaCl 150 mM, pH7.4) and fixed in 4 % (w/v) paraformaldehyde for 30 minutes at room temperature. The paraformaldehyde was then removed and replaced with TBS and the cells were stored at 4°C until required for analyses. Apoptotic cell death was assessed by monitoring DNA fragmentation, using the DeadEnd colorimetric apoptosis system (Promega Corporation, Madison, USA). Slices were permeabilised with Triton X-100 (0.1 % v/v), proteinase-k (1μg/ml) in TBS and refixed in 4 % paraformaldehyde for 10 min. Slides were incubated in equilibration buffer (composition; 200mM potassium cacodylate (pH 6.6 at 25°C), 25mM Tris-HCL (pH 6.6 at 25°C), 0.2mM DTT, 0.25mg/ml BSA, 2.5mM cobalt chloride) for 10 min. A reaction buffer (TdT; 98µl equilibration buffer, 1µl biotinylated nucleotide mix, 1µl TdT enzyme) was applied for one hour at 37°C in order to incorporate the biotinylated nucleotide to the 3'–OH DNA ends of fragmented DNA strands. Horseradish-peroxidase-labelled streptavidin was then bound to the biotinylated nucleotide (100 μl; 1:100 dilution in PBS for 1 hr at RT) and this was detected using a DAB solution. Incubation proceeded until cells had taken on a stained appearance (approximately 10 min). The slides were washed in deionised water, dehydrated through graded alcohols and mounted on slides with DPX mounting medium. Cells were then viewed under light microscopy (Nikon Laboplot, Nikon Instech Co., Ltd, Kanagawa, Japan) at approximately x100 magnification, where nuclei of TUNEL positive cells stained dark purple.

2.21 Immunostaining

Frozen sections were thawed to room temperature, washed in PBS for 5min fixed in 4% formaldehyde in PBS (w/v) for 30min. They were then washed three in PBS and permeabilized in 0.1% Triton in PBS (v/v) for 5min. Sections were washed as before and refixed in 4% paraformaldehyde for 15min after which they were washed. Sections were then exposed to 2% H_2O_2 for 5min and then washed in PBS as before. Sections were blocked in 5% normal goat serum (Vector Laboratories) in PBS (v/v) for 30min. The blocking serum was removed and sections were incubated overnight at 4°C in a humidified chamber with primary antibody (TrkB 1:50, Santa Cruz; p75NTR 1:110, Sigma). Sections were washed in PBS and incubated for one hour at room temperature in biotinylated goat anti-rabbit IgG (1:200; Sigma). Slides were washed in PBS and exposed to ABC signal amplification system (Vectastain, Vector Laboratories) for 30min. Sections were briefly washed before being exposed to DAB stain for 5min. Sections were then counterstained with methyl green, washed briefly in water to remove excess stain and then dehydrated and sealed. Slides were examined using a Nikon light microscope. The total number of cells and the number of positively stained cells were counted in the CA1, CA3 and dentate gyrus.

2.22 Statistical Analysis

Data are expressed as means ± SEM. In the majority of experiments three-way and two-way analysis of variance (ANOVA) were performed to determine whether there were significant differences between groups using the statistical package GBSTAT. Where appropriate the student's t-test for independent means was used to establish significant differences between groups. When these analyses indicated significant differences between conditions, *post-hoc* Student's Newman-Keuls test analysis was used to determine which conditions were significantly different from each other.

Chapter 3

The effect of lipopolysaccharide on neurotrophin receptor expression and signalling

3.1 Introduction

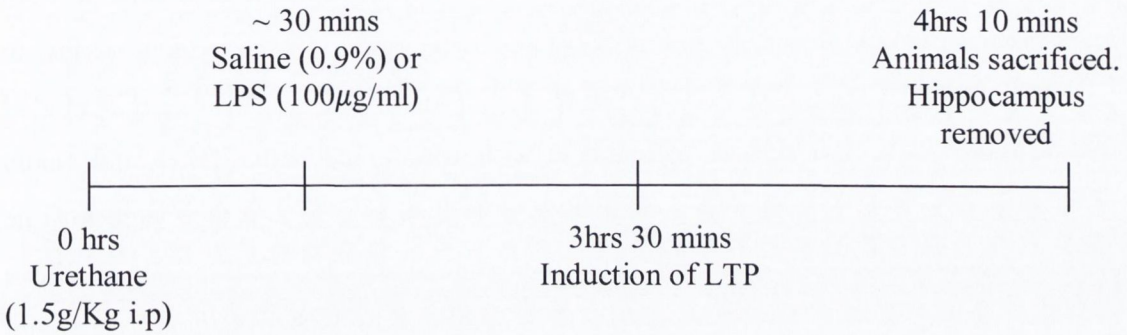
Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria and is known to induce a profound inflammatory and immunostimulatory response when introduced to the body. Peripheral injection of LPS has been shown to induce functional impairments in the CNS. In 2000, Vereker *et al.* investigated the effect of intraperitoneal (i.p.) injection of LPS on synaptic function in the rat hippocampus. They reported that the systemic injection of LPS caused an impairment in LTP in the dentate gyrus, a finding which has been supported by several other groups (Shaw *et al.*, 2005; Barry *et al.*, 2005; Kelly *et al.*, 2003; Hauss-Wegrzyniak *et al.*, 2002). Systemic injection of LPS has also been shown to cause deficits in spatial learning in the Morris water maze (Shaw *et al.*, 2005). The effects of LPS on synaptic function are thought to originate from the neurodegenerative effects it elicits through its activation of the inflammatory cytokines such as IL-1β and TNF-α.

Recently it has been proposed that LPS can effect neurotrophin concentration in the CNS as well as receptor expression and that the compromised neurotrophin action may be involved in the functional impairment observed upon LPS injection. Guan and Fang (2006) found that i.p. injection of LPS significantly decreased BDNF in the hippocampus, frontal cortex, parietal cortex, temporal cortex and the occipital cortex. NGF was also significantly decreased in the hippocampus and all of the previously mentioned cortical areas, except the occipital cortex. NT-3 concentration was also decreased in the frontal cortex. Watt and Paden (2001) found that there was a strong correlation between increased p75NTR immunoreactivity and LPS administration in the rat neural lobe. LPS has also been shown to increase expression of TrkA, TrkB and TrkC in macrophages (Caroleo *et al.*, 2001; Barouch *et al.*, 2001) while not affecting p75NTR expression. Interestingly, Caroleo and colleagues found that neutralization of endogenous NGF by NGF antibody lead to the overexpression of p75NTR on macrophages and resulted in apoptosis. If, like Guan and Fang reported, there is a decrease in NGF concentration in the brain following LPS administration then perhaps the overexpression of p75NTR may be responsible for some of the neurodegeneration induced.

The purpose of this study was to assess whether the change in neurotrophin receptor expression was associated with degenerative processes following systemic LPS administration and to try and determine the signalling pathway utilised by the p75NTR.

3.2 Materials and Methods

3.2.1 Experimental Timeline



3.2.2Treatment

Briefly, rats were anaesthetised with urethane (1.5g/kg i.p.) until loss of consciousness occurred, evidenced by the absence of the pedal reflex. Rats were subsequently injected with 300μl of 100μg/ml Escherichia Coli LPS or 300μl of saline (0.9% w/v).

3.2.3 Induction of LTP *in vivo*

Three hours following LPS injection LTP was induced. Rats were placed in a stereotaxic frame and a bipolar stimulating electrode was placed in the perforant path of the dentate gyrus while a recording electrode was placed in the granule cells of the dorsal body of the region. Test shocks were delivered at 30 sec intervals and recorded for 10 minutes in order to establish a stable baseline. This was proceeded by delivery of three High-frequency trains of stimuli (250Hz for 200msec) at 30 sec intervals after which recording resumed at test shock frequency for 40 minutes. Upon cessation of recording animals were sacrificed by decapitation and the tissue removed. The removed tissue was sliced and stored at -80°C for further analysis.

3.2.4 Immunostaining

Frozen sections were thawed to room temperature, washed in PBS for 5min fixed in 4% formaldehyde in PBS (w/v) for 30min. They were then washed three in PBS and permeabilized in 0.1% Triton in PBS (v/v) for 5min. Sections were washed as before and refixed in 4% paraformaldehyde for 15min after which they were washed. Sections were then exposed to 2% H_2O_2 for 5min and then washed in PBS as before. Sections were blocked in 5% normal goat serum (Vector Laboratories) in PBS (v/v) for 30min. The blocking serum was removed and sections were incubated overnight at 4°C in a humidified chamber with primary antibody (TrkB 1:50, Santa Cruz; p75NTR 1:110, Sigma). Sections were washed in PBS and incubated for one hour at room temperature in biotinylated goat anti-rabbit IgG (1:200; Sigma). Slides were washed in PBS and exposed to ABC signal amplification system (Vectastain, Vector Laboratories) for 30min. Sections were briefly washed before being exposed to DAB stain for 5min. Sections were then counterstained with methyl green, washed briefly in water to remove excess stain and then dehydrated and sealed. Slides were examined using a Nikon light microscope. The total number of cells and the number of positively stained cells were counted in the CA1, CA3 and dentate gyrus.

3.2.5 Terminal deoxinucleotidyltransferase-mediated biotinylated UTP nick end labelling (TUNEL)

Slides were washed in Tris Buffered Saline (TBS; Tris-HCl 20mM, NaCl 150 mM, pH7.4) and fixed in 4 % (w/v) paraformaldehyde for 30 minutes at room temperature. The paraformaldehyde was then removed and replaced with TBS and the cells were stored at 4ºC until required for analyses. Apoptotic cell death was assessed by monitoring DNA fragmentation, using the DeadEnd colorimetric apoptosis system (Promega Corporation, Madison, USA). Slices were permeabilised with Triton X-100 (0.1 % v/v), proteinase-k (1μg/ml) in TBS and refixed in 4 % paraformaldehyde for 10 min. Slides were incubated in equilibration buffer (composition; 200mM potassium cacodylate (pH 6.6 at 25ºC), 25mM Tris-HCL (pH 6.6 at 25ºC), 0.2mM DTT, 0.25mg/ml BSA, 2.5mM cobalt chloride) for 10 min. A reaction buffer (TdT; 98µl equilibration buffer, 1µl biotinylated nucleotide mix, 1µl TdT enzyme) was applied for one hour at 37ºC in order to incorporate the biotinylated nucleotide to the 3'–OH DNA ends of fragmented DNA strands.

Horseradish-peroxidase-labelled streptavidin was then bound to the biotinylated nucleotide (100 μl; 1:100 dilution in PBS for 1 hr at RT) and this was detected using a DAB solution. Incubation proceeded until cells had taken on a stained appearance (approximately 10 min). The slides were washed in deionised water, dehydrated through graded alcohols and mounted on slides with DPX mounting medium. Cells were then viewed under light microscopy (Nikon Laboplot, Nikon Instech Co., Ltd, Kanagawa, Japan) at approximately x100 magnification, where nuclei of TUNEL positive cells stained dark purple.

3.2.6 *In vitro* incubations ± tyrphostin AG879 ± NGF

Samples of hippocampus or dentate gyrus were preincubated in oxygenated Krebs solution in the presence or absence of the Trk inhibitor tyrphostin AG879 (1mM) for 15min at 37°C with continuous oxygenation. Following this initial incubation, the samples were further incubated for 25min at 37°C with oxygen in the presence or absence of NGF (100ng/ml) resulting in four treatments, control, NGF alone Tyrphostin alone and NGF/Tyrphostin. This was a preliminary study carried out to ensure that the concentrations of inhibitor employed were correct. This protocol was repeated using rats that had been pre-treated with either saline (0.9%), LPS (300μl of 100μg/ml). Following incubation, the samples were equalized for protein concentration according to the method of Bradford (1976). Equal volume of sample buffer was added and the samples were boiled for 2-3min and used for gel electrophoresis and western immunoblotting.

3.2.7 Analysis of ERK activation in LPS treated rats

ERK activity was assessed using gel electrophoresis and western immunoblotting as described in section 2.10. Membranes were incubated with 5% BSA/TBS-T overnight to block any unspecific binding. Membranes were given three 10 minutes washes with TBS-T after which they were incubated for two hours at room temperature in primary antibody (anti-phosphoERK (Santa Cruz) 1:3000 in 2% BSA/TBS-T; anti-ERK2 (Santa Cruz) 1:1000 in 2% BSA/TBS-T). Following primary antibody incubation membranes were washed with TBS-T (three 10 minute washes) and were subsequently incubated with secondary antibody for one hour at room temperature (1:1000 anti-mouse IgG (Sigma) for detection of both pERK and ERK2). Membranes were washed in TBS-T as before. The protein bands were

detected using Supersignal (Amersham) before being exposed to photographic film (Hyperfilm).

3.2.8 Analysis of JNK activity in LPS treated rats

JNK activity was assessed as described in section in section 2.14. Briefly, blots were blocked overnight in 5% BSA/TBS-T. Membranes were washed and incubated with primary antibody (anti-phosphoJNK IgG (Santa Cruz) 1:200 in 2% BSA/TBS-T; anti-JNK1 1:300 dilution in 2% BSA/TBS-T) for two hours at room temperature. Blots were washed and incubated with secondary antibody for one hour at room temperature (anti-mouse IgG (Sigma) 1:400 for both phospho-JNK and JNK1, in 2% BSA/TBS-T. Membranes were washed in TBS-T as before. The protein bands were detected using Supersignal (Amersham) before being exposed to photographic film (Hyperfilm).

3.2.9 Densitometric Analysis

All protein bands were quantitated by densitometric analysis using the ZERO-DScan Image Analysis System (Scanalytics, Fairfax, USA) or the Gel Doc It Imaging System (UVP, Medical Supply Company, Ireland) in conjuction with LabWorks (Lablogics Inc, Mission Viejo, California, USA).

3.3 Results

3.3.1 LPS injection i.p. increases p75NTR expression in the dentate gyrus and decreases TrkB expression in the CA1 and CA3 *in vivo*

Neutrotrophin receptor expression in the dentate gyrus, CA1 and CA3 following LPS administration (100μg/Kg) was assessed by immunostaining. p75NTR was significantly increased in the dentate gyrus of LPS treated rats (Figure 3.1A & 3.2A; **$p<0.001$, students *t*-test for independent means; 17.38736 ± 0.44 and 37.22 ± 5.17 for saline and LPS treated rats respectively). No changes in p75NTR expression in the CA1 and CA3 were observed. TrkB expression was decreased significantly in the CA1 (Figure 3.1A & 3.2A; **$p<0.001$, students *t*-test for independent means; 42.7 ± 3.1 and 19.21 ± 3.29 for saline and LPS rats respectively) and CA3 (*$p<0.05$, students *t*-test for independent means; 43.28 ± 4.5 and 20.71 ± 4.81 for saline and LPS rats respectively) following LPS administration with no changes observed in the dentate gyrus. Results are expressed as the mean number of positively-stained cells expressed as a percentage of total cell number ± SEM.

3.3.2 LPS injection i.p. increases cell death in the dentate gyrus *in vivo*

Cell death in the dentate gyrus following LPS injection was assessed by TUNEL staining. TUNEL staining measures DNA fragmentation indicating apoptosis Analysis revealed that LPS increased the number of positively stained TUNEL cells when compared to saline controls (Figure 3.3).

3.3.3 LPS injection i.p. impairs LTP in the rat dentate gyrus *in vivo*

The ability of LPS treated rats to sustain LTP in the perforant path granule cell synapses following high-frequency stimulation of the perforant path was assessed. Delivery of high-frequency stimulation resulted in an immediate increase in the slope of epsp in both groups of rats (Figure 3.4 & 3.5). Results are means ± SEM (n=6). Slopes are expressed as a percentage of the normalized slopes in the 5 minutes prior to high-frequency stimulation. Statistical analysis of the % epsp slope 2 minutes after high-frequency stimulation revealed no significant differences between saline and LPS treated rats (128.18 ± 0.75 and 126.13 ± 3.04 respectively). Statistical analysis of the % epsp slope 50min after high-frequency stimulation revealed an extremely significant decrease in the % epsp slope in LPS treated rats,

when compared with saline-treated rats (Figure 3.5C, **p< 0.001; 2-way ANOVA, *post-hoc* Newmann Keuls, n=6; 114.26 ± 0.77 and 100.72 ± 0.623 for saline and LPS-treated rats respectively).

3.3.4 LPS treatment increases JNK activity *in vivo*

The effect of LPS on JNK activity was assessed using western immunoblotting. LPS administration increased JNK activity in the hippocampus when compared to saline controls (195.31 ± 24.09 and 133.82 ± 11.05 respectively (Figure 3.6). Results are expressed as a ratio of phosphorylated JNK/ total JNK expression (mean ± SEM, n=6), students unpaired t-test, *p<0.05 (p=0.0381).

3.3.5 Tyrphostin effectively blocks Trk receptor activity

The effectiveness of the Trk receptor inhibitor, tyrphostin was assessed using *in vitro* incubations. ERK phosphorylation is known to occur downstream of Trk receptor activation. As a result ERK phosphorylation was used as a read out of Trk receptor activity. Upon incubation with NGF (100ng/ml), ERK phosphorylation was increased significantly when compared to saline controls (Figure 3.7B; *p<0.05, ANOVA; 3869.440 ± 340.5 and 4477.090 ± 424.2 for saline and NGF treated incubations respectively, mean ± SEM, n=6. Incubation with the Trk inhibitor tyrphostin resulted in decreased basal ERK phosphorylation indicating that Trk activation was blocked (*p<0.05, ANOVA; 3869.440 ± 340.5 and 2487.050 ± 392.6 for saline and tyrphostin treated incubations respectively, mean ± SEM, n=6 (Figure 3.7B). Tyrphostin also blocked the NGF induced increase in ERK activity further illustrating its effectiveness in blocking Trk receptor activity (*p<0.05, ANOVA; 4477.090 ± 424.2 and 3673.250 ± 319.1 for NGF and NGF/tyrphostin treated incubations respectively, mean ± SEM, n=6 (Figure 3.7B).

3.3.6 The effect of LPS on p75NTR signalling

LPS administration upregulates the expression of the p75NTR. Knowing that tyrphostin successfully blocks Trk receptor activity I hypothesized that incubation with NGF and tyrphostin in LPS treated slices would activate p75NTR apoptotic signalling. JNK is known to be activated downstream of p75NTR (Friedman, 2000) and was used as a marker of p75NTR apoptotic signalling. Similarly, ERK is activated downstream of Trk receptor signalling and has been

shown to have an opposing effect to JNK (Xia *et al.*, 1995). Therefore, I hypothesized that there would be a downregulation in ERK activity in LPS treated animals. Densitometric analysis revealed a significant increase in JNK activity in tissue taken from animals that had been treated with LPS (100μg/ml) (Figure 3.9; *$p<0.05$, 3-way ANOVA, *post hoc* Newmann Keuls; 78.69 ± 24.89% and 237.4 ± 58.4% for control saline and LPS treated animals respectively). Incubation of control and LPS treated tissue with NGF (100ng/ml) had no effect on JNK activity (88.45 ± 27.79% and 209 ± 56.24% for saline and LPS treated animals respectively). Inhibition of the Trk receptor by tyrphostin AG879 (1mM) had no effect on JNK activity in either saline or LPS treated tissue (100 ± 17.52% and 218 ± 59.7% for saline and LPS treated animals respectively). Finally, co-incubation with both NGF and tyrphostin had no effect on JNK activity in either saine or LPS treated animals (78.23 ± 4.6% and 227.1 ± 45.94% for saline and LPS treated animals respectively). Results are expressed as mean percentage ± SEM of phosphorylated JNK1/ total JNK1 expression (n=2-4) (Figure 3.9). Densitometric analysis revealed no significant difference in ERK activity in tissue taken from animals that had been treated with either saline (0.9%) w/v) or LPS (100μg/ml) (Figure 3.8; 3-way ANOVA, 94.75 ± 4.5% and 140 ± 37.31% for control saline and LPS treated animals respectively). Incubation of control and LPS treated tissue with NGF (100ng/ml) had no effect on ERK activity (133.5 ± 21.24% and 172 ± 41.8% for saline and LPS treated animals respectively). Inhibition of the Trk receptor by tyrphostin AG879 (1mM) had no effect on ERK activity in either saline or LPS treated tissue (100 ± 27.9% and 163.8 ± 34.67% for saline and LPS treated animals respectively). Finally, co-incubation with NGF and tyrphostin had no effect on ERK activity in either saline or LPS treated animals (114 ± 32.73% and 176.4 ± 49.93% for saline and LPS treated animals respectively). Results are expressed as mean percentage ± SEM of phosphorylated p42ERK/ total ERK expression (n=2-3) (Figure 3.8).

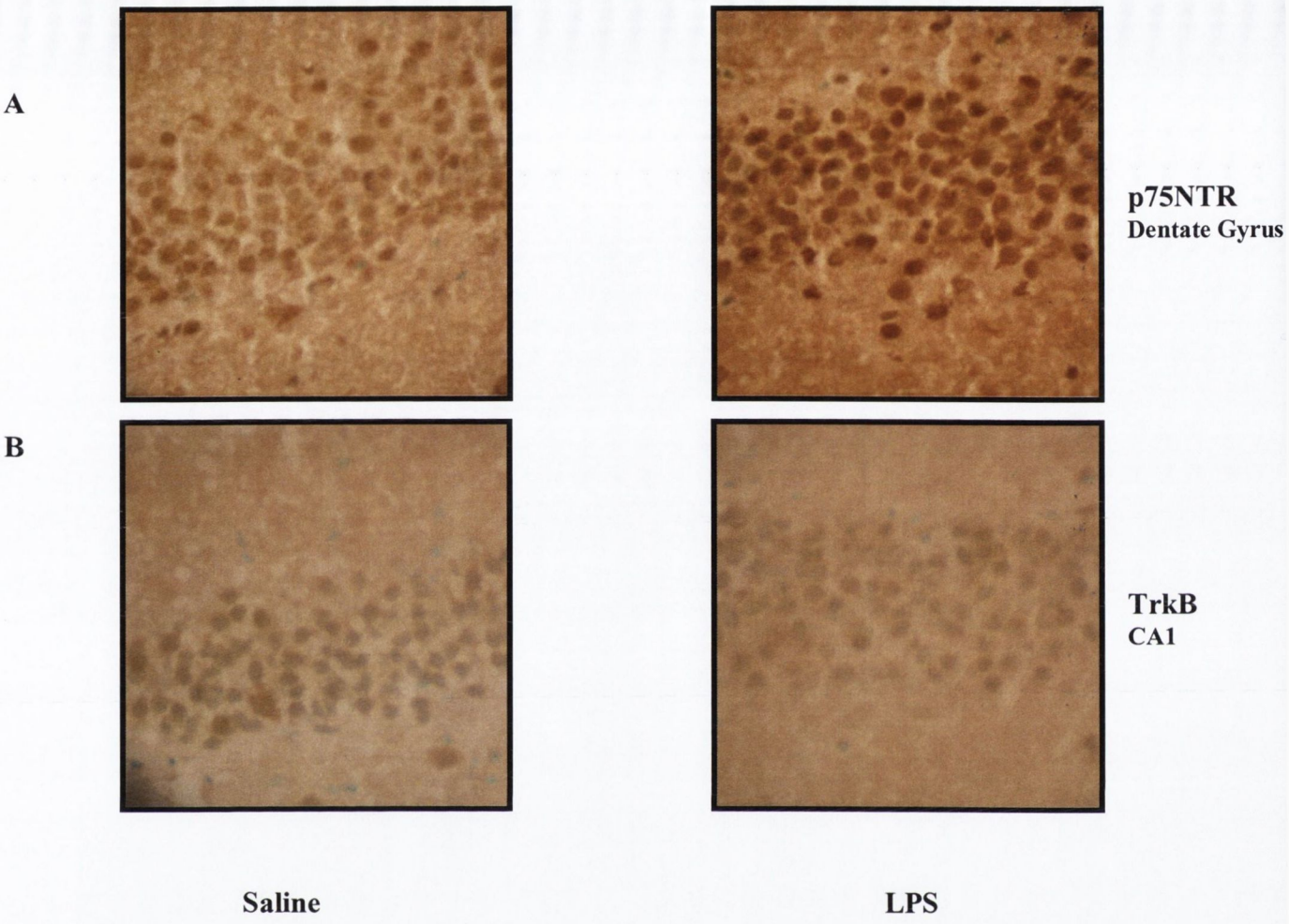
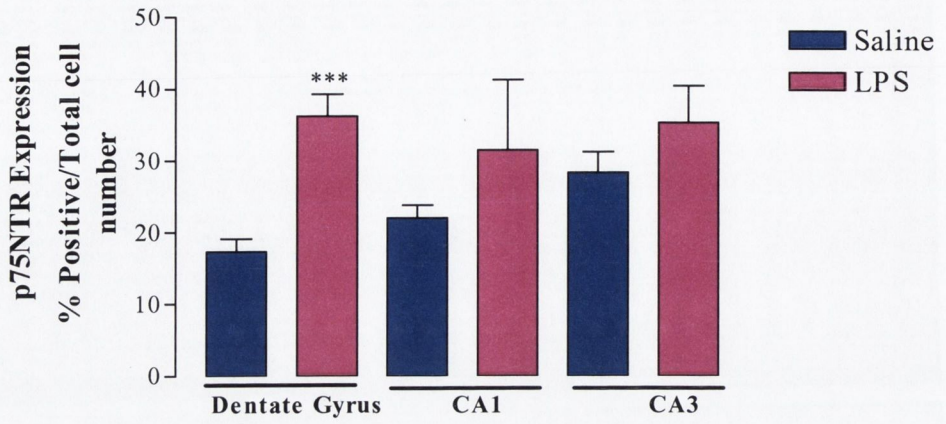


Figure 3.1 LPS increases p75NTR expression in the dentate gyrus and decreases TrkB expression in the CA1 and CA3 regions of the rat hippocampus *in vivo*

A. Immunostaining revealed that LPS administration (300µl of 100µg/ml i.p.) significantly increased p75NTR expression in the dentate gyrus of the rat when compared to saline controls. (n=2; three independent observations).

B. The expression of TrkB following LPS administration was assessed. Statistical analysis revealed a significant decrease in receptor expression in the CA1 and CA3 regions of the hippocampus when compare to saline controls. (n=2; three independent observations).

A



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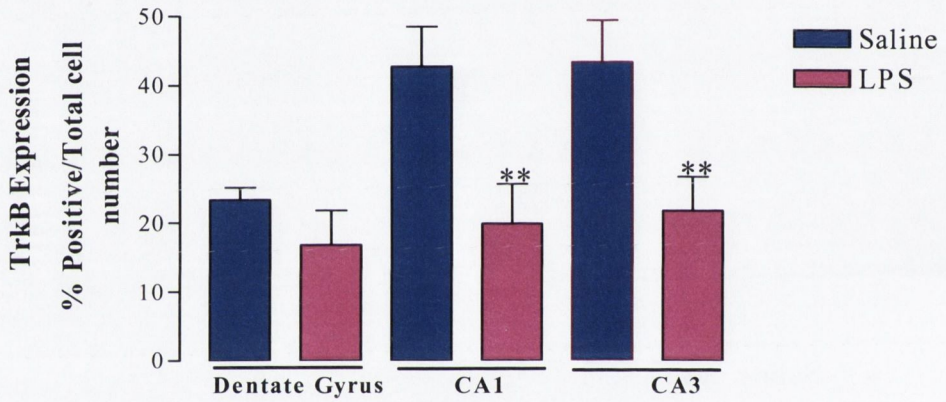
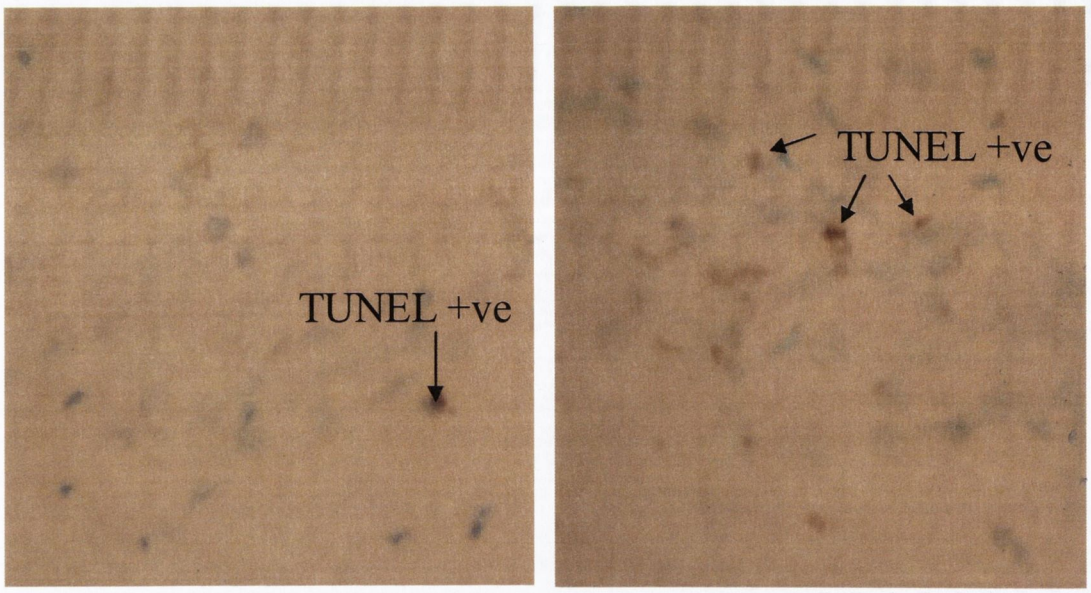


Figure 3.2 LPS increases p75NTR expression in the dentate gyrus and decreases TrkB expression in the CA1 and CA3 regions of the rat hippocampus *in vivo*

A. Immunostaining revealed that LPS administration (300µl of 100µg/ml i.p.) significantly increased p75NTR expression in the dentate gyrus of the rat when compared to saline controls (**$p<0.001$; students *t*-test for independent means). (n=2; three independent observations). Results are expressed as the percentage of positively stained cells/total cell number (mean ± SEM).

B. The expression of TrkB following LPS administration was assessed. Statistical analysis revealed a significant decrease in receptor expression in the CA1 and CA3 regions of the hippocampus when compare to saline controls (**$p<0.01$ for CA1 and *$p<0.05$ for CA3; students *t*-test for independent means). Results are expressed as the percentage of positively stained cells/total cell number (mean ± SEM).



Saline **LPS**

Figure 3.3 LPS administration induces apoptosis in the dentate gyrus *in vivo*

Three hours following either saline (0.9% w/v) or LPS (300µl of 100µg/ml) injection i.p. animals were sacrificed. LPS injection increased the number of TUNEL positive cells observed in the dentate gyrus when compared to saline controls indicating that an increased number of cells were undergoing apoptosis following LPS administration.

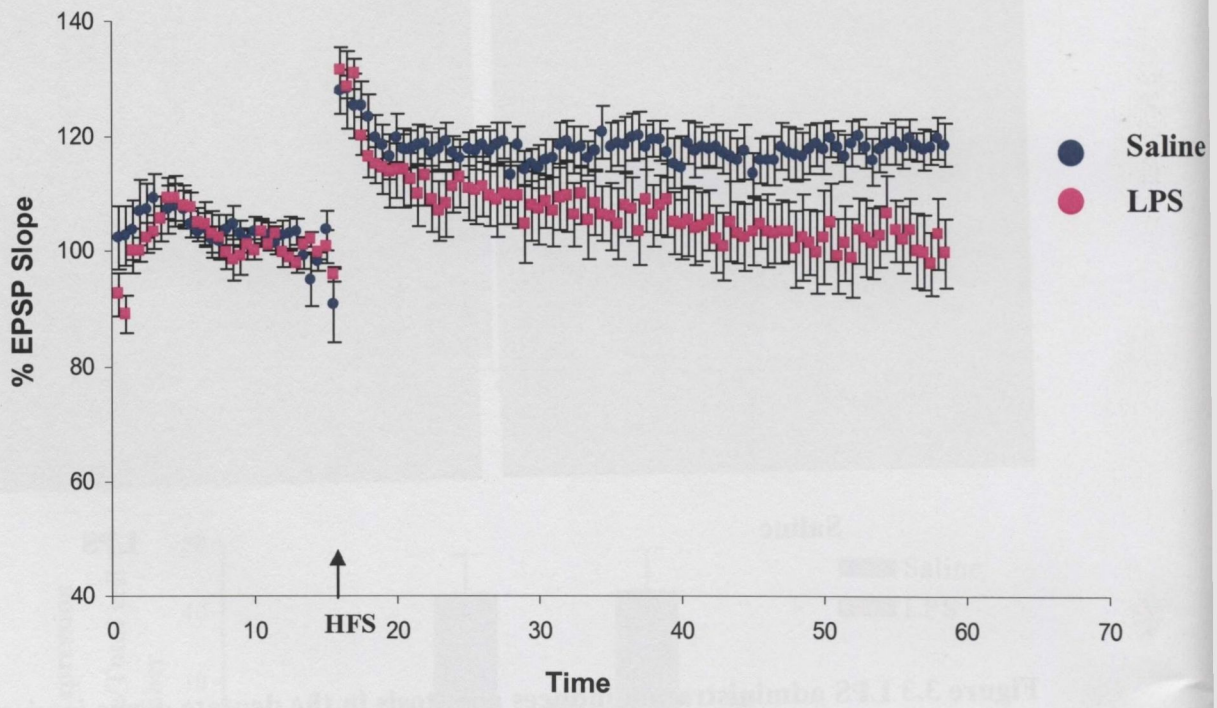
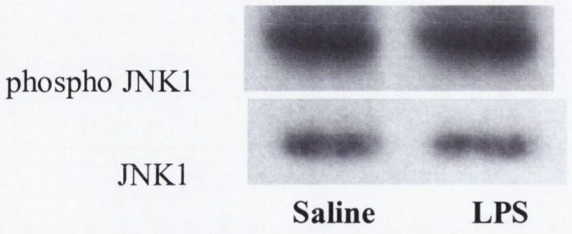


Figure 3.4 Expression of LTP in saline and LPS treated rats *in vivo*

Test shocks were given at 30 sec intervals for a 10min control prior to tetanization. High frequency stimulation (3 trains of stimuli of 200Hz for 200msec at 20sec intervals) was delivered to the perforant path. Recording at test shock frequency resumed for 40min following tetanus. LTP was maintained by saline treated rats; however those treated with LPS failed to sustain LTP. Results are expressed as mean EPSP slope normalized with respect to the EPSP slope recorded in the 5 min immediately prior to tetanic stimulation. Results are expressed as means ± SEM of 6 independent observations for both treatment groups.

A



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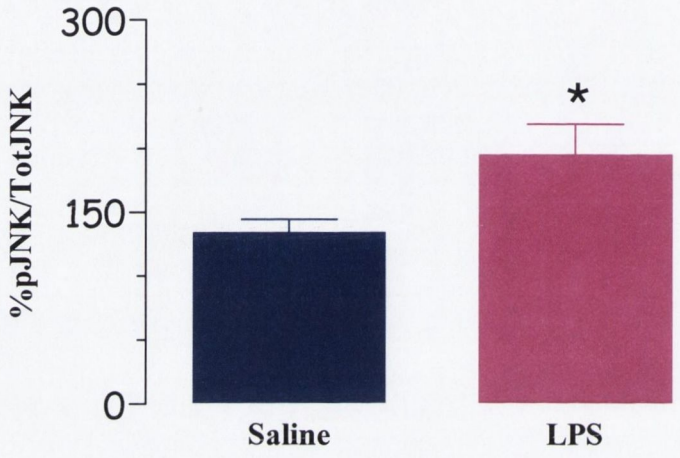
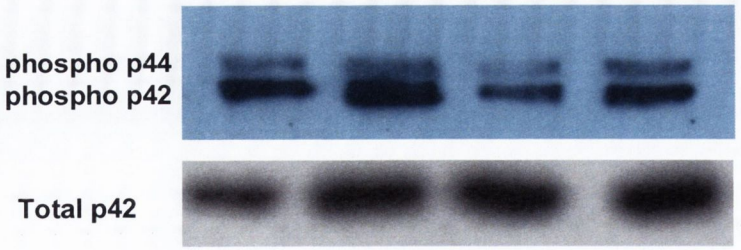


Figure 3.6 LPS increases JNK activity *in vivo*

A Sample western immunoblot illustrating increased JNK activity in rats treated with LPS

B Rats were injected i.p. with either LPS (300µl, 100µg/Kg) or saline (0.9%) three hours prior to recording LTP. Phosphorylation of JNK and total JNK expression were assessed by western immunoblot. Injection of LPS significantly increased JNK activity when compared to saline controls (195.31 ± 24.09 and 133.82 ± 11.05 respectively). Results are expressed as a ratio of phosphorylated JNK/ total JNK expression (mean ± SEM, n=6), students unpaired t-test, *$p<0.05$ ($p=0.0381$).

A



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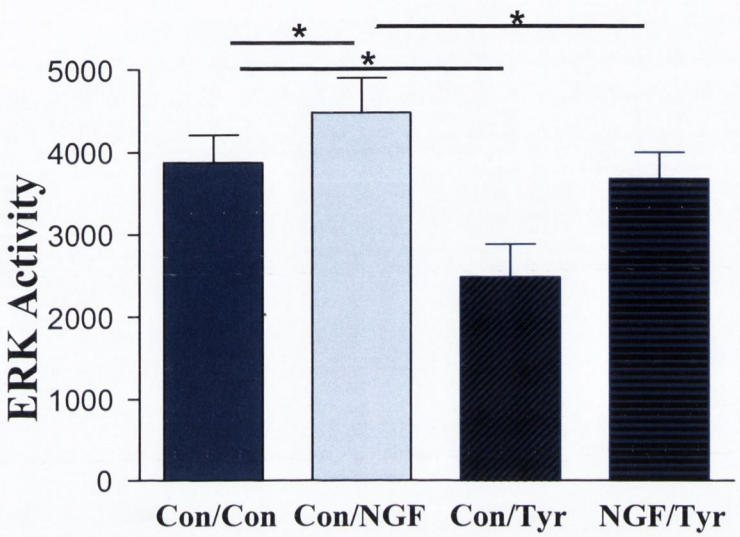
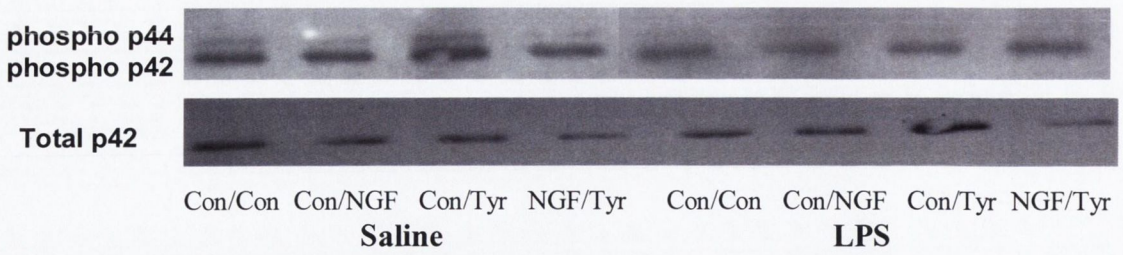


Figure 3.7 The effect of the Trk inhibitor tyrphostin AG879 on ERK activity in hippocampal slices

A. Sample western immunoblot illustrating ERK phosphorylation in hippocampal slices treated with either NGF or the Trk inhibitor tyrphostin AG879

B. Hippocampal slices were incubated in the presence and absence of the Trk inhibitor tyrphostin AG879 (1mM) and in the presence and absence of NGF (100ng/ml) at 37°C. Phosphorylation of ERK was significantly increased following incubation with NGF (*$p<0.05$; one-way ANOVA). Incubation with tyrphostin alone blocked ERK phosphorylation (*$p<0.05$; one-way ANOVA). Incubation with NGF and tyrphostin attenuated the NGF induced increase in ERK phosphorylation (*$p<0.05$; one-way ANOVA). Results are expressed as arbitrary units (mean ± SEM, n=6).

A



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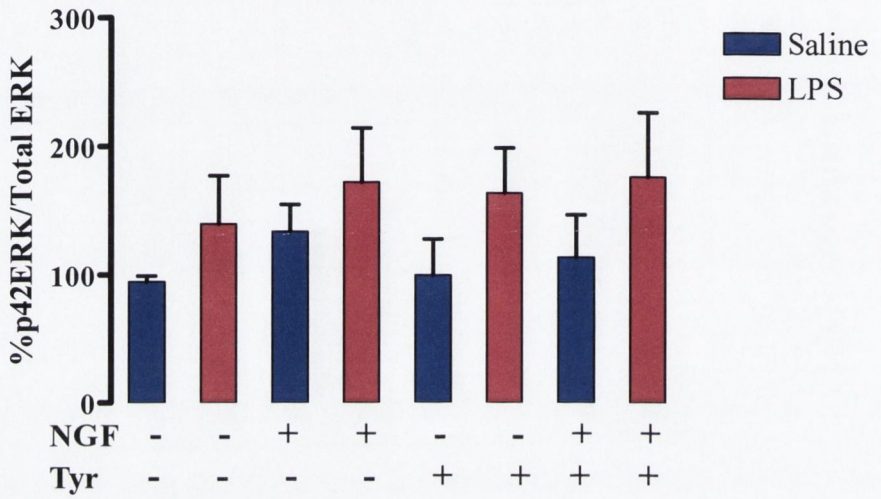


Figure 3.8 The effect of the Trk inhibitor tyrphostin AG879 on ERK activity in saline and LPS hippocampal slices

A. Sample western immunoblot illustrating ERK activity in saline and LPS treated hippocampal slices incubated with either NGF or the Trk inhibitor tyrphostin AG879.

B. Hippocampal slices were incubated in the presence and absence of the Trk inhibitor tyrphostin AG879 (1mM) and in the presence and absence of NGF (100ng/ml) at 37°C. Phosphorylation of ERK and total ERK expression were assessed by western immunoblot. Densitometric analysis revealed no significant differences between any of the groups. Results are expressed as a ratio of phosphorylated ERK/ total ERK expression (mean ± SEM, n=2-3).

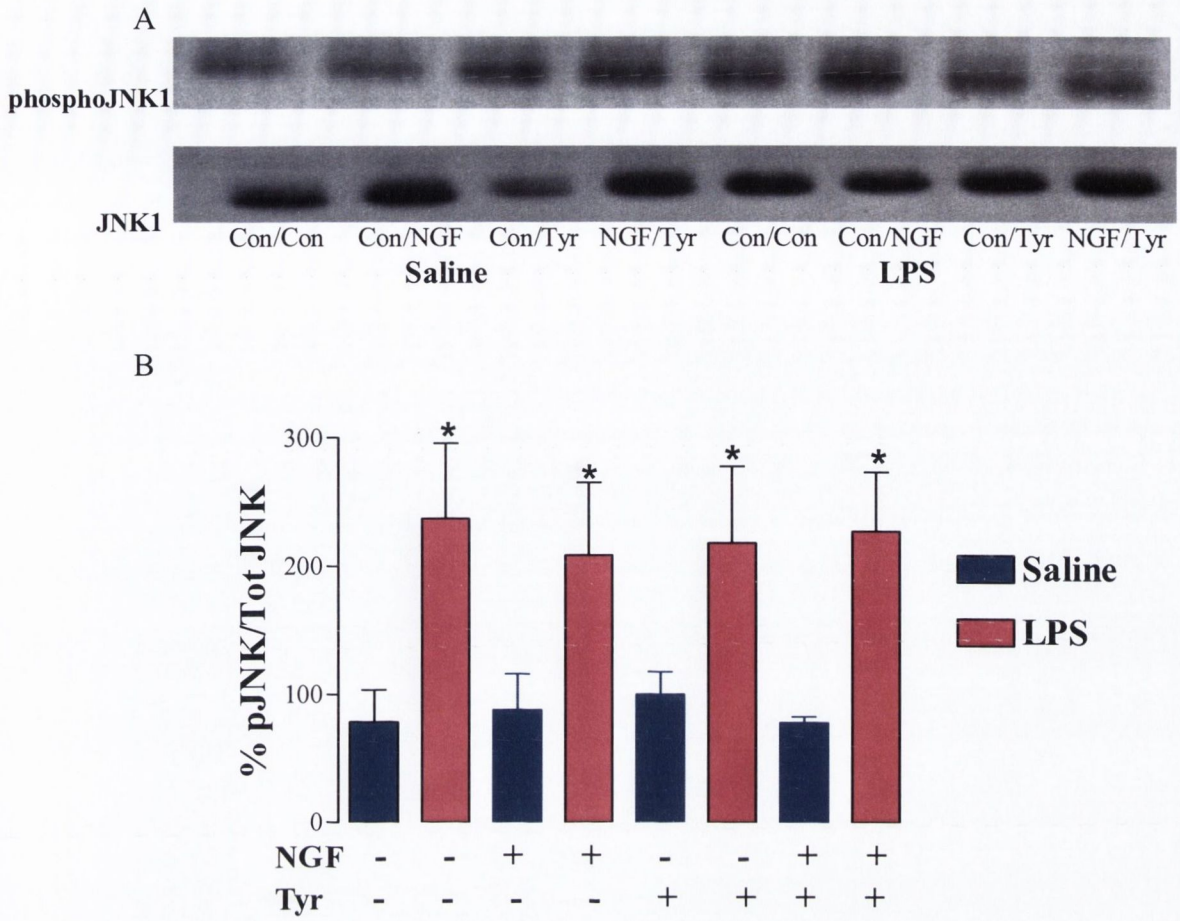


Figure 3.9 The effect of the Trk inhibitor tyrphostin AG879 on LPS induced JNK activation.

A. Sample western immunoblot illustrating JNK activity in saline and LPS treated hippocampal slices incubated with either NGF or the Trk inhibitor tyrphostin AG879.

B. Hippocampal slices were incubated in the presence and absence of the Trk inhibitor tyrphostin AG879 (1mM) and in the presence and absence of NGF (100ng/ml) at 37°C. Phosphorylation of JNK and total JNK expression were assessed by western immunoblot. Densitometric analysis revealed a significant increase in JNK activity following LPS administration; *$p<0.05$, 3-way ANOVA, *post hoc* Newman Keuls. Results are expressed as a ratio of phosphorylated JNK/ total JNK expression (mean ± SEM, n=2-4).

3.4 Discussion

The primary objective of this study was to assess whether the peripheral administration of LPS effects neurotrophin receptor expression in the hippocampus. This study also aimed to establish whether the differential changes in receptor expression following LPS injection may contribute to the neuronal degeneration observed following LPS administration and subsequently lead to impairments in synaptic plasticity and cognition.

Immunohistochemistry revealed that the peripheral administration of LPS impacted neurotrophin receptor expression in the hippocampus. Previous studies have demonstrated that the peripheral administration of the endotoxin, LPS increases p75NTR expression in the rat neural lobe (Watt and Paden, 2001). In this study a decrease in TrkB receptor expression was observed in the CA1 and CA3 regions of the hippocampus. This coincided with an increase in p75NTR expression in the dentate gyrus. No change in TrkA receptor expression was observed in any region of the hippocampus (data not included). p75NTR plays a dichotomous biological role in that it is capable of signalling for cell survival and cell death. It is postulated that the balance between life and death signalling is controlled by the ratio of receptor expression between Trk and p75NTR (Barrett and Bartlett, 1994). p75NTR as a co-receptor of Trk is a prosurvival receptor (Mahadeo *et al.*, 1994) However, p75NTR expression in the absence of Trk can induce apoptosis (Harrington *et al.*, 2002). Evidence from this study demonstrates that LPS alters the ratio between the expression of Trk and p75NTR and tips the balance towards p75NTR apoptotic signalling. Numerous studies have used experimental models such as ischemia, axotomy and seizures to induce increased expression of the p75NTR (Greferath *et al.*, 2002; Johnson *et al.*, 1999; Roux *et al.*, 1999) and all have shown a strong association with its expression and neuronal degeneration.

The peripheral administration of LPS has been shown to impair synaptic function (Shaw *et al.*, 2001, 2005). LTP is a form of synaptic plasticity that is induced experimentally and is thought to mimic learning. This study clearly demonstrates that LPS administration impairs LTP in the dentate gyrus of the rat. Analysis of the percentage epsp slope in the two minutes following High-frequency stimulation indicates that the induction of LTP is not affected by LPS and is

equivalent to saline controls. However, while the increase in percentage epsp slope was sustained by Saline-treated rats those treated with LPS were unable to sustain the increase and the slope gradually declined to baseline. This impairment of LTP by LPS has been demonstrated by several groups (Barry *et al.*, 2005; Kelly *et al.*, 2003; Hauss-Wegrzyniak *et al.*, 2002) and has been attributed by many to neurodegeneration propagated by an LPS induced increase in inflammatory cytokines such as interleukin- 1β (IL-1β), although the precise mechanisms are unknown. Evidence from this study suggests a role for the p75NTR in the LPS induced impairment of LTP. Increased p75NTR expression has been strongly correlated to impairments in synaptic function. Greferath *et al.* (2000) reported that p75NTR knock out mice had improved spatial learning performance when compared to their wild type counterparts.

An investigation into the role of mitogen activated protein kinase (MAPK) family members in neuronal apoptosis was carried out by Xia and co-workers (1995). They reported that NGF withdrawal from PC12 cells resulted in the sustained activation of JNK, the inhibition of ERK and ultimately neuronal apoptosis. They proposed that the balance between JNK and ERK activity may be important in determining cell fate. In this study, an increase in JNK activity following LPS administration was observed. JNK is a proapoptotic signalling molecule and has been shown to be activated downstream of p75NTR apoptotic signalling (Yoon *et al.* 1998). A study by Friedman in 2000 reported that JNK signalling mediated p75NTR induced apoptosis in hippocampal neurons in a ligand dependent manner. Therefore it can be suggested that the increase in JNK activity may be due to increased p75NTR signalling. Using *in vitro* methods this study attempted to elucidate the signalling pathways utilised by the p75NTR in the absence of Trk receptor signalling in both control animals and those treated with LPS in the hippocampus. The Trk inhibitor tyrphostin AG879 was used to silence Trk signalling. The rationale was that by inhibiting the Trk receptors and incubating hippocampal slices with NGF p75NTR signalling in the absence of Trk would occur i.e. p75NTR mediated apoptotic signalling. Tyrphostin was found to successfully inhibit Trk signalling. However, I failed to elucidate whether or not p75NTR activation was responsible for the JNK activity observed in LPS treated animals. It would appear that JNK signalling was maximally activated by LPS before

incubation with tyrphostin and NGF hence there were no observable changes in JNK activity between the groups. The problem with isolating JNK or any of the other downstream apoptotic signalling molecules of the p75NTR such as ceramide or NF-κB is that these molecules are activated by several other pathways including those activated by the inflammatory cytokines. Several studies have proposed a relationship between the inflammatory cytokines and the p75NTR. Boyle *et al.* (2005) reported that the proinflammatory cytokine tumour necrosis factor (TNF-α) mediated Schwann cell death by upregulating p75NTR expression in axotomised mouse sciatic nerves. Similarly, Bläsing and colleagues (2005) demonstrated that the inflammatory cytokines interferon-γ (IFN-γ), IL-1β and TNF-α upregulated p75NTR expression as well as NGF, NT-3 and NT-4 immunoreactivity in the skin. The interplay between cytokines and the neurotrophins was reported in the early nineties. Gadient *et al.* (1990) reported that IL-1β and TNF-α synergistically stimulated the release of NGF from cultured rat astrocytes. Similarly, NGF has been shown to regulate TNF- α production in mouse macrophages. Evidence from these papers makes it plausible to suggest that the increase in p75NTR expression post LPS administration may be associated with the neurodegeneration observed.

This study examined the effect of LPS administration on ERK activity in the hippocampus. ERK is a signalling molecule that is activated downstream of Trk receptor activation. It was hypothesized that the peripheral administration of LPS would result in a decrease in ERK activity and would be concomitant with decreased Trk receptor expression. The increase in JNK activity following LPS administration further supported the hypothesis that a decrease in ERK activity would be observed. While a decrease in TrkB receptor expression was observed along with an increase in JNK activity, LPS did not appear to effect ERK activity when compared to saline controls. *In vitro*, no changes in ERK activity were observed in any of the groups. This was not expected as we had previously observed an increase in ERK activity in Saline-treated hippocampal slices incubated with NGF.

This study demonstrates that LPS injection i.p. can manipulate neurotrophin receptor expression *in vivo*. LPS increases p75NTR expression in the dentate gyrus and decreases TrkB expression in the CA1 and CA3 *in vivo*. The differential

neurotrophin receptor expression induced by LPS administration was accompanied by an impairment in synaptic function as evidenced by the inability of LPS treated rats to sustain LTP *in vivo*. LPS increased neuronal cell death in the dentate gyrus *in vivo*. Interestingly, this was the region in which increased p75NTR expression was observed suggesting that p75NTR expression may be involved in LPS mediated cell death. JNK activity, which is associated with apoptotic signalling and is known to be activated downstream of the p75NTR, was increased in LPS treated animals when compared to saline controls. Overall the evidence presented in this study implicates a role for differential neurotrophin receptor expression and signalling in the neurodegeneration observed following LPS administration.

Chapter 4

The effect of kainic acid on neurotrophin receptor expression and signalling

4.1 Introduction

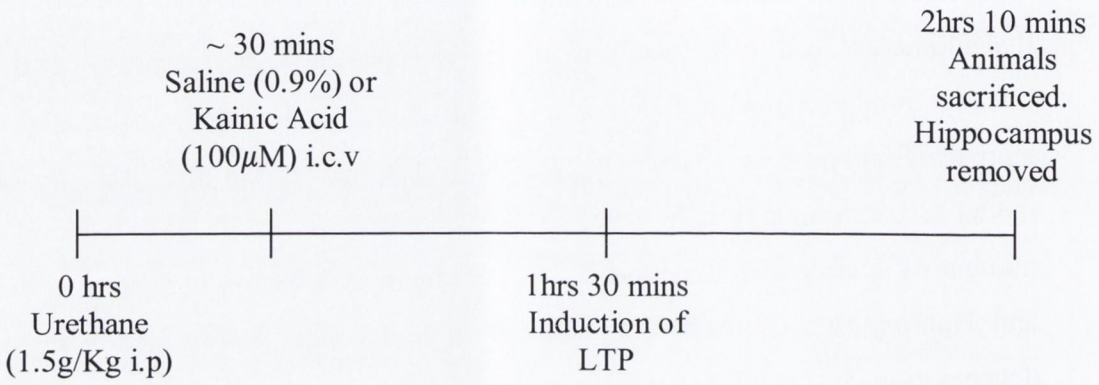
Kainic acid is a naturally occurring excitatory amino acid. It is a potent agonist of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate class of ionic glutamate receptors. Activation of glutamate receptors results in an influx of calcium and sodium ions into neurons. High concentrations of intracellular calcium can initiate intracellular signalling cascades that ultimately lead to the death of susceptible neurons. Kainic acid is frequently used to simulate temporal lobe epilepsy and induce excitotoxicity in experimental animals (Ben-Ari, 1984; Tasker *et al.*, 2002). It has proved to be a useful research model as excitotoxicity is believed to play a central role in the pathogenesis of a number of neurological disorders such as Alzheimer's disease, Parkinson's disease and Huntington's chorea. Administration of kainic acid results in the selective degeneration of several neuronal subtypes with those in the CA3 and dentate hilar region of the hippocampus being particularly susceptible. Kainic acid administration also produces profound deficits in memory and learning as assessed by the Morris water maze and object recognition tasks (Holmes *et al.*, 2002; Gobbo and O'Mara, 2004, 2005).

Roux *et al.* (1999) observed increased p75NTR expression following pilocarpine-induced seizures and reported its intrinsic involvement in apoptotic signalling. Results from Yi *et al.* (2003) and Oh *et al.* (2000) clearly implicate p75NTR activity in kainic acid induced neurodegeneration in both the basal forebrain and the hippocampus. Both groups report an increase in p75NTR expression following kainic acid administration and that this increase in expression is closely correlated to apoptotic cell signalling.

The purpose of this study was to investigate the effect of kainic acid administration on hippocampal synaptic function as assessed by the induction of LTP and to investigate whether any impairment in synaptic function is associated with altered neurotrophin receptor expression in the hippocampus.

4.2 Materials and Methods

4.2.1 Experimental Timeline



4.2.2 Treatment

Briefly, rats were anaesthetised with urethane (1.5g/kg i.p.) until loss of consciousness occurred, evidenced by the absence of the pedal reflex. Rats were subsequently injected intracerebroventricularly (i.c.v.) with 5μl of 100μM kainic acid or 5μl of saline (0.9% w/v).

4.2.3 Induction of LTP *in vivo*

One hour following kainic acid injection LTP was induced. Rats were placed in a stereotaxic frame and a bipolar stimulating electrode was placed in the perforant path of the dentate gyrus while a recording electrode was placed in the dorsal cell body of the region. Test shocks were delivered at 30 sec intervals and recorded for 10 minutes in order to establish a stable baseline. This was proceeded by delivery of three High-frequency trains of stimuli (250Hz for 200msec) at 30 sec intervals after which recording resumed at test shock frequency for 40 minutes. Upon cessation of recording animals were sacrificed by decapitation and the tissue removed. The removed tissue was sliced and stored at -80°C for further analysis.

4.2.4 *In vitro* incubations with kainic acid

Samples of hippocampus were incubated with kainic acid (100μM) or saline (0.9% w/v) for one hour in oxygenated Krebs solution. Following incubation, the samples were equalized for protein concentration according to the method of Bradford (1976). Equal volume of sample buffer was added and the samples were boiled for 2-3min and used for gel electrophoresis and western immunoblotting.

4.2.5 *In vitro* incubations ± tyrphostin AG879 ± NGF

Samples of hippocampus were incubated with kainic acid (100μM) or saline (0.9% w/v) for one hour in oxygenated Krebs solution. The slices were then incubated in the presence or absence of the Trk inhibitor tyrphostin AG879 (1mM) for 15min at 37°C with continuous oxygenation. The samples were further incubated for 25min at 37°C with oxygen in the presence or absence of NGF (100ng/ml) resulting in four treatments, control, NGF alone, tyrphostin alone and NGF/Tyrphostin. Following incubation, the samples were equalized for protein concentration according to the method of Bradford (1976). Equal volume of sample buffer was added and the samples were boiled for 2-3min and used for gel electrophoresis and western immunoblotting.

4.2.6 Analysis of ERK and JNK activation in kainic acid-treated rats

ERK and JNK activity was assessed using gel electrophoresis and western immunoblotting as described in section 2.14. Membranes were incubated with 5% BSA/TBS-T overnight to block any unspecific binding. Membranes were given three 10 minutes washes with TBS-T after which they were incubated for two hours at room temperature in primary antibody (anti-phosphoERK (Santa Cruz) 1:3000 in 2% BSA/TBS-T; anti-ERK2 (Santa Cruz) 1:1000 in 2% BSA/TBS-T; anti-phosphoJNK (Santa Cruz) 1:200 in 2% BSA/TBS-T). Following primary antibody incubation membranes were washed with TBS-T (three 10 minute washes) and were subsequently incubated with secondary antibody for one hour at room temperature (1:1000 anti-mouse IgG (Sigma) for detection of pERK, ERK2, pJNK and JNK1). Membranes were washed in TBS-T as before. The protein bands were detected using Supersignal (Amersham) before being exposed to photographic film (Hyperfilm).

4.2.7 Analysis of neurotrophin receptor expression

Neurotrophin receptor expression was assessed using gel electrophoresis and western immunoblotting. As before, the blots were blocked overnight in 5% BSA/TBS-T. Primary antibody was incubated for 2 hours (anti-p75NTR (Upstate Signalling) 1:200 2% BSA/TBS-T; anti-TrkA (Santa Cruz) 1:100 2% BSA/TBS-T). Following a 30min wash in TBS-T (3x10min), membranes were incubated with secondary antibody for one hour (anti-rabbit IgG (Sigma) 1:500). Blots were washed and the receptors were detected using Supersignal (Amersham). Receptor expression was quantified using densitometric analysis.

4.2.8 Densitometric analysis

All protein bands were quantitated by densitometric analysis using the ZERO-DScan Image Analysis System (Scanalytics, Fairfax, USA) or the Gel Doc It Imaging System (UVP, Medical Supply Company, Ireland) in conjuction with LabWorks (Lablogics Inc, Mission Viejo, California, USA).

4.3 Results

4.3.1 Effect of kainic acid on LTP *in vivo*

The ability of kainic acid-treated rats to sustain LTP in the perforant path-granule cell synapses following High-frequency stimulation was assessed. There was an increase in epsp slope immediately after tetanic stimulation in the saline-treated rats (n=4) which was not observed in the kainic acid-treated rats (n=4) (Figure 4.1). The mean percentage epsp slopes (± SEM) in the last 5 mins of recording was compared 117.3± 0.3% and 83.74 ± 0.73% for saline and kainic acid-treated rats respectively; **p<0.01, 2-way ANOVA, *post-hoc* Newmann Keuls, n=4) (Figure 4.2B).

4.3.2 Effect of kainic acid on p75NTR expression *in vivo*

Expression of p75NTR in the hippocampus following treatment with saline or kainic acid was analysed by gel electrophoresis and immunoblotting. p75NTR expression was increased in the hippocampus of kainic acid-treated rats compared with saline-treated rats as shown in Fig 4.3A. Densitometric analysis revealed that this increase was statistically significant **p<0.001; students t-test for independent means (p=0.0052). Results are expressed as a percentage of p75NTR expresion/actin expression (mean ± SEM, n=4) 52.56 ± 1.4% and 46.49 ± 0.81% for kainic acid and saline-treated rats respectively (Figure 4.3B).

4.3.3 Effect of kainic acid on TrkA expression *in vivo*

The expression of TrkA receptor expression in the hippocampus following kainic acid administration was assessed. Statistical analysis revealed that kainic acid administration had no effect on TrkA receptor expression . Results are expressed as a percentage of TrkA expresion/actin expression (mean ± SEM, n=4) 118.9 ± 9.938% and 112.2 ± 15.77% for kainic acid and saline-treated rats respectively (Figure 4.4B).

4.3.4 Effect of kainic acid on ERK activity *in vivo*

The effect of kainic acid on ERK activity was assessed using gel electrophoresis and immunoblotting. ERK activity was decreased in the hippocampus of kainic acid-treated rats compared with saline-treated rats as shown in Figure 4.5A. Densitometric analysis revealed that this decrease was statistically

significant *p<0.05; students t test for independent means. Results are expressed as a percentage of pERK/total ERK expression (mean ± SEM, n=4) 0.48 ± 0.04% and 0.76 ± 0.07% for kainic acid and saline-treated rats respectively (Figure 4.5B).

4.3.5 Effect of kainic acid on JNK activity *in vivo*

JNK activity following kainic acid administration was assessed using gel electrophoresis and western immunoblotting. Figure 4.6A shows a representative immunoblot displaying that kainic acid treatment markedly increases JNK activity in the hippocampus. Densitometric analysis of the immunoblots showed this increase to be statistically significant ***p<0.0001; students t-test for independent means. Results are expressed as a percentage of phosphorylated JNK/total JNK expression (mean ± SEM, n=4), 36.79 ± 1.19% and 6.28 ± 2.09% respectively for kainic acid-treated rats and saline-treated rats respectively (Figure 4.6B).

4.3.6 Effect of kainic acid on p75NTR expression *in vitro*

p75NTR expression in the hippocampus *in vitro* was assessed using gel electrophoresis and western immunoblotting. Densitometric analysis of the immunoblots revealed that kainic acid treatment significantly increases p75NTR expression when compared to saline controls (114.9 ± 25.95 and 50.08 ± 11.92 respectively, Figure 4.7B). Results are expressed as the means ± SEM, n=6, students t-test for independent means, *p<0.05 (p=0.0465).

4.3.7 The effect of tyrphostin on ERK activity following incubation with kainic acid *in vitro*

ERK activity in hippocampal slices incubated with either saline or kainic acid and subsequently treated with either NGF or tyrphostin or both was assessed using western immunoblotting. Densitometric analysis revealed no significant difference in ERK activity in hippocampal slices that had been treated with either saline (0.9%) w/v) or kainic acid (100μM) (3-way ANOVA, 131.8 ± 41.1% and 100.4 ± 12.98% for control saline and kainic acid-treated slices respectively). Incubation of control and kainic acid-treated hippocampal slices with NGF (100ng/ml) had no effect on ERK activity (105.3 ± 16.69% and 100.3 ± 17.69% for saline and kainic acid-treated slices respectively). Inhibition of the Trk receptor by tyrphostin AG879 (1mM) had no effect on ERK activity in either saline or kainic

acid-treated slices (87.32 ± 11.68% and 83.95 ± 27.66% for saline and kainic acid-treated slices respectively). Finally, co-incubation with NGF and tyrphostin had no effect on ERK activity in either saline or kainic acid-treated slices (92.94 ± 20.51% and 73.19 ± 2.409% for saline and kainic acid-treated slices respectively). Results are expressed as mean percentage ± SEM of phosphorylated p42ERK/total ERK expression (n=3-4) (Figure 4.8).

4.3.8 The effect of tyrphostin on JNK activity following incubation with kainic acid *in vitro*

JNK activity was assessed using western immunoblotting (Figure 4.7A). Densitometric analysis revealed a significant increase in JNK activity in hippocampal slices that had been treated with kainic acid (100μM) when compared to those that had been treated with saline (0.9% w/v) (3-way ANOVA, *post-hoc* Newmann Keuls; 163.2 ± 25.04% and 284.4 ± 31.04% for control saline and kainic acid-treated slices respectively). Incubation of saline-treated slices with NGF (100ng/ml) increased JNK activity when compared to saline only controls while it had no effect on kainic acid-treated slices(*$p<0.05$, 3-way ANOVA, *post-hoc* Fisher's LSD; 305.7 ± 29.03% and 241.1 ± 45.62% for saline and kainic acid-treated slices respectively). Inhibition of the Trk receptor by tyrphostin AG879 (1mM) had no effect on JNK activity in either saline or kainic acid-treated slices (267.5 ± 35.39% and 248.1 ± 42.85% for saline and kainic acid-treated slices respectively). Finally, co-incubation with NGF and tyrphostin had no effect on JNK activity in saline-treated slices but did significantly decrease JNK activity in kainic acid-treated slices when compared to kainic acid only controls (*$p<0.05$, 3-way ANOVA, *post-hoc* Newmann Keuls; 145 ± 19.19% and 143 ± 14.33% for saline and kainic acid-treated slices respectively). Results are expressed as mean percentage ± SEM of phosphorylated JNK1/ total JNK1 expression (n=2-4) (Figure 4.9B).

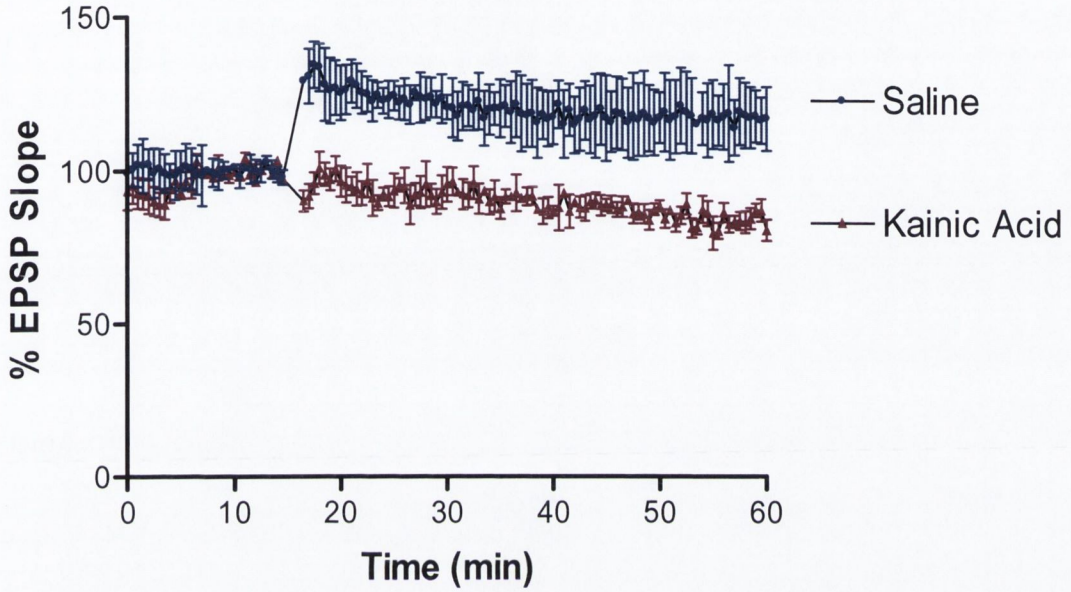


Figure 4.1 Expression of LTP in saline and kainic acid treated rats *in vivo*

Test shocks were given at 30 sec intervals for a 10min control prior to tetanization. High frequency stimulation (3 trains of stimuli of 200Hz for 200msec at 20sec intervals) was delivered to the perforant path. Recording at test shock frequency resumed for 40min following tetanus. LTP was maintained by saline-treated rats; however those treated with kainic acid failed to potentiate. Results are expressed as mean EPSP slope normalized with respect to the EPSP slope recorded in the 5 min immediately prior to tetanic stimulation. Results are expressed as means ± SEM of 4 independent observations for both treatment groups.

A

B

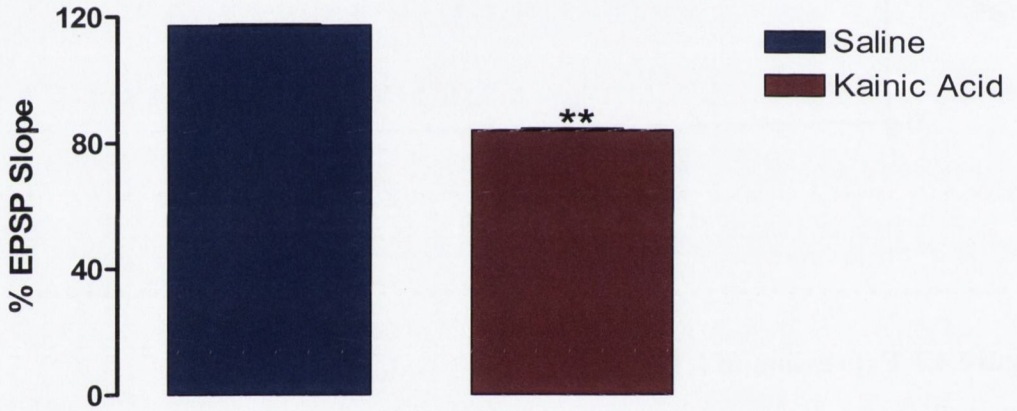


Figure 4.2 Expression of LTP in saline and kainic acid treated rats *in vivo*

The ability of rats treated with kainic acid to maintain LTP was assessed. High frequency stimulation (HFS; 3 trains of stimuli of 200Hz for 200msec at 20sec intervals) was delivered to the perforant path. Recording at test shock frequency resumed for 40min following tetanus. A significant increase in percentage epsp slope was observed in saline-treated rats following high frequency stimulation. This increase in epsp slope was maintained for the duration of recording by saline-treated animals. However, those treated with kainic acid failed to sustain LTP to the cessation of recording (Figure 4.2B**$p<0.01$, 2-way ANOVA, *post-hoc* Newmann Keuls, n=4).

A.

Saline **Kainic Acid**

B.

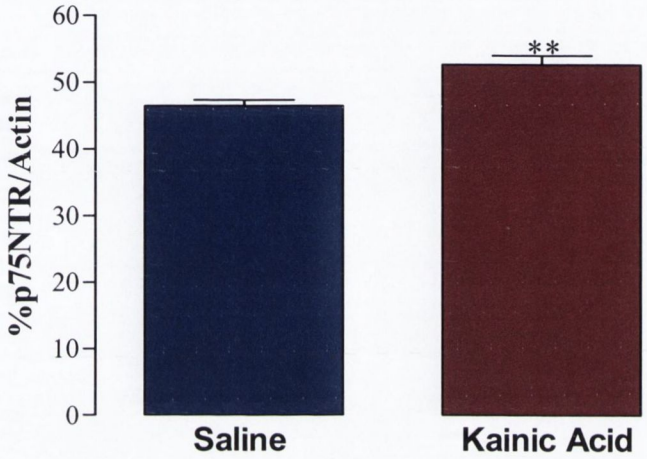


Figure 4.3 Kainic acid administration increases p75NTR expression *in vivo*

A. Sample western immunoblot illustrating increased p75NTR expression in rats treated with kainic acid

B. Rats were injected i.c.v with either kainic acid (100μM) or saline (0.9%) one hour prior to recording LTP. p75NTR expression was assessed by western immunoblot analysis. Administration of kainic acid significantly increased p75NTR expression when compared to saline controls (52.56 ± 1.441 and 46.49 ± 0.8077 respectively). Results are expressed as a percentage of p75NTR expresion/actin expression (mean ± SEM, n=4), students unpaired t-test, **$p<0.001$ ($p=0.0052$).

A

Saline **Kainic Acid**

B

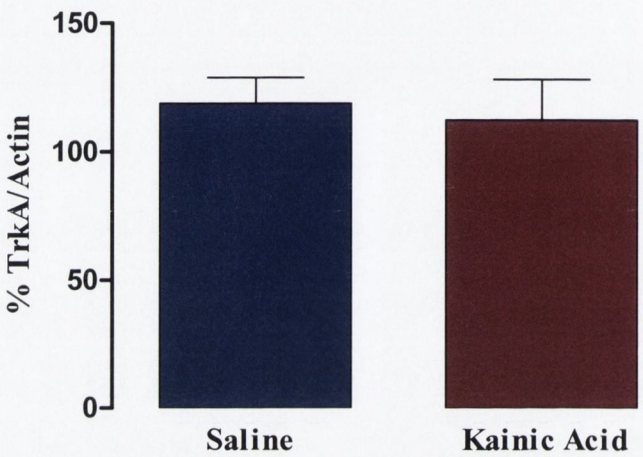
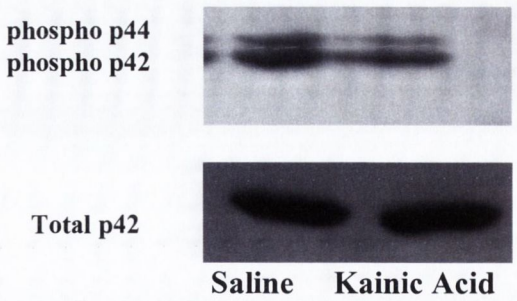


Figure 4.4 Kainic acid administration has no effect on TrkA expression *in vivo*

A. Sample western immunoblot illustrating increased TrkA expression in rats treated with kainic acid

B. Rats were injected i.c.v with either kainic acid (100μM) or saline (0.9%) one hour prior to recording LTP. TrkA expression was assessed by western immunoblot analysis. Administration of kainic acid had no effect on TrkA expression when compared to saline controls. Results are expressed as a percentage of TrkA expresion/actin expression (mean ± SEM, n=4).

A



B

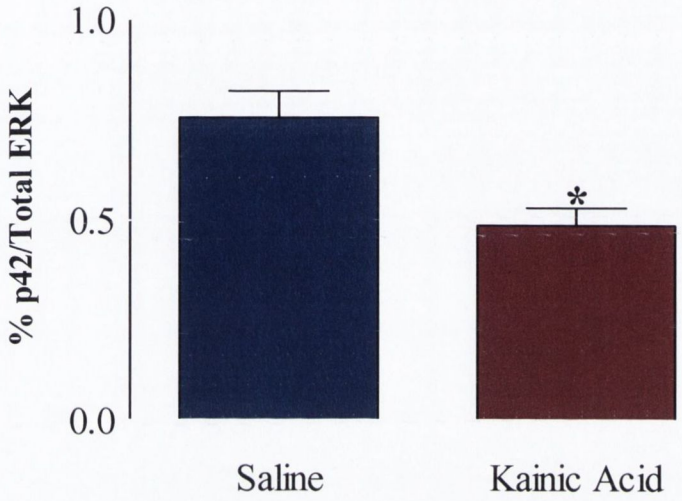
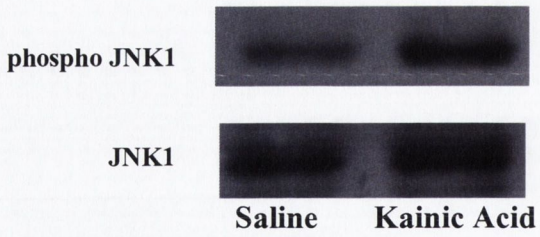


Figure 4.5 Kainic acid administration decreases ERK activity *in vivo*

A. Sample western immunoblot illustrating decreased ERK activity in animals treated with kainic acid

B. Rats were injected i.c.v with either kainic acid (100μM) or saline (0.9%) one hour prior to recording LTP. Phosphorylation of ERK and total ERK expression were assessed by western immunoblot. Administration of kainic acid significantly decreased ERK activity when compared to saline controls. Results are expressed as a ratio of phosphorylated ERK/ total ERK expression (mean ± SEM, n=4), students unpaired t-test, *$p<0.05$ ($p=0.0138$).

A



B

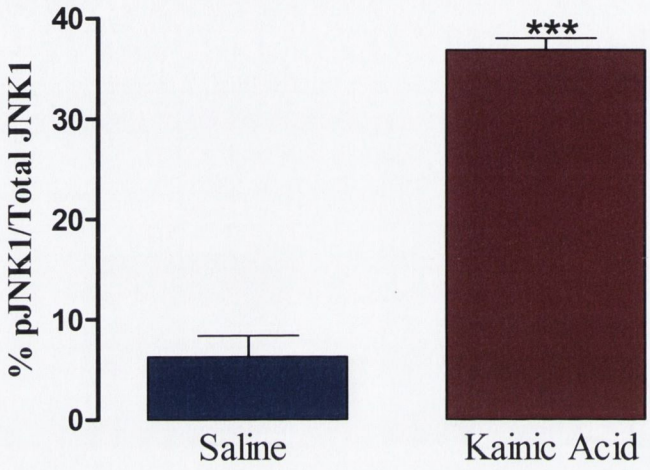


Figure 4.6 Kainic acid administration increases JNK activity *in vivo*

A. Sample western immunoblot illustrating increased JNK activity in rats treated with kainic acid

B. Rats were injected i.c.v with either kainic acid (100μM) or saline (0.9%) one hour prior to recording LTP. Phosphorylation of JNK and total JNK expression were assessed by western immunoblot. Administration of kainic acid significantly increased JNK activity when compared to saline controls. Results are expressed as a ratio of phosphorylated JNK/ total JNK expression (mean ± SEM, n=4), students unpaired t-test, ***$p<0.0001$.

A

B

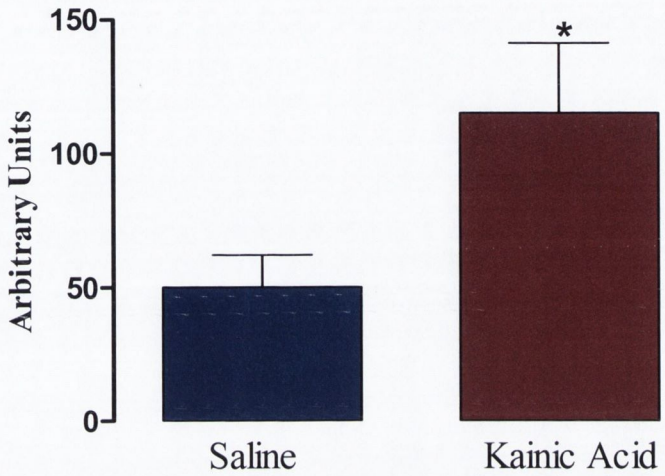
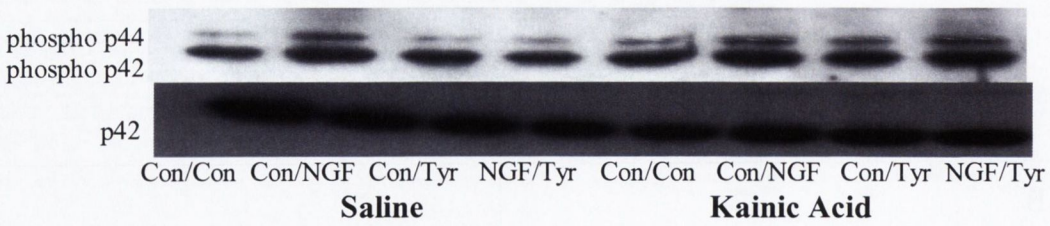


Figure 4.7 Kainic acid administration increases p75NTR expression *in vitro*

A. Sample western immunoblot illustrating increased p75NTR expression in rats treated with kainic acid

B. Samples were incubated with either kainic acid (100μM) or saline (0.9%) for one hour at 37°C . p75NTR expression was assessed by western immunoblot analysis. Treatment with kainic acid significantly increased p75NTR expression when compared to saline controls (114.9 ± 25.95 and 50.08 ± 11.92 respectively). Results are expressed as the means ± SEM, n=6, students unpaired t-test, *$p<0.05$ ($p=0.0465$).

A



B

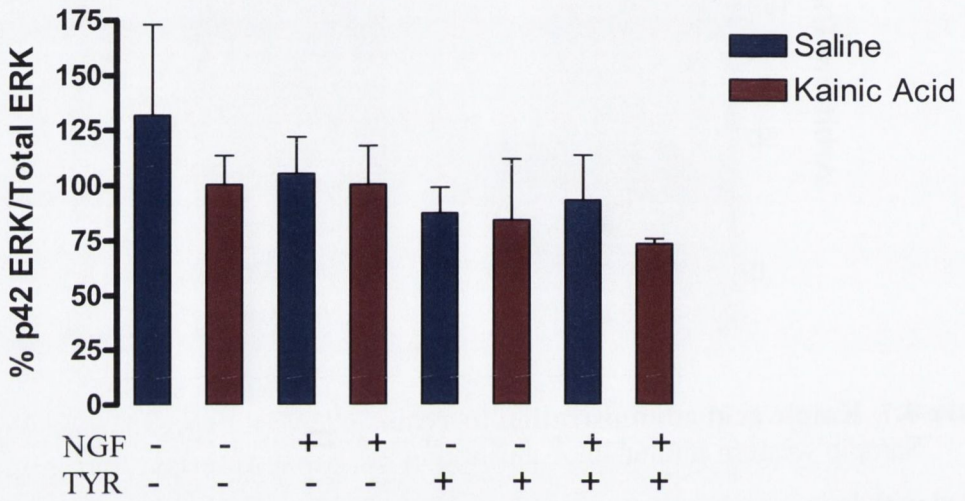
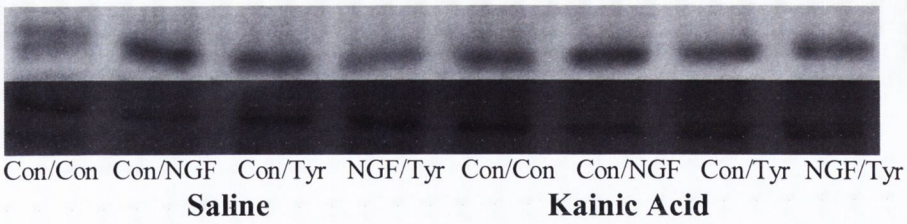


Figure 4.8 The effect of tyrphostin on ERK activity following incubation with kainic acid *in vitro*

A. Sample western immunoblot illustrating the effect of tyrphostin on ERK activity following incubation with kainic acid *in vitro*

B. Samples were incubated with either kainic acid (100μM) or saline (0.9%) for one hour at 37°C . The slices were subsequently incubated with either NGF or tyrphostin or both. ERK activity was assessed by western immunoblot analysis. Densitometric analysis revealed no significant differences between any of the groups. Results are expressed as the means ± SEM, n=4, 3-way ANOVA.

A



B

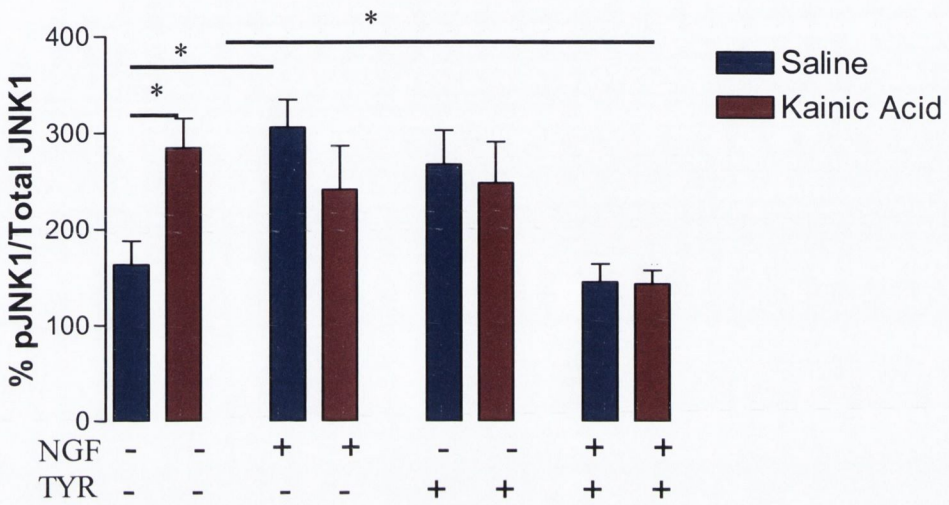


Figure 4.9 The effect of tyrphostin on JNK activity following incubation with kainic acid *in vitro*

A. Sample western immunoblot illustrating the effect of tyrphostin on JNK activity following incubation with kainic acid *in vitro*

B. Samples were incubated with either kainic acid (100μM) or saline (0.9%) for one hour at 37°C . The slices were subsequently incubated with either NGF or tyrphostin or both. JNK activity was assessed by western immunoblot analysis. Densitometric analysis revealed a significant increase in JNK activity following incubation with kainic acid when compared with saline controls (*$p<0.05$). It was observed that this kainic acid increase in JNK activity was blocked following incubation with NGF and tyrphostin (*$p<0.05$). Results are expressed as the means ± SEM, n= 2-4, 3-way ANOVA.

4.4 Discussion

The primary objective of this study was to investigate the effect of kainic acid administration on neurotrophin receptor expression in the hippocampus. Furthermore this study attempted to determine whether any changes in receptor expression observed may contribute to the degenerative effects of kainic acid. This study also examined the possible signalling pathways utilised by the p75NTR.

This study demonstrates that the administration of kainic acid either *in vivo* or *in vitro* upregulates the expression of the p75NTR. This evidence supports the findings of Oh *et al.* (2000) and Yi *et al.* (2003) both of whom reported an increase in p75NTR expression following kainic acid injection. Kainic acid injection produces lesions in the brain and has been shown to induce selective neurodegeneration of specific regions in the hippocampus, namely the CA1, CA3 and dentate hilar regions (Oh et al, 2000; Shetty *et al.*, 2003; Yi *et al.*, 2003). Kainic acid is a glutamate analog and activates the kainate/AMPA subtype of glutamate receptor. Kainic acid-induced neurotoxicity has been attributed to increased activation of these receptors and increased intracellular calcium concentrations although the precise mechanisms are not known. Evidence from this study suggests that increased p75NTR expression is implicated in kainic acid-induced neuronal degeneration. Increased p75NTR expression is associated with pro apoptotic signalling (Frade *et al.*, 1996; Barrett and Bartlett, 1994). Previous studies using pilocarpine-induced seizures (Roux *et al.*, 1999) demonstrated a strong correlation between the p75NTR and neuronal death. The results presented by both Oh *et al.* and Yi *et al.* showed a strong correlation between expression of the p75NTR and apoptosis in kainic acid-treated animals.

In this study, kainic acid-administration was shown to have a profound effect on synaptic function. Injection of kainic acid (100μM) i.c.v. one hour prior to LTP recording, completely abolished the ability of rats to induce LTP. Saline and kainic acid-treated animals had similar baseline epsp slopes with no significant difference between groups prior to High-frequency stimulation. Analysis of the percentage epsp slope in the two minutes following High-frequency stimulation indicates that the induction of LTP is inhibited by kainic acid administration. High-frequency stimulation increased the percentage epsp slope of saline-treated animals

by approximately 20% indicating a strengthening in synaptic efficacy. Upon high-frequency stimulation the percentage epsp slope of kainic acid-treated animals fell below baseline level indicating a severe impairment in synaptic function. Saline animals maintained the increase in epsp slope until the cessation of recording. The epsp slope of kainic acid-treated animals steadily deteriorated over the recording period ultimately being approximately 20% below baseline. The impairment in LTP induced by kainic acid administration can be attributed to the selective neurodegeneration it instigates in the specific subpopulations of neurons in the hippocampus (Oh et al, 2000; Shetty *et al.*, 2003; Yi *et al.*, 2003). Since the p75NTR is implicated in kainic acid-induced neurodegeneration the observed impairment in LTP may be due to the increased expression of the p75NTR.

A study by Xia and co-workers (1995) established that JNK signalling was critically involved in the apoptosis of PC12 cells upon NGF withdrawal. They reported that NGF withdrawal from PC12 cells resulted in the sustained activation of JNK, the inhibition of ERK and ultimately neuronal apoptosis. They proposed that the balance between JNK and ERK activity may be important in determining cell fate.

JNK is a stress activated protein kinase (SAPK) and is a proapoptotic signalling molecule. Yang *et al.* (1997) reported the absence of kainic acid induced apoptosis in the hippocampus of mice lacking the JNK3 gene, determining that JNK signalling is critical to the pathogenesis of glutamate neurotoxicity. This study found an increase in JNK activity following kainic acid administration both *in vivo* and *in vitro*. JNK has previously been shown to be activated downstream of p75NTR apoptotic signalling (Yoon *et al.* 1998). The increase in JNK activity is concomitant with increased p75NTR expression supporting the hypothesis that increased p75NTR expression is associated with apoptotic signalling. *In vivo,* increased JNK activity was associated with decreased ERK activity, mimicking the results reported by Xia *et al.*.

With the knowledge that p75NTR could be successfully upregulated by kainic acid administration I returned to using *in vitro* methods in an attempt to isolate the signalling pathways utilised by the p75NTR in the absence of Trk

receptor signalling in the hippocampus. The Trk inhibitor tyrphostin AG849 was used to silence Trk signalling. The hypothesis was that by inhibiting the Trk receptors and incubating hippocampal slices with NGF, p75NTR signalling in the absence of Trk would occur i.e. p75NTR mediated apoptotic signalling. Previous studies using tyrphostin had shown it to be successful in inhibiting Trk signalling. Analysis of ERK activity revealed no significant changes between any of the groups. As with the previous *in vitro* and *in vivo* work kainic acid was found to increase JNK activity when compared to saline controls. The hypothesis was that by blocking trk signalling and incubating with NGF that p75NTR signalling would occur and there would be increased JNK activity. However, it was observed that blocking the Trk receptor and incubating with NGF resulted in decreased JNK activity when compared to kainic acid only treated slices. This was not expected and suggests that the p75NTR receptor may actually be playing a neuroprotective role in kainic acid-induced excitotoxicity. Previous studies in culture have implicated a neuroprotective role for the p75NTR (Kume *et al.*, 2000; Culmsee *et al.*, 2002). Culmsee *et al.* (2002) investigated the neuroprotective role of the p75NTR in glutamate-induced toxicity. In hippocampal cells lacking p75NTR activation of TrkA was not detected. Moreover, NGF induced neuroprotection against glutamate toxicity was abolished indicating the p75NTR in supporting NGF TrkA signalling pathways mediating survival. This result needs to be examined in further detail as it is by no means conclusive and if indeed the p75NTR is playing a neuroprotective role following injury *in vivo* the mechanism needs to be elucidated.

In this study I have found a successful model by which I can manipulate p75NTR both *in vivo* and *in vitro.* Kainic acid increases p75NTR expression both *in vitro* and *in vivo* when compared to saline controls. This increase in p75NTR was accompanied by a severe impairment in synaptic function as evidenced by the inability of kainic acid-treated rats to induce LTP *in vivo.* There was an increase in JNK activity both *in vivo* and *in vitro* indicating increased cell death signalling which was concomitant with decreased ERK activity *in vivo.* The *in vivo* evidence suggested that the injury-induced expression of the p75NTR was having a neurodegenerative effect in the hippocampus. However, the results from the *in vitro* studies suggested otherwise. JNK activity was abrogated in kainic acid-treated slices by incubating with NGF and the Trk inhibitor tyrphostin AG879 when compared

kainic acid-treated controls. p75NTR may apoptotically signalling via one of its other pathways such as that involving ceramide. It is clear that further work is required to fully elucidate the role of the p75NTR following kainic acid-induced injury.

Chapter 5

The effect of lipopolysaccharide administration on rats deficient in NGF and TrkA

5.1 Introduction

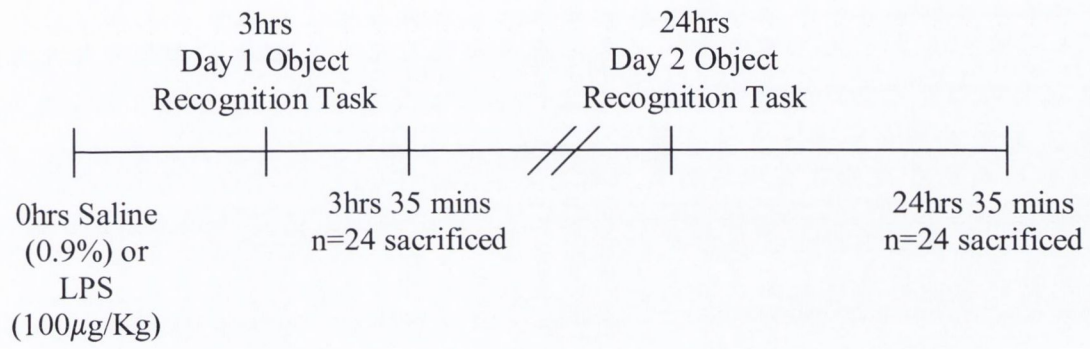
The neurotrophins are classically regarded as the mediators of growth, differentiation and maintenance of the developing nervous system. It was not until the work of Lohof and colleagues, in the early 1990's that the neurotrophins were able to shed this classic image and emerge as key modulators of the mature nervous system. Both BDNF and NGF and their corresponding receptors, TrkB and TrkA have been shown to play a fundamental role in the induction and maintenance of LTP (Korte *et al.*, 1996; Kelly *et al.*, 1998; Gooney and Lynch, 2001). The neurotrophins have been demonstrated to be intrinsic to the successful completion of spatial tasks such as the water Morris maze and the object preference task (Linnarsson *et al.*, 1997; Scali *et al.*, 1994). In 1998, it was reported by Kelly *et al.* that the NGF and its receptor, TrkA, played a role in LTP. This was demonstrated by using an inbred strain of genetically hypertensive (GH) rat which had been previously been shown to be NGF deficient in the peripheral nervous system (Messina and Bell, 1991). Kelly and co-workers ascertained that this NGF deficiency was prevalent in the central nervous system as shown by decreased levels of NGF in the dentate gyrus of the GH rats. It was also determined that there was a corresponding deficiency in TrkA receptor expression in the dentate gyrus of the GH rats.

In Chapter 3 it was shown that an i.p. injection of LPS increased the expression of the p75NTR in the dentate gyrus and decreased the expression of Trk B in the CA1 and CA3 regions of the hippocampus and that this differential expression of receptors is concomitant with an impairment in synaptic function as assessed by LTP. Several studies have shown that the peripheral administration of LPS results in diminished neurotrophin levels and a reduction in neurotrophin receptor expression in several regions of the central nervous system including the hippocampus (Guang and Fang, 2005; Tanaka *et al.*, 2006). These alterations in both ligand and receptor expression levels are believed to contribute to the neurodegenerative effects of LPS. As previously mentioned the p75NTR has a conflicting binary function; it can induce the activation of both apoptotic and pro-survival signalling pathways. It is widely believed that its function depends on the expression of co-receptors. In 1994, Barrett and Bartlett postulated that the differential expression of TrkA was responsible for the diverse functions of the

p75NTR. When co-expressed the p75NTR enhances TrkA survival signalling (Mahadeo *et al.*, 1994) but when the ratio of p75NTR expression is greater than TrkA expression apoptotic signalling occurs (Bono *et al.*, 1999). The majority of research conducted in order to examine pro-apoptotic signalling induced by the p75NTR has been carried out *in vitro* using cell lines and culture. In an attempt to isolate p75NTR signalling pathways *in vivo*, I employed the use of the Trk deficient GH rats. The hypothesis was that LPS administration to the GH rats would increase p75NTR expression as per my previous experiment and that the absence of Trk receptors would facilitate p75NTR apoptotic signalling.

5.2 Methods

5.2.1 Experimental Timeline



5.2.2 Animals

Two strains of animal were used in this experiment; normotensive (N) New Zealand Otago Wistar rats and genetically hypertensive (GH) New Zealand Otago Wistar rats.

The N and GH rats were obtained from breeding colonies in the BRU (a gift from Prof. Bell). The GH strain of rat was originally bred in the Department of Medicine in the University of Otago, New Zealand. It was developed as a model of essential hypertension by brother-sister matings of successive generations of Wistar rats that displayed high blood pressures (~ 140mmHg systolic blood pressure). The N strain was bred from the ancestral stock from which the GH rats were originally bred and serve as controls for the GH rats. All N and GH rats were male and aged between 3 and 4 months.

5.2.3 Treatment

Rats were injected with either Escherichia Coli LPS (100μg/Kg) or saline (0.9% w/v) three hours prior to the commencement of the novel object preference task on day one.

5.2.4 Object Recognition Task

For the object recognition task, the exploration criteria were based strictly on active exploration. The rats must be seen to have touched to object with at least their nose for it to be deemed as true exploration. The discrimination between familiar and new objects is measured by the time spent exploring and not on the basis of the rat's first choice. The measurement of the time spent exploring each object is recorded and subsequently expressed as a percentage of the total exploration time.

During the one-day experiment, rats were individually placed in the open field. All rats had either been injected with saline or LPS. The rats were allowed to explore two objects for three 5min time periods (**Figure 5.1A**). Between each time period the rats were removed from the open field and placed in a holding cage for 5min. The time spent exploring each of the two objects was recorded. The whole experiment lasted for approximately 30min. All rats were sacrificed 5min after their final exposure to the objects.

The two day experiment consisted of rats performing the day one protocol as described above. These animals were not sacrificed at the end of day 1. On day 2, the rats were given a single 5min exposure to one familiar and one novel object (**Figure 5.1B**). The rats were sacrificed 5min after their day 2 exposure. The time spent actively exploring each object was recorded on both days.

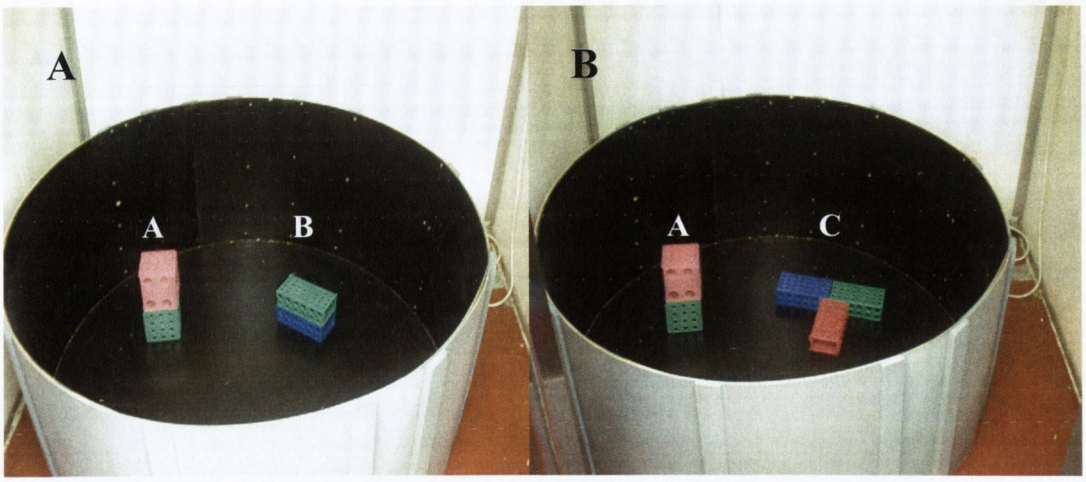


Figure 5.1 Object Recognition Task

A. Day one apparatus for the object recognition task. Two objects A and B are placed into the arena. Rats are introduced to the arena and the time spent actively exploring each object is recorded

B. Day two apparatus for the object recognition task. Object B is replaced with novel object C. Rats are introduced to the arena and the time spent actively exploring each object is recorded

5.2.5 Analysis of ERK and JNK activation in N and GH rats treated with saline or LPS

ERK and JNK activity was assessed using gel electrophoresis and western immunoblotting as described in section 2.14. Membranes were incubated with 5% BSA/TBS-T overnight to block any unspecific binding. Membranes were given three 10 minutes washes with TBS-T after which they were incubated for two hours at room temperature in primary antibody (anti-phosphoERK (Santa Cruz) 1:3000 in 2% BSA/TBS-T; anti-ERK2 (Santa Cruz) 1:1000 in 2% BSA/TBS-T; anti-phosphoJNK (Santa Cruz) 1:200 in 2% BSA/TBS-T). Following primary antibody incubation membranes were washed with TBS-T (three 10 minute washes) and were subsequently incubated with secondary antibody for one hour at room temperature (1:1000 anti-mouse IgG (Sigma) for detection of pERK, ERK2, pJNK and JNK1). Membranes were washed in TBS-T as before. The protein bands were detected

using Supersignal (Amersham) before being exposed to photographic film (Hyperfilm).

5.2.6 Analysis of actin expression

Western blotting was used to assess actin expression in all groups. Membranes were blocked in 5% BSA/TBS-T overnight and washed in TBS-T. Primary antibody was incubated for 2 hours (1:100 (Cell signalling) 2% BSA/TBS-T) after which the membranes were washed in TBS-T. Membranes were subsequently incubated with secondary antibody, for one hour (1:500 anti-rabbit IgG (Sigma)). The protein bands were detected using Supersignal (Amersham) before being exposed to photographic film (Hyperfilm) and were quantified using densitometric analysis.

5.2.7 Analysis of neurotrophin receptor expression

Neurotrophin receptor expression was assessed using gel electrophoresis and western immunoblotting. As before, the blots were blocked overnight in 5% BSA/TBS-T. Primary antibody was incubated for 2 overnight at 4°C (anti-p75NTR (generous gift from Dr.Phil Barker; 1:1000 in TBS-T containing 2% BSA); anti-TrkA (Upstate Cell Signalling) 1:800 2% BSA/TBS-T). Following a 30min wash in TBS-T (3x10min), membranes were incubated with secondary antibody for one hour (donkey anti-rabbit IgG (Sigma) 1:1000). Blots were washed and the receptors were detected using Supersignal (Amersham). Receptor expression was quantified using densitometric analysis.

5.3 Results

5.3.1 Decreased NGF concentration and TrkA receptor expression impairs recognition memory in the rat as does the peripheral injection of LPS

The performance of N and GH rats treated with either saline or LPS in the object recognition task was assessed. As explained in section 5.2.3 the object recognition task takes place over two days and assesses the ability of animals to recognise the presence of a novel object. N animals treated with saline explored objects A and B equally on day one (Figure 5.2A). They successfully observed the presence of the novel object C on day two as demonstrated by the significantly greater amount of time spent it than the familiar object A (**$p<0.01$, 2-way ANOVA, post-hoc Newmann Keuls; 69.83 ± 3.3% and 30.17 ± 3.3% for objects C and A respectively). There was no significant difference in the amount of time exploring objects A and B in N rats treated with LPS (Figure 5.2B). However, LPS administration inhibited the ability for normotensive rats to recognise the novel object C on day 2 (45.62 ± 5.7% and 54.38 ± 5.7% for object A and C respectively, n=6). GH rats treated with saline showed a slight preference for the novel object C on day two (Figure 5.2C), this was not significant. LPS further abrogated the ability of the GH rats to recognise the novel object (Figure 5.2D). Results are expressed as a percentage of the time taken exploring each object/total exploration time.

5.3.2 TrkA expression in the dentate gyrus of N rats is decreased following peripheral LPS injection and is decreased in the dentate gyrus of GH control rats

The hippocampii and dentate gyri of N and GH rats sacrificed on day one and day two were assessed for TrkA expression. Peripheral injection of LPS (100μg/Kg) significantly decreased the expression of TrkA in the dentate gyrus of N rats when compared to N saline-treated controls on day one of the object recognition task (*$p<0.05$; 2-way ANOVA, *post-hoc* Newmann Keuls; 172.3 ± 50.15 and 63.34 ± 12.1 for N saline and LPS-treated rats respectively, n=4-5, Figure 5.3B). Densitometric analysis also revealed that GH saline-treated rats were significantly deficient in TrkA expression when compared to N saline-treated animals (*$p<0.05$; 2-way ANOVA, *post-hoc* Newmann Keuls; 172.3 ± 50.15 and 76.51 ± 20.5 for N saline and GH saline-treated rats respectively, n=4-5, Figure 5.3B). LPS administration had no effect on TrkA expression in GH rats (103 ± 13.4, n=5,

Figure 5.3B). There were no statistical differences between any of the groups in the hippocampus (79.68 ± 9.234% and 53.31 ± 12.5% for N saline and LPS-treated rats respectively and 61.36 ± 6.412% and 56.21 ± 8.567% for GH saline and LPS-treated rats respectively, Figure 5.3D). The LPS-induced decrease of TrkA expression in the dentate gyrus of N LPS-treated rats was not observed on day 2 of the object recognition task (7.750 ± 3.751% and 8.067 ± 2.389% for N saline and LPS-treated rats respectively, Figure 5.4B). Statistical analysis also revealed no differences in the dentate gyrus between N rats treated with saline or LPS and GH rats treated with saline or LPS on day 2 of the object recognition task (5.781 ± 1.486% and 11.66 ± 4.1% for GH saline and LPS-treated rats respectively, Figure 5.4B). However, a significant decrease in TrkA expression in the hippocampus of GH saline-treated rats was observed when compared with N saline-treated controls (*$p<0.05$, 2-way ANOVA, *post-hoc* Newmann Keuls; 214.6 ± 50.9% and 75.56 ± 16.90% for N saline-treated and GH saline-treated rats respectively, n=3-4, Figure 5.4D). LPS did not have an effect on TrkA expression in either the N or the GH rats on day 2 of the object recognition task (97.78 ± 27.41% and 101 ± 16.9% for N LPS-treated and GH LPS-treated rats respectively, n=5, Figure 5.4D). Results are expressed as a percentage of TrkA expression/actin expression (mean ± SEM).

5.3.3 p75NTR expression is increased in the dentate gyrus of N rats following peripheral LPS injection

The hippocampii and dentate gyrii of N and GH rats sacrificed on day one and day two were assessed for p75NTR expression. Peripheral injection of LPS (100μg/Kg) did not significantly effect receptor expression in the hippocampii and dentate gyrii of N and GH rats on day one (Figure 5.5). On day two, an increase in p75NTR expression in the dentate gyrus of N rats treated with LPS was observed (*$p<0.05$, 2-way ANOVA, *post-hoc* Newmann Keuls; 85.88 ± 21.34 and 153.7 ± 16.3 for N saline and LPS-treated rats respectively (Figure 5.6)). No changes in receptor expression in the dentate gyrus of GH rats were observed. There were no changes in p75NTR expression in the hippocampus of N and GH rats treated with saline or LPS (Figure 5.6)

5.3.4 ERK activity in N and GH rats treated with LPS

ERK activity in the dentate gyrii and hippocampii of N and GH rats sacrificed on day one and day two was examined. Analysis revealed that there were no significant changes in ERK activity in any of the groups in either the dentate gyrus or the hippocampus on day one (Figure 5.7B, C, E & F). There was no significant difference in p44 or p42 ERK activity in the dentate gyrus of N and GH rats treated with either saline or LPS on day two of the object recognition task. The activation of the p44 ERK isoform was significantly decreased in the hippocampus of GH rats treated with LPS on day two (* $p<0.05$; 2-way ANOVA, post-hoc Newmann Keuls; 22.69 ± 2.8% and 12.09 ± 2.3% for GH saline and LPS-treated rats respectively, Figure 5.8E). There was no significant difference in p42 ERK activity in the hippocampus of any of the groups on day two. Results are expressed as a mean percentage of phosphorylated ERK isoform/total ERK ± SEM, n=5-6.

5.3.5 JNK activity in N and GH rats treated with LPS

JNK activity in the dentate gyrii and hippocampii of N and GH rats sacrificed on day one and day two was examined. Analysis revealed that there were no significant changes in JNK activity in any of the groups in either the dentate gyrus or the hippocampus on day one (Figure 5.9B & D). There was no significant difference in JNK activity on day two of the object recognition task in either the dentate or the hippocampus in N or GH rats treated with saline or LPS (Figure 5.10B & D). Results are expressed as a mean percentage of phosphorylated JNK/total JNK ± SEM, n=4-6).

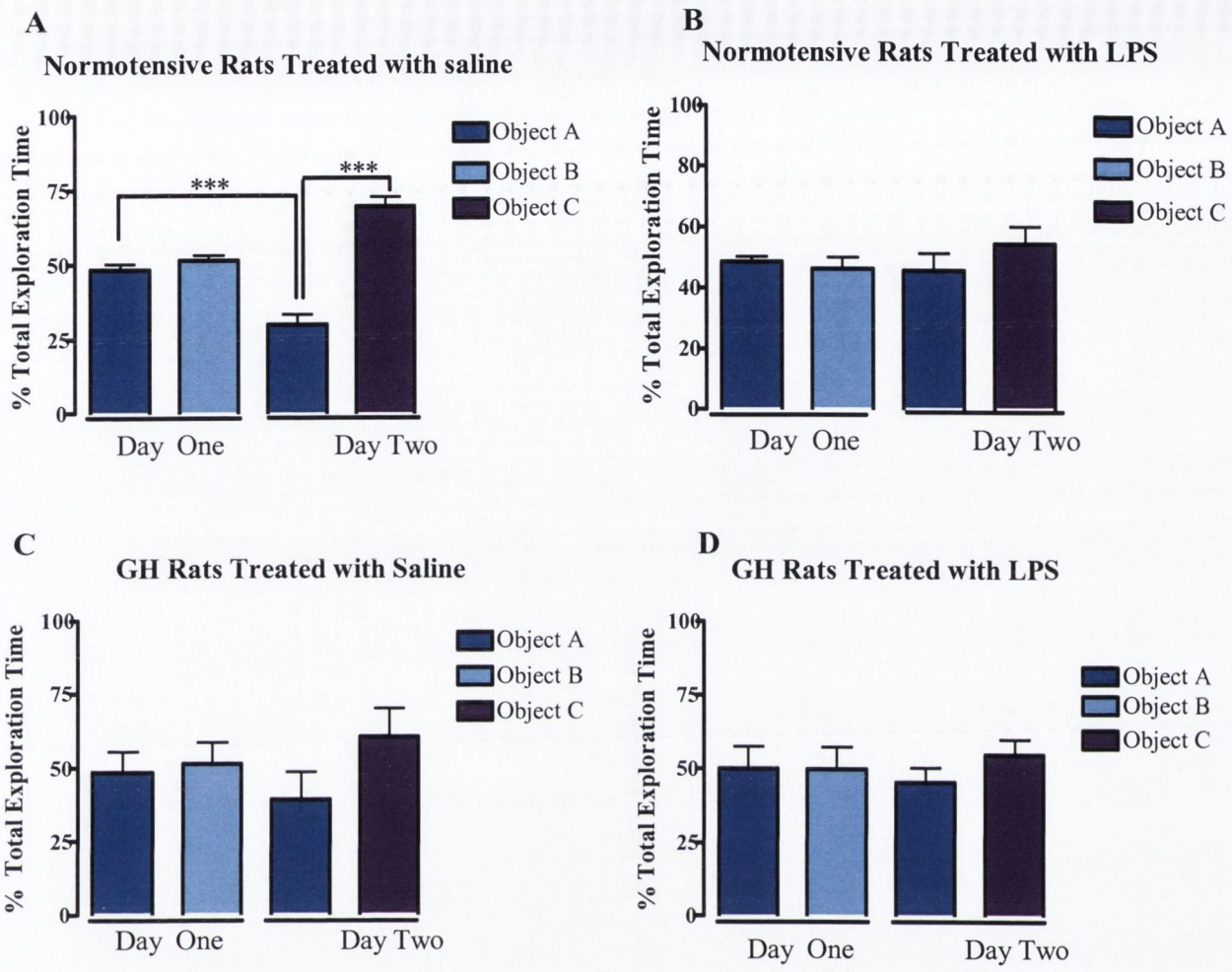


Figure 5.2 Decreased NGF concentration and TrkA receptor expression impairs recognition memory in the rat as does the peripheral injection of LPS

Rats were injected i.p. with either LPS (300µl) or saline (0.9%) 3 hours prior to performing the object recognition task on day one. Results are expressed as a percentage of the total exploration time (mean ± SEM, n=6). All groups explored objects A and B equally on day one. Normotensive rats treated with saline (**A**) successfully recognised the presence of a novel object on day 2 and spent significantly more time actively exploring Object C than the familiar object A (***$p<0.001$; 2-way ANOVA). The ability of normotensive rats to recognise the novel objected was impaired by treatment with LPS (**B**). GH rats treated with saline spent slightly more time exploring the novel object on day 2 (**C**), however this was not significant and was further impaired by treatment with LPS (**D**).

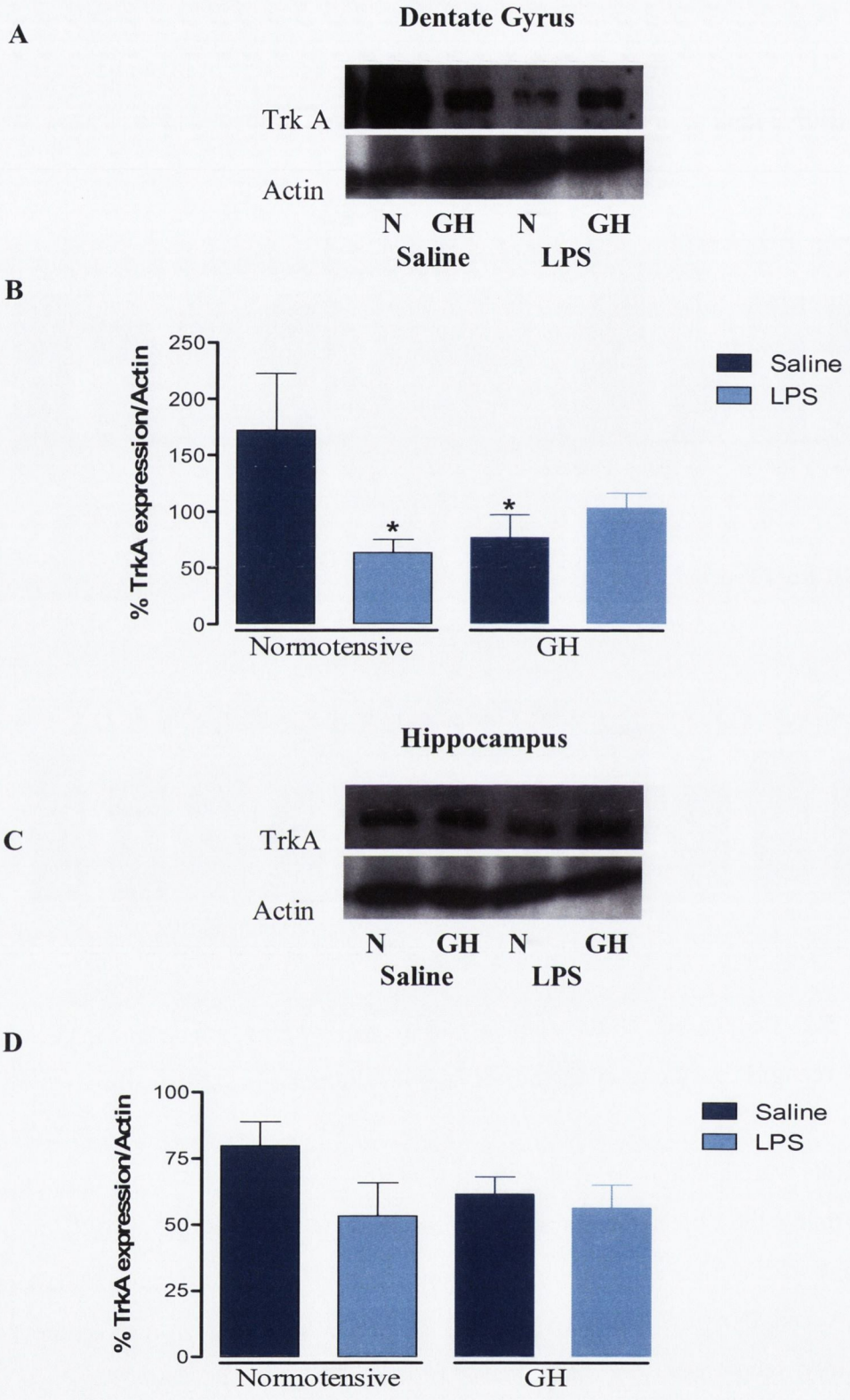


Figure 5.3 TrkA expression in N and GH rats treated with saline or LPS on day one of the object recognition task

A. Sample western immunoblots illustrating TrkA expression and actin expression in the dentate gyrus of normotensive and GH rats treated with saline and LPS on day one of the object recognition task.

B. Densitometric analysis revealed a significant decrease in TrkA expression in normotensive rats treated with saline (*$p<0.05$; 2-way ANOVA, *post-hoc* Newmann Keuls). It also revealed a significant deficit in TrkA expression in the control GH rats when compared to the control N rats (*$p<0.05$; 2-way ANOVA, *post-hoc* Newmann Keuls). Results are expressed as a percentage of TrkA expression/actin expression (mean ± SEM, n=4-5).

C. Sample western immunoblots illustrating TrkA expression and actin expression in the hippocampus of normotensive and GH rats treated with saline and LPS on Day one of the object recognition task.

D. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage TrkA expression/actin expression (mean ± SEM, n=4-6), 2-way ANOVA.

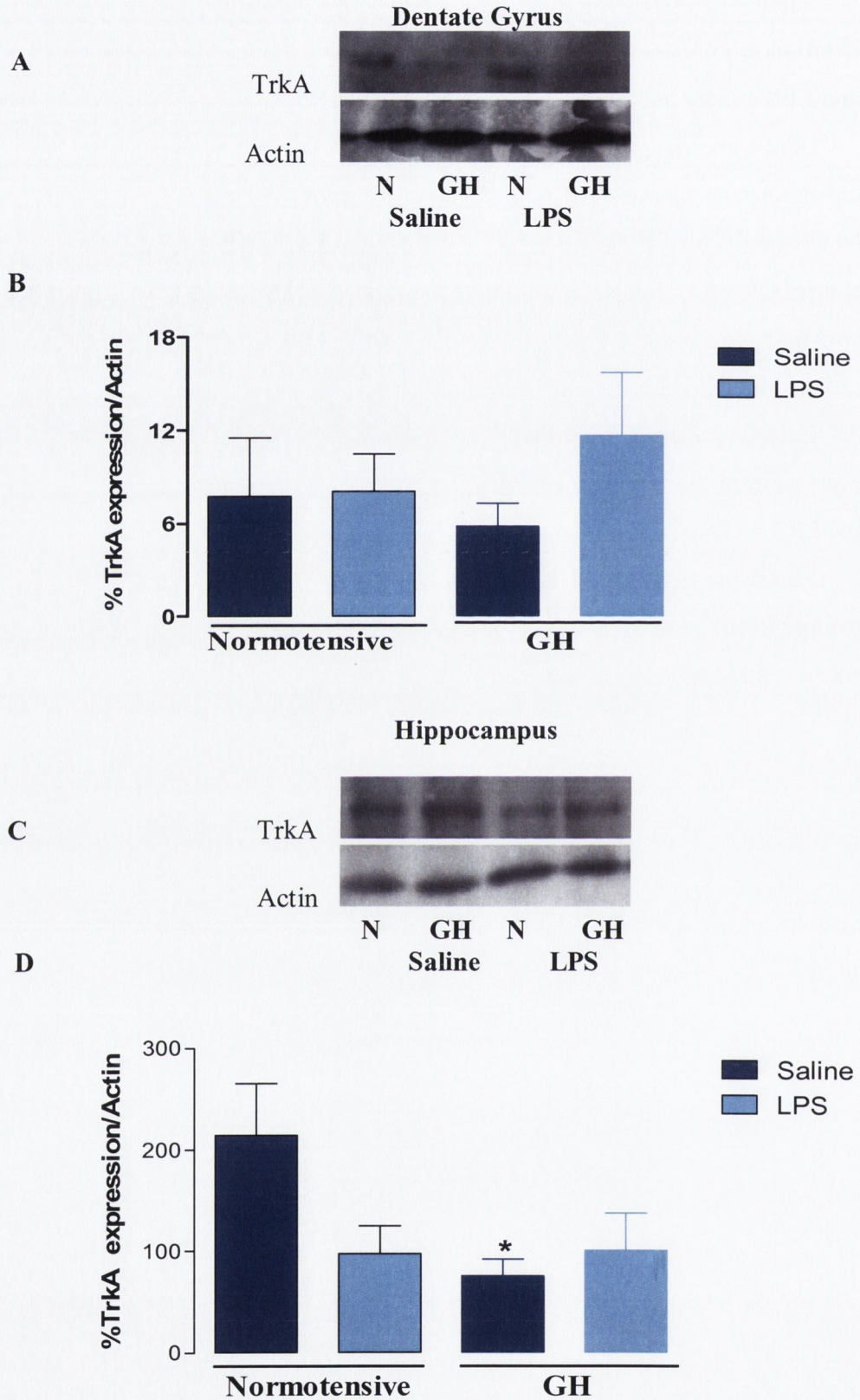


Figure 5.4 TrkA expression in N and GH rats treated with saline or LPS on day two of the object recognition task

A. Sample western immunoblots illustrating TrkA expression and actin expression in the dentate gyrus of normotensive and GH rats treated with saline and LPS on Day two of the object recognition task.

B. Densitometric analysis revealed no significant difference in TrkA expression in between any of the groups. Results are expressed as a percentage of TrkA expression/actin expression (mean ± SEM, n=4-5).

C. Sample western immunoblots illustrating TrkA expression and actin expression in the hippocampus of normotensive and GH rats treated with saline and LPS on Day two of the object recognition task.

D. Densitometric analysis revealed a significant decrease in TrkA expresssion in the hippocampus of control GH rats when compared to N controls. LPS administration did not have any affect on TrkA expression in either N or GH rats. Results are expressed as a percentage of TrkA expression/actinexpression (mean ± SEM, n=3-5), 2-way ANOVA, *post-hoc* Newmann Keuls.

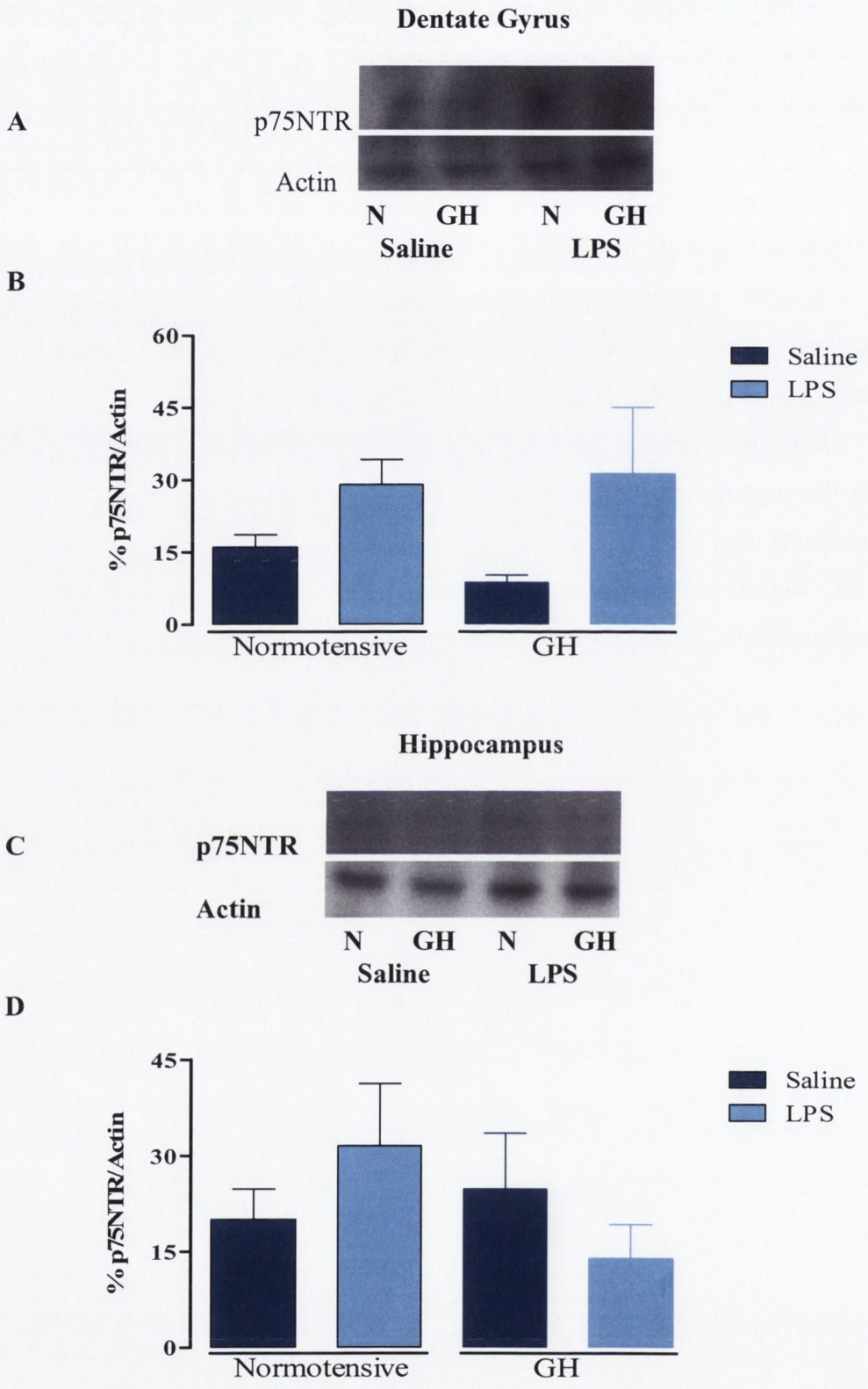


Figure 5.5 p75NTR expression in N and GH rats treated with saline or LPS on day one of the object recognition task

A. Sample western immunoblot illustrating p75NTR and actin expression in the dentate gyrus of normotensive and GH rats treated with saline and LPS on day one of the object recognition task.

B. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage p75NTR expression/actin expression (mean ± SEM, n=3-5), 2-way ANOVA.

C. Sample western immunoblot illustrating p75NTR and actin expression in the hippocampus of normotensive and GH rats treated with saline and LPS on day one of the object recognition task.

D. p75NTR expression was assessed by western immunoblot. Densitometric analysis revealed no significant difference in receptor expression between any of the groups. Results are expressed as a percentage p75NTR expression/actin expression (mean ± SEM, n=4-6), 2-way ANOVA.

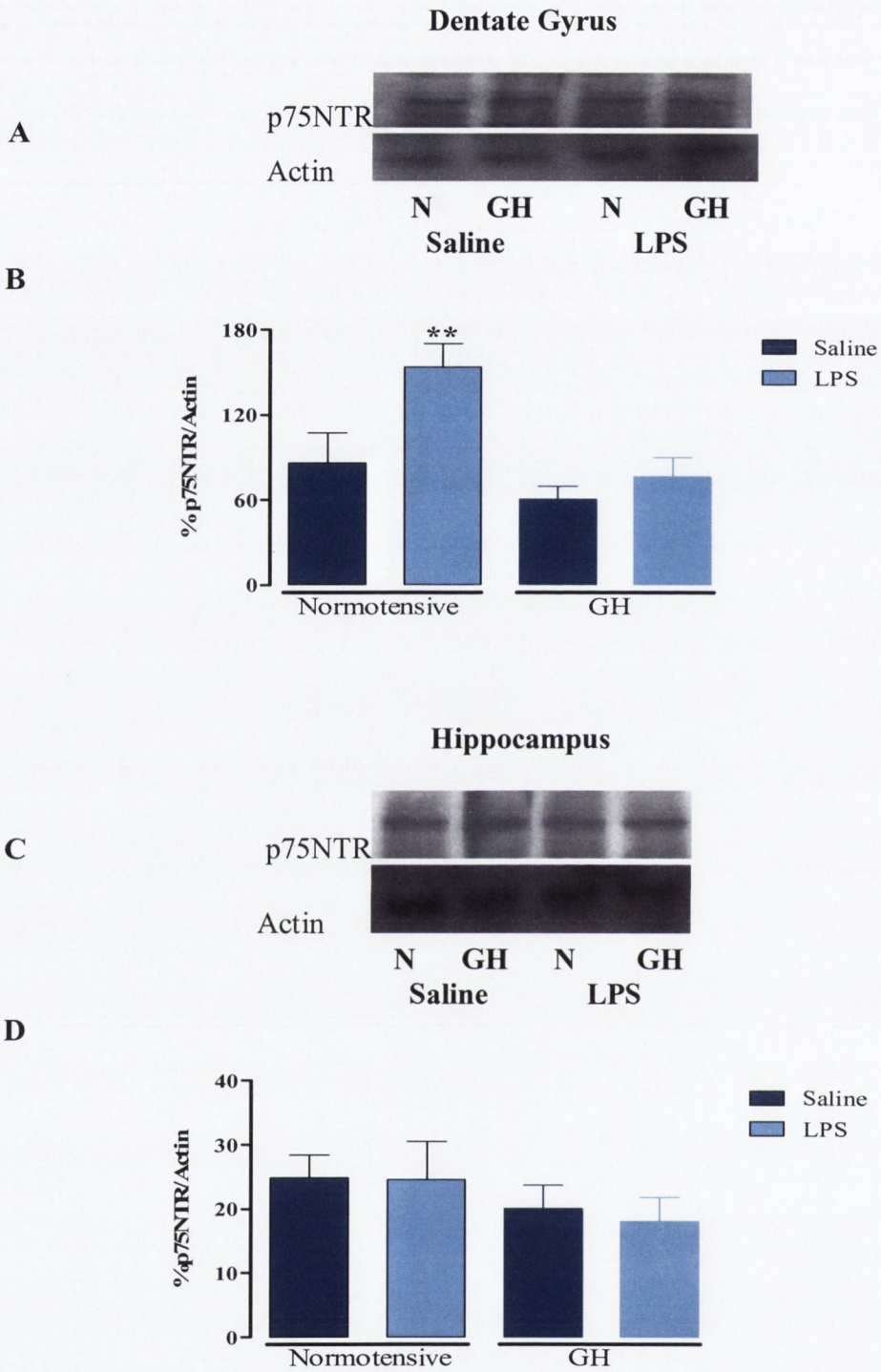


Figure 5.6 p75NTR expression in N and GH rats treated with saline or LPS on day two of the object recognition task

A. Sample western immunoblot illustrating p75NTR and actin expression in the dentate gyrus of normotensive and GH rats treated with saline and LPS on day two of the object recognition task.

B. Rats were injected i.p. with either LPS (300µl, 100µg/Kg) or saline (0.9%) three hours prior to performing the object recognition task. p75NTR expression was

assessed using gel electrophoresis and western immunoblot. Densitometric analysis revealed a significant increase in p75NTR expression in normotensive rats treated with LPS when compared with saline controls(**$p<0.01$, 2-way ANOVA, *post-hoc* Newmann Keuls). Results are expressed as a percentage of p75NTR expression/actin expression (mean ± SEM, n=4-6).

C. Sample western immunoblot illustrating p75NTR and actin expression in the hippocampus of normotensive and GH rats treated with saline and LPS on day two of the object recognition task.

D. Rats were injected i.p. with either LPS (300µl, 100µg/Kg) or saline (0.9%) three hours prior to performing the object recognition task. p75NTR expression was assessed using gel electrophoresis and western immunoblot. Densitometric analysis revealed no significant differences between groups. Results are expressed as a percentage of p75NTR expression/actin expression (mean ± SEM, n=6), 2-way ANOVA.

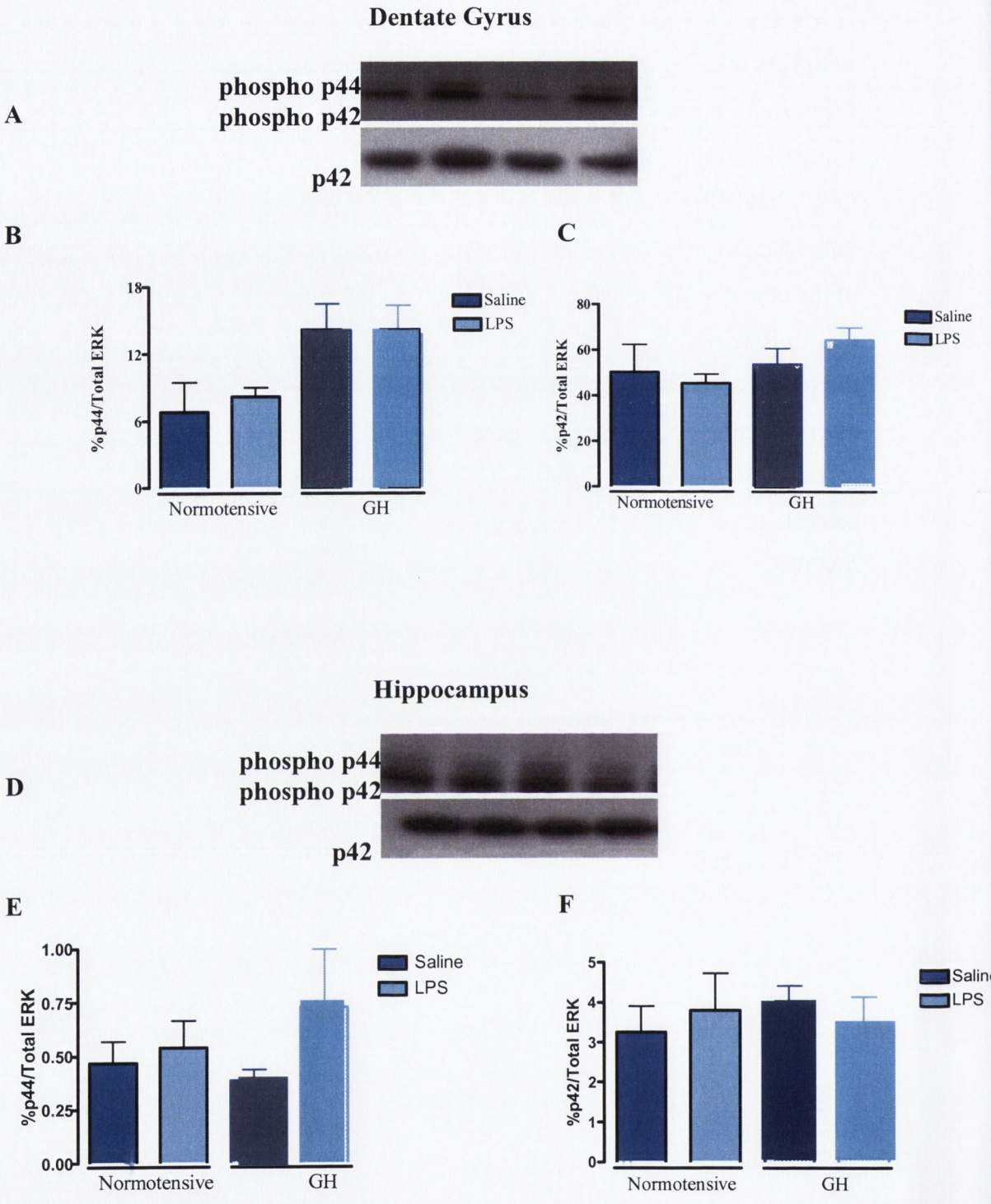


Figure 5.7 ERK activity in N and GH rats treated with saline or LPS on day one of the object recognition task

A. Sample western immunoblot illustrating phosphorylated ERK and total ERK expression in the dentate gyrus of normotensive and GH rats treated with saline and LPS on day one of the object recognition task.

B. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage of p44ERK/ total ERK expression (mean ± SEM, n=5-6), 2-way ANOVA.

C. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage of p42ERK/ total ERK expression (mean ± SEM, n=4-6), 2-way ANOVA.

D. Sample western immunoblot illustrating ERK activity in the hippocampus of normotensive and GH rats treated with saline and LPS on day one of the object recognition task.

E. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a p44ERK/ total ERK expression (mean ± SEM, n=5-6), 2-way ANOVA.

F. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage of p42ERK/ total ERK expression (mean ± SEM, n=5-6), 2-way ANOVA.

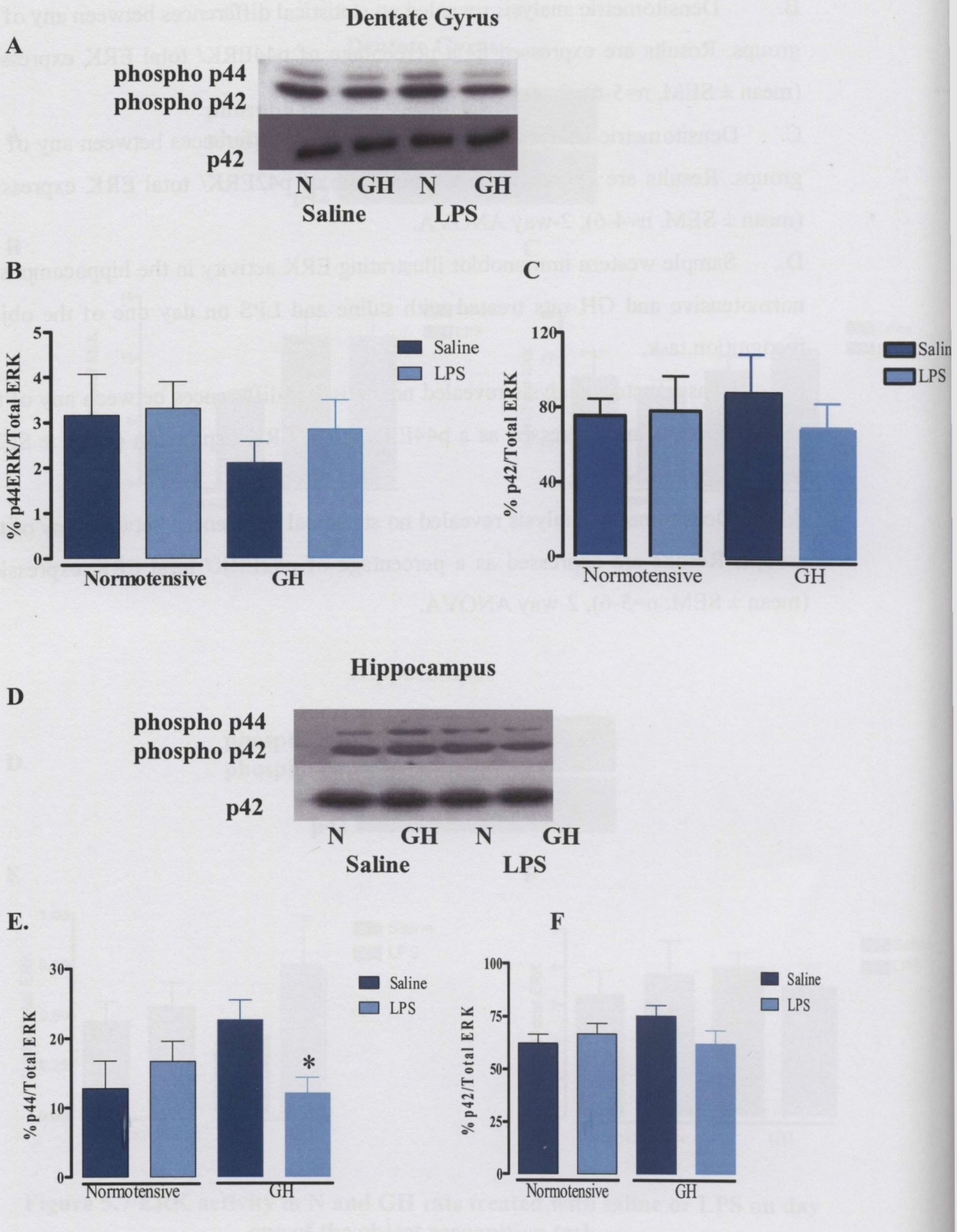


Figure 5.8 ERK activity in N and GH rats treated with saline or LPS on day two of the object recognition task

A. Sample western immunoblot illustrating phosphorylated ERK and total ERK expression in the dentate gyrus of normotensive and GH rats treated with saline and LPS on day two of the object recognition task.

B. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage of phosphorylated p44ERK/ total ERK expression (mean ± SEM, n=6), 2-way ANOVA.

C. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage of phosphorylated p42ERK/ total ERK expression (mean ± SEM, n=6), 2-way ANOVA.

D. Sample western immunoblot illustrating ERK activity and total ERK expression in the hippocampus of normotensive and GH rats treated with saline and LPS on day two of the object recognition task.

E. Densitometric analysis revealed a significant decrease in p44ERK activity in the hippocampus of LPS-treated GH rats (*$p<0.05$; 2-way ANOVA, *post-hoc* Newmann Keuls). Results are expressed as a percentage of p44ERK/ total ERK expression (mean ± SEM, n=6), 2-way ANOVA.

F. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage of p42ERK/ total ERK expression (mean ± SEM, n=6), 2-way ANOVA.

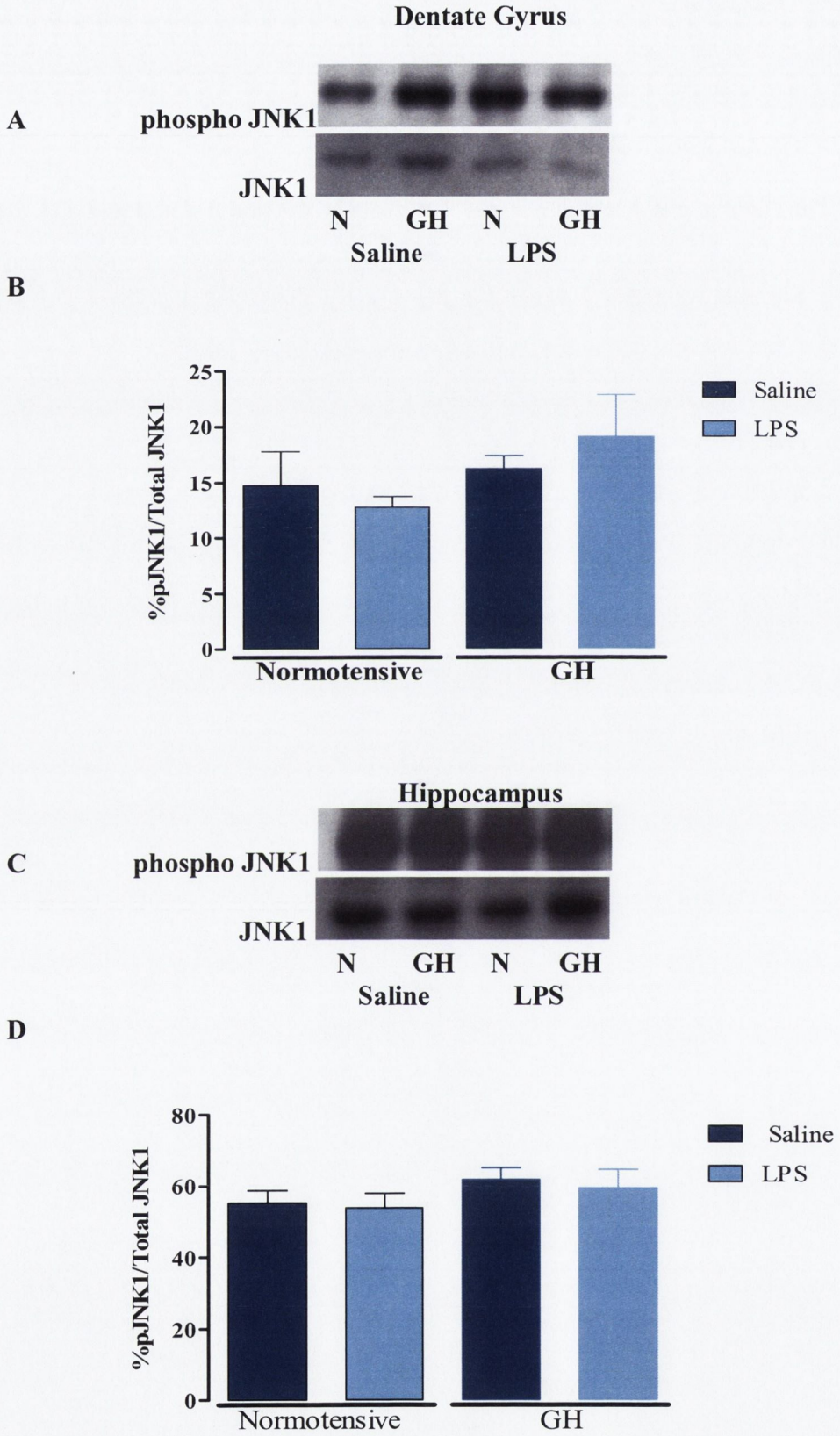


Figure 5.9 JNK activity in N and GH rats treated with saline or LPS on day one of the object recognition task

A. Sample western immunoblot illustrating phosphorylated JNK1 and total JNK1 expression in the dentate gyrus of normotensive and GH rats treated with saline and LPS on day one of the object recognition task.

B. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage of phosphorylated JNK1/ total JNK1 expression (mean ± SEM, n=3-6), 2-way ANOVA.

C. Sample western immunoblot illustrating phosphorylated JNK1 and total JNK1 expression in the hippocampus of normotensive and GH rats treated with saline and LPS on day one of the object recognition task.

D. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage of phosphorylated JNK1/ total JNK1 expression (mean ± SEM, n=5-6), 2-way ANOVA.

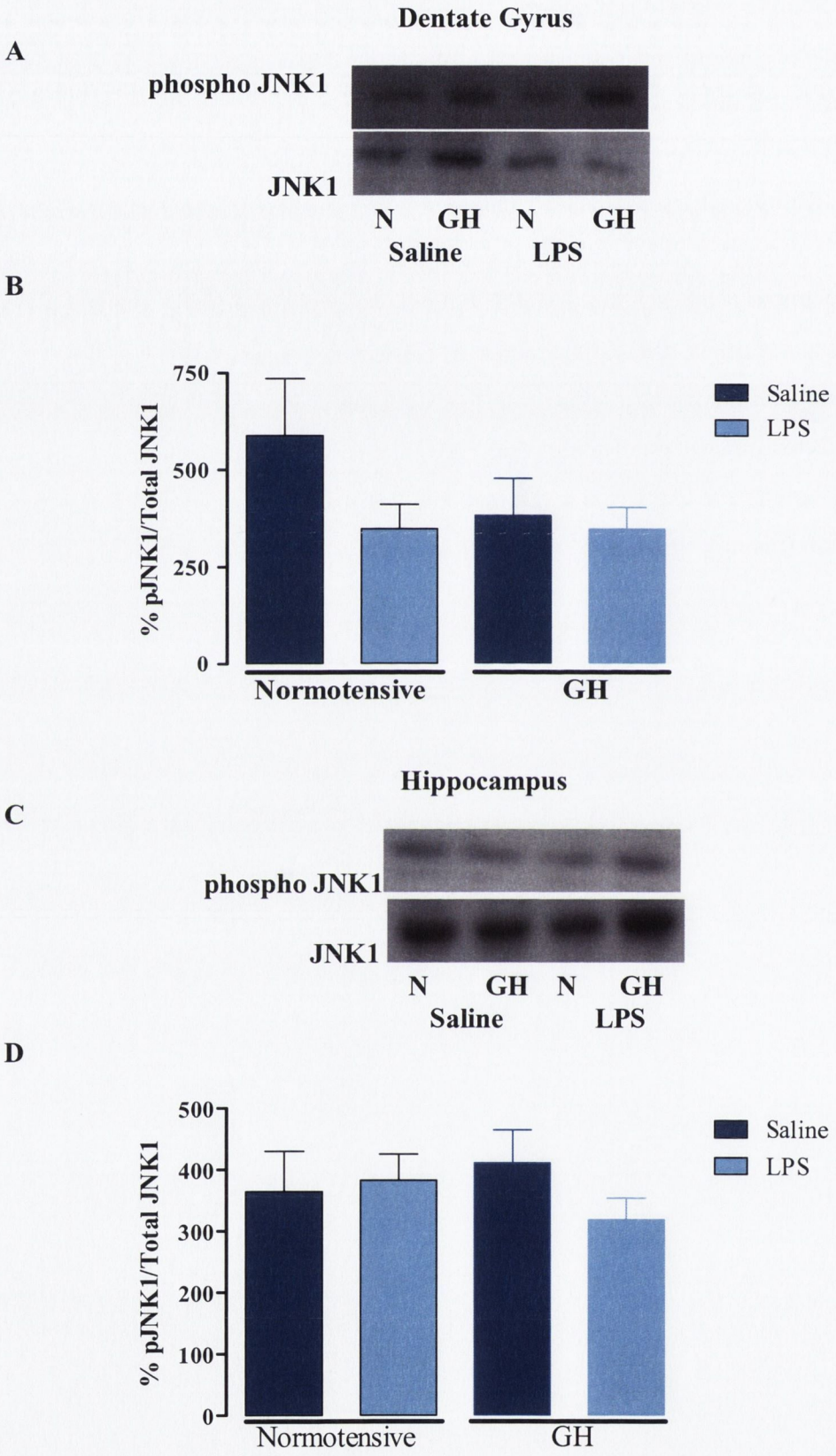


Figure 5.10 JNK activity in N and GH rats treated with saline or LPS on day two of the object recognition task

A. Sample western immunoblot illustrating phosphorylated JNK1 and total JNK1 expression in the dentate gyrus of normotensive and GH rats treated with saline and LPS on day two of the object recognition task.

B. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentage of phosphorylated JNK1/ total JNK1 expression (mean ± SEM, n=6), 2-way ANOVA.

C. Sample western immunoblot illustrating phosphorylated JNK1 and total JNK1 expression in the hippocampus of normotensive and GH rats treated with saline and LPS on day two of the object recognition task.

D. Densitometric analysis revealed no statistical differences between any of the groups. Results are expressed as a percentageof phosphorylated JNK1/total JNK1 expression (mean ± SEM, n=6), 2-way ANOVA.

5.4 Discussion

Barrett and Bartlett (1994) postulated that the ratio of expression between TrkA and p75NTR would determine cell fate, with a higher proportion of Trk receptor expression favouring cell survival and a higher proportion of p75NTR expression favouring cell death. p75NTR expression is greatly reduced in a variety of cell types in adulthood but its expression is induced following neuronal injury. The biological implications of this increase in expression are unclear but a number of studies have shown that this injury-induced expression is tightly correlated with the degenerative effects following the trauma (Casha *et al.*, 2001; Greferath *et al.*, 2001; Roux *et al.*, 1999; Ernfors *et al.*, 1989). We have shown that peripheral injection of LPS increases p75NTR expression in the dentate gyrus and is associated with apoptotic signalling and neuronal death. In an attempt to isolate p75NTR signalling *in vivo* I employed the use of GH rats that are deficient in NGF and TrkA. The hypothesis was that LPS administration to the GH rats would increase p75NTR expression as per my previous experiment and that the absence of Trk receptors would facilitate p75NTR apoptotic signalling.

Both LPS administration and a deficiency in NGF and TrkA have been previously demonstrated to negatively impact on synaptic function as assessed by LTP (Barry *et al.*, 2005; Hauss-Wegrzyniak *et al.*, 2002; Kelly *et al.*, 2003; Maguire *et al.*, 1999; Kelly *et al.*, 1998). This study examined the effect of LPS administration and a deficiency in NGF and TrkA on cognitive function by assessing the ability of animals to recognise the presence of a novel object. LPS has previously been shown to impair several types of hippocampal dependent learning including spatial navigation in the Morris water maze (Shaw *et al.*, 2005; Shaw *et al.*, 2001; Hauss-Wegrzyniak *et al.*, 2000) and T-maze spontaneous alteration task (Hauss-Wegrzyniak *et al.*, 2000). It has previously been reported that chronic intracerebroventricular LPS administration has no effect on object recognition memory (Hauss-Wegrzyniak *et al.*, 2000). In this study we found that acute peripheral LPS injection impaired the ability of N rats to recognise the presence of a novel object. GH rats treated with saline were unable to identify the presence of a novel object. This result is consistent with the findings of Kelly *et al.* (1998, 2000) indicating that NGF and TrkA deficiency is playing a key role in this impairment of

cognitive function. LPS administration further abrogated the performance of GH rats in the novel object preference task.

Western immunoblotting confirmed that GH rats are deficient in TrkA expression in the dentate gyrus. It was observed that TrkA receptor expression in the dentate gyrus on day two of the object recognition task was not significantly different to N controls. The differing levels of receptor expression may be explained by the high levels of variation in TrkA expression in the N control group on day two. Clarification of this inconsistency in expression levels could be achieved by increasing the n number in each of the groups, subsequently reducing the variation. One other explanation is that there may be an increase in basal TrkA expression in the GH animals following exposure to the recognition task. The level of neurotrophin expression is known to be activity-dependent and several studies have shown that neurotrophin expression is sensitive to electrical activity. Normal physiological activity that is capable of inducing LTP increases BDNF and NGF mRNA (Castraen *et al.*, 1993). Neuronal depolarization by glutamate also increases the level of BDNF and NGF mRNA as well as protein release (Zafra *et al.*, 1991; Kelly *et al.*, 2000). Furthermore we have shown that successful completion of the object recognition task is concomitant with increased NGF concentrations and increased TrkA phosphorylation (Hennigan *et al.*, unpublished data). Although the GH animals did not successfully complete the task it is plausible that this is the case, training in the task could result in an activity-dependent increase the release of NGF and possibly in a subsequent increase in TrkA receptor expression. It would be interesting to probe further into this possibility. If the GH animals were repeatedly exposed to a learning paradigm or enriched environment would their basal levels of NGF and TrkA increase in an activity dependent manner? It was observed that there was a significant decrease in TrkA expression in the hippocampus on day two of the task. This was a surprising result that warrants further investigation, as it suggests a shift in the profile of receptor expression following training in the object recognition task.

LPS administration was found to significantly decrease TrkA expression levels on day one in dentate gyrus of N rats with no effect on expression levels in the hippocampus. This LPS-induced deficit in receptor expression was ameliorated

by day two of the object recognition task. These data indicate that the LPS-induced decrease in TrkA receptor expression effects the acquisition of object recognition memory. Interestingly, it has been shown that peripheral administration of LPS results in significantly decreased levels NGF in the hippocampus (Guan and Fang, 2006) which would also contribute to the observed impairment in function. Studies have shown that application of exogenous NGF can ameliorate impairments in LTP and recognition memory caused by neurotrophin deficits and the pathogenesis of disease (Kelly *et al.*, 1999; De Rosa *et al.*, 2005).

I have previously shown that peripheral LPS administration significantly increases p75NTR expression in the dentate gyrus *in vivo*. These data show an increase in p75NTR expression in both the dentate gyrus and hippocampus of N rats on day one. Statistical analysis deemed this increase to be insignificant. An increase in n number would reduce the variation in the groups and elucidate the full extent of this LPS-induced increase in receptor expression. p75NTR expression was significantly increased in the dentate gyrii of N rats on day two of the object recognition task. Here, as has previously been demonstrated, LPS has been shown to impair the successful completion of a learning task (Tanaka *et al.*, 2006; Shaw *et a.*., 2001). These negative effects have been attributed by many to neurodegeneration propagated by an LPS-induced increase in inflammatory cytokines such as interleukin- 1β (IL-1β), although the precise mechanisms are unknown. Evidence from this study suggests a role for the p75NTR in the LPS-induced impairment in recognition memory. Increased p75NTR expression has been strongly correlated with impairments in synaptic function. Greferath *et al.* (2000) reported that p75NTR knock out mice had improved spatial learning performance when compared to their wild type counterparts. Several studies have proposed a relationship between the inflammatory cytokines and the p75NTR. Boyle *et al.* (2005) reported that the proinflammatory cytokine tumour necrosis factor (TNF-α) mediated Schwann cell death by upregulating p75NTR expression in axotomised mouse sciatic nerves. Conti *et al.* (2002) observed concomitant induction of IL-1β and p75NTR in an experimental model of diabetic neuropathy. Similarly, Bläsing and colleagues (2005) demonstrated that the inflammatory cytokines interferon-γ (IFN-γ), IL-1β and TNF-α upregulated p75NTR expression as well as NGF, NT-3 and NT-4 immunoreactivity in the skin. The interplay between cytokines and the

neurotrophins was reported in the early nineties. Gadient *et al.* (1990) reported that IL-1β and TNF-α synergistically stimulated the release of NGF from cultured rat astrocytes. It is also interesting to note that the interleukin 1 receptor-associated kinase (IRAK) can be recruited by the p75NTR resulting in NF-κB activation (Mamidipudi and Wooten, 2002). Evidence from these papers makes it plausible to suggest that the increase in p75NTR expression post LPS administration may be associated with the neurodegeneration observed.

Barrett and Bartlett (1994) originally hypothesized that the differential expression of the neurotrophin receptors would determine neuronal fate ie. when co-expressed the p75NTR enhances TrkA survival signalling (Mahadeo *et al.*, 1994) but when the ratio of p75NTR expression is greater than TrkA expression apoptotic signalling occurs (Bono *et al.*, 1999). In this study we have created a two-day profile of differential receptor expression by using a group of inbred rats, with decreased expression of TrkA that have been previously shown to be deficient in NGF (Kelly *et al.*, 1998) and a group of rats that have been treated with bacterial endotoxin, which I have previously shown to increase p75NTR expression. The hypothesis of this study was that administration of LPS to NGF and Trk deficient GH rats would increase p75NTR expression and facilitate its apoptotic signalling. Analysis of p75NTR expression in GH rats treated with saline or LPS revealed no significant difference between the groups on either day of the recognition task suggesting that the impairment in recognition memory in these animals is not as a result of p75NTR-induced neurodegeneration. This was a disappointing result as it meant that using the GH rats would not facilitate the investigation of p75NTR mediated apoptotic signalling *in vivo*. However it is possible that the ratio of p75NTR expression to TrkA expression is greater as a result of their integral deficiency in TrkA.

To gain an understanding of the molecular impact of the differential expression of the neurotrophin receptors I examined proteins involved the downstream signalling pathways of TrkA and p75NTR.

Activation of TrkA by NGF induces the activation of several signalling pathways including the Ras/extracellular regulated kinase (ERK) protein kinase

pathway, the phosphatidylinositol-3-OH kinase (PI3K)/Akt kinase pathway and phospholipase C-γ1 (PLC- γ1), which culminate in the activation of transcription factors. TrkA activation of ERK has been shown to play a key role in neuronal survival (Anderson and Tolkovsky, 1999) and in synaptic plasticity (McGahon et al., 1999; English and Sweatt, 1997). In this study no change in the activity of either the p44 or the p42 isoform in either the hippocampus or the dentate gyrus was observed on day one of the recognition task. Interestingly, we observed a significant decrease in p44ERK activity in the hippocampus of GH rats treated with LPS on day two of the learning task while no change in the activity of the p42 isoform was detected. These data may explain the further impairment in recognition memory in GH rats treated with LPS. It is clear that the LPS impairment observed in both N and GH rats is occurring via two distinct signalling mechanisms. It is unclear as to why LPS is differentially affecting the isoforms of ERK and this finding needs further investigation.

JNK is a stress activated protein kinase (SAPK). JNK activation can occur as a consequence of p75NTR activation and can induce neuronal apoptosis (Friedman, 2000; Yoon *et al.*, 1998). Interestingly, a balance between ERK and JNK signalling has been shown to play a role in determining cell fate (Xia *et al.*, 1995). They reported that NGF withdrawal from PC12 cells resulted in the sustained activation of JNK, the inhibition of ERK and ultimately neuronal apoptosis. They proposed that the balance between JNK and ERK activity may be important in determining cell fate. I have previously shown that LPS administration increases JNK activity, a finding which was consistent with other studies (Barry *et al.*, 2005; Vereker *et al.*, 2000) and we have shown that LPS can upregulate the expression of p75NTR. Therefore it was expected that an increase in JNK activity would be observed. However, in this study no changes in JNK activity in either the dentate gyrii or hippocampii of any of the groups were observed. Despite the fact that the animals were handled and habituated for several days prior to performing the task it is possible that the control animals stress levels were slightly elevated carrying out the task thereby disguising any LPS induced changes that may have occurred. Ideally these experiments would be repeated using naïve animals as another form of control so that any task performing-induced changes could be detected and distinguished from LPS-induced changes.

This study demonstrates that neurotrophins and their receptors play a fundamental role in recognition learning in the rat. Increased expression of p75NTR and decreased expression of TrkA in N rats treated with LPS confirms my previous finding that LPS can manipulate neurotrophin receptor expression in the rat denate gyrus. Furthermore this differential expression of neurotrophin receptors was accompanied by a profound impairment in cognitive function as evidenced by the inability of rats treated with LPS to recognise the presence of a novel object. GH rats that are deficient in NGF and TrkA are cognitively impaired as evidenced by their inability to identify a novel object. This impairment was further abrogated by the administration of LPS. Though these impairments in cognitive function are concomitant with changes in neurotrophin receptor expression it is apparent that they occur by two distinct mechanisms.

Chapter 6

An investigation into the role of increased p75NTR expression in functional impairment observed post neuronal insult

6.1 Introduction

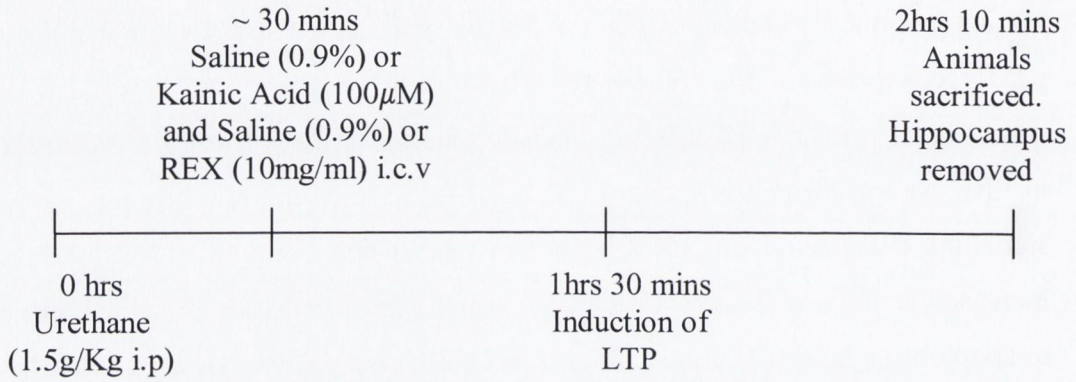
Kainic acid administration induces the selective degeneration of neuronal populations in the hippocampus (Oh *et al.*, 2000; Shetty *et al.*, 2003; Yi *et al.*, 2003). Several studies have implicated the p75NTR as a mediator of some of the neurodegenerative effects observed post-kainic acid injection (Volosin *et al.*, 2006; Yi *et al.*, 2003; Oh *et al.*, 2000). Indeed evidence from chapter 4 supports the findings of these studies. Following kainic acid administration an increase in p75NTR expression was observed. This increase in receptor expression was concomitant with an impairment in synaptic function as assessed by LTP, a decrease in ERK activity, which is known to be involved in neurotrophin mediated survival signalling (Encinas *et al.*, 1999; Hetman *et al.*, 1999; Creedon *et al.*, 1997) and increased activity of the stress activated protein kinase JNK which is a contributor to apoptotic signalling (Linggi *et al.*, 2005; Okuno *et al.*, 2004; Xia *et al.*, 1995).

Studies have shown that inhibition of the p75NTR prevents neuronal apoptosis induced by ligand binding (Friedman, 2000). Therefore, the objective of this study was to assess the implication of p75NTR expression in the impairment of synaptic function and neurodegenerative processes.

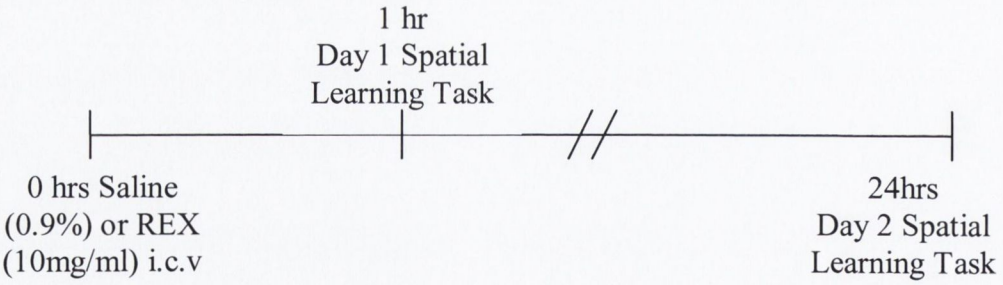
6.2 Materials and Methods

6.2.1 Experimental Timeline

A Effect of Kainic Acid and the p75NTR inhibitor REX on LTP



B Effect of the p75NTR inhibitor REX on Spatial Learning



6.2.2 Treatment

Briefly, rats were anaesthetised with urethane (1.5g/kg i.p.) until loss of consciousness occurred, evidenced by the absence of the pedal reflex. Rats were subsequently given an injection into the 3rd ventricle (coordinates: 0.5mm lateral and 2.5mm posterior to bregma) of either kainic acid (5μl, 100μM; Sigma) or saline

(5µl, 0.9%) which were co-administered with either the p75NTR inhibitor REX (5µl, 10mg/ml; generous gift from Dr. Louis Reichardt, University of California, San Francisco, USA) or physiological saline (5µl, 0.9%) one hour prior to tetanic stimulation.

6.2.3 Induction of LTP *in vivo*

One hour following kainic acid injection LTP was induced. Rats were placed in a stereotaxic frame and a bipolar stimulating electrode was placed in the perforant path while a recording electrode was placed in the dorsal cell body of the dentate gyrus. Test shocks were delivered at 30 sec intervals and recorded for 10 minutes in order to establish a stable baseline. This was proceeded by delivery of three High-frequency trains of stimuli (250Hz for 200msec) at 30 sec intervals after which recording resumed at test shock frequency for 40 minutes. Upon cessation of recording animals were sacrificed by decapitation and the hippocampus removed, sliced and stored at -80°C for further analysis.

6.2.4 Surgical procedure and drug delivery

Rats were anaesthetized with ketamine (100µg/kg; BioResources Unit, Trinity College, Dublin, Ireland) and xylazine (10mg/kg; Rompun®, BioResources Unit, Trinity College, Dublin, Ireland) and supplemented throughout the surgical procedure as necessary. A single hole was drilled in the skull over the left ventricle (coordinates, bregma, 0.9 mm; midline, 1.3 mm), and a cannula (Plastics 1, Bilaney Consultants Ltd., Kent, UK) was lowered slowly into the ventricle to a depth of 3.6 mm below the brain surface. A guide cannula (Plastics 1, Bilaney Consultants Ltd., Kent, UK) was held in place and fixed to the skull with dental cement (Prestige Dental Products, West Yorkshire, UK). The incision was closed with surgical staples (Promed, Kerry, Ireland). Rats were given 7-10 days to fully recover before being tested. The p75NTR inhibitor REX (5µl, ~10mg/ml) or vehicle (normal rabbit serum; 5µl) were administered via the implanted cannula one hour prior to performing the spatial learning task.

6.2.5 Spatial learning

Rats were implanted with cannulae as outlined in section 2.6 and allowed to recover for 7-10 days before being handled daily for 5 days and habituated to an open field (height, 0.5m; diameter, 0.9m). On day 1 the rats were injected with either the p75NTR inhibitor REX (5µl, ~10mg/ml) or normal rabbit serum (5 µl, 0.9% (w/v)) i.c.v. one hour prior to commencement of the spatial learning task. Rats were given three 5-min trials (inter-trial interval 5 min) during which they were allowed to explore three novel objects constructed from Lego. The time spent actively exploring each of the objects was recorded. On day 2 one of the objects was displaced and rats were placed in the arena for a single 5min trial and allowed to explore. The time spent exploring each of the objects was recorded. For spatial learning assessment, the exploration criteria were based strictly on active exploration. The rats must be seen to have touched to object with at least their nose for it to be deemed as true exploration.

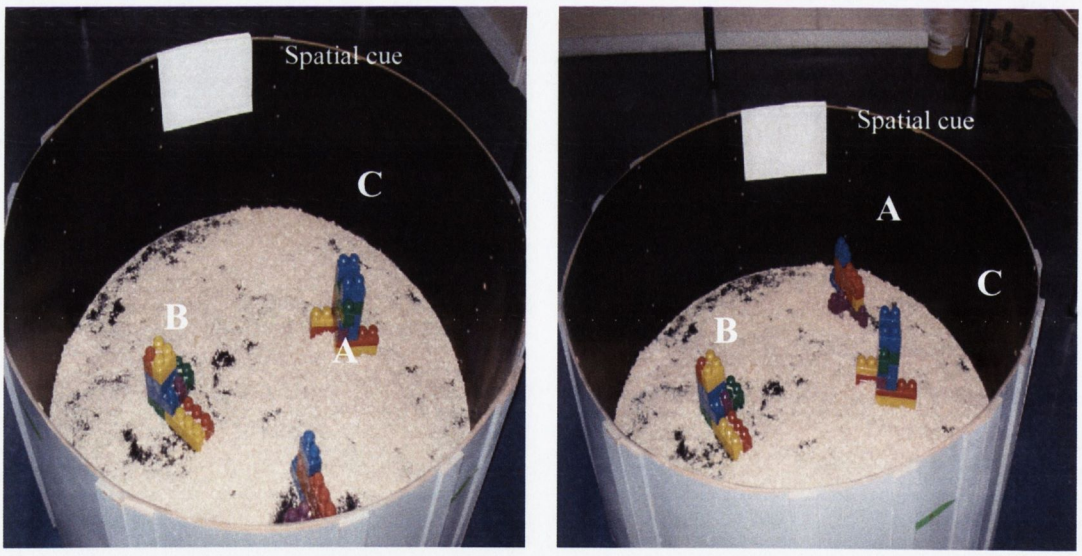


Figure 6.1 Spatial learning task

6.2.6 Analysis of ERK activation in kainic acid-treated rats

ERK activity was assessed using gel electrophoresis and western immunoblotting as described in section 2.14. Membranes were incubated with 5% BSA/TBS-T overnight to block any non-specific binding. Membranes were given three 10 minutes washes with TBS-T after which they were incubated for two hours

at room temperature in primary antibody (anti-phosphoERK (Santa Cruz) 1:3000 in 2% BSA/TBS-T; anti-ERK2 (Santa Cruz) 1:1000 in 2% BSA/TBS-T). Following primary antibody incubation membranes were washed with TBS-T (three 10 minute washes) and were subsequently incubated with secondary antibody for one hour at room temperature (1:1000 anti-mouse IgG (Sigma) for detection of pERK, ERK2). Membranes were washed in TBS-T as before. The protein bands were detected using Supersignal (Amersham) before being exposed to photographic film (Hyperfilm).

6.2.7 Analysis of neurotrophin receptor expression

Neurotrophin receptor expression was assessed using gel electrophoresis and western immunoblotting. As before, the blots were blocked overnight in 5% BSA/TBS-T. Primary antibody was incubated for 2 overnight at 4°C (anti-p75NTR (generous gift from Dr.Phil Barker; 1:1000 in TBS-T containing 2% BSA); anti-TrkA (Upstate Cell Signalling) 1:800 2% BSA/TBS-T). Following a 30min wash in TBS-T (3x10min), membranes were incubated with secondary antibody for one hour (donkey anti-rabbit IgG (Sigma) 1:1000). Blots were washed and the receptors were detected using Supersignal (Amersham). Receptor expression was quantified using densitometric analysis.

6.2.8 Proneurotrophin expression

ProNGF and proBDNF expression was examined by western immunoblot. Non-specific binding was blocked by incubation of the nitrocellulose membrane in TBS-T containing 5% BSA overnight at 4°C. The membrane was washed three times for 10min in TBS-T and incubated with the primary antibody (rabbit polyclonal anti-proNGF; Alomone; 1:500 in TBS-T containing 2% BSA; rabbit polyclonal anti-proBDNF; Alomone; 1:500 in TBS-T containing 2% BSA) for 2 hours at room temperature. The membrane was washed three times for 10min in TBS-T and incubated with the secondary antibody (donkey anti-rabbit IgG-HRP; Sigma; 1:1000 in TBS-T containing 2% BSA) for 1hr at room temperature. The membrane was washed three times for 10mins in TBS-T prior to incubation with SuperSignal for 5min. The membrane was then exposed to the photographic film images were developed as before.

6.2.9 Mature neurotrophin expression

NGF and BDNF expression was examined by western immunoblot. Non-specific binding was blocked by incubation of the nitrocellulose membrane in TBS-T containing 5% BSA overnight at 4°C. The membrane was washed three times for 10min in TBS-T and incubated with the primary antibody (rabbit polyclonal anti-NGF; Alomone; 1:500 in TBS-T containing 2% BSA; rabbit polyclonal anti-BDNF; Alomone; 1:500 in TBS-T containing 2% BSA) for 2 hours at room temperature. The membrane was washed three times for 10min in TBS-T and incubated with the secondary antibody (donkey anti-rabbit IgG-HRP; Sigma; 1:1000 in TBS-T containing 2% BSA) for 1hr at room temperature. The membrane was washed three times for 10mins in TBS-T prior to incubation with SuperSignal for 5min. The membrane was then exposed to the photographic film images were developed as before. Blots were stripped with an antibody stripping solution (1:10 dilution in dH_2O; ReBlot Plus Strong Antibody Stripping Solution; Chemicon) and reprobed for actin expression to confirm equal loading of protein.

6.2.10 Densitometric analysis

All protein bands were quantitated by densitometric analysis using the ZERO-DScan Image Analysis System (Scanalytics, Fairfax, USA) or the Gel Doc It Imaging System (UVP, Medical Supply Company, Ireland) in conjuction with LabWorks (Lablogics Inc, Mission Viejo, California, USA).

6.3 Results

6.3.1 The effect of REX on LTP in saline and kainic acid-treated rats

Previous studies have demonstrated that the administration of kainic acid (100μM) i.c.v. leads to a significant impairment in LTP and a significant increase in p75NTR expression. In order to determine whether the kainic acid-induced impairment of LTP was dependent on increased p75NTR expression, rats were co-injected with either saline or kainic acid and the p75NTR inhibitor REX one hour prior to LTP recording. Delivery of High-frequency stimulation resulted in an immediate increase in the slope of epsp in all groups of rats (n=5) (Figure 6.2). Saline-treated rats maintained this increase in epsp slope until the end of recording (Figure 6.3**C**). The increase in epsp slope of kainic acid-treated rats was transient and by the end of recording there was significant decrease in percentage epsp slope when compared to saline-treated rats (Figure 6.3C)**$p<0.01$; 3-way ANOVA, *post-hoc* Newmann Keuls). Slopes are expressed as a percentage of the normalized slopes in the 5 minutes prior to High-frequency stimulation. The mean percentage epsp slopes (± SEM) in the last 10 mins of recording was compared 120.2 ± 0.64% and 107.1 ± 2.654% for saline and kainic acid-treated rats respectively (Figure 6.3C). Co-administration of the p75NTR inhibitor REX with kainic acid failed to ameliorate the kainic acid-induced impairment in LTP (Figure 6.3C; **$p<0.01$; 3-way ANOVA, *post-hoc* Newmann Keuls; 98.28 ± 0.42, mean percentage epsp slopes ± SEM in the last 10 mins of recording). However, co-administration of REX with saline lead to a significant increase in percentage epsp slope when compared to saline only controls (Figure 6.3C; **$p<0.01$; 3-way ANOVA, *post-hoc* Newmann Keuls; 147.7 ± 1.9, mean percentage epsp slopes ± SEM in the last 10 mins of recording), suggesting that the p75NTR is exerting a tonic suppression of LTP.

6.3.2 Administration of the p75NTR inhibitor REX enhances spatial learning in the rat

The p75NTR inhibitor REX (5μl, ~10mg/ml) or control vehicle (5μl) was administered i.c.v. via an implanted cannula one hour prior to performing the spatial learning task on day one. The spatial learning task takes place over two days and assesses the ability of rats to recognise that an object has been displaced. Both control treated and REX treated rats explored all three objects equally on day one (Figure 6.4 **A** and **B**; Control A; 34.99 ± 4.6%, B; 42.08 ± 3.4%, C; 31.70 ± 3.4%;

REX A; 35.28 ± 4.6%, B; 37.97 ± 4.5%, C; 28.5 ± 2.9%). Results are expressed as a percentage of the total exploration time (mean ± SEM, n=6-8). On day two object A was displaced and rats were placed in the arena and allowed to explore as outlined in section 6.2.4. Both control and REX treated animals successfully recognised that object A had been displaced and spent significantly more time exploring it than objects B and C (**$p<0.01$, 3-way ANOVA, *post-hoc* Newmann Keuls; Control A; 45.59 ± 4.4%, B; 27.02 ± 2.0%, C; 23.97 ± 3.0%; REX A; 62.53 ± 5.1%, B; 24.23 ± 4.1%, C; 15.22 ± 2.3%). Statistical analysis also revealed that animals treated with REX spent significantly more of their exploration time exploring the displaced object than controls (++ $p<0.01$, 3way ANOVA, *post-hoc* Newmann Keuls).

6.3.3 Effect of kainic acid administration on p75NTR expression *in vivo*

Expression of p75NTR in the hippocampus following treatment with saline or kainic acid in the presence and absence of the p75NTR inhibitor REX was analysed by gel electrophoresis and immunoblotting. P75NTR expression was increased in the hippocampus of kainic acid-treated rats compared with saline-treated rats as shown in Fig 6.5A. Densitometric analysis revealed that this increase was statistically significant (*$p<0.05$; 2-way ANOVA, *post-hoc* Newmann Keuls). Results are expressed as a percentage of p75NTR expresion/actin expression (mean ± SEM, n=5-6) 166.0 ± 22.59% and 370.3 ± 71.73% for saline and kainic acid-treated rats respectively (Figure 6.5B). There was no significant difference in p75NTR expression in any of the other groups (199.6 ± 34.05% and 216.3 ± 56.13% (mean ± SEM, n=5) for saline/REX and kainic acid/REX treated rats respectively).

6.3.4 The effect of kainic acid administration and the p75NTR inhibitor REX on TrkA expression *in vivo*

The effect of kainic acid and the p75NTR inhibitor REX on TrkA receptor expression was assessed by gel electrophoresis and immunoblotting. TrkA receptor expression was decreased in kainic acid-treated rats both in the presence and absence of REX and in saline-treated animals in the presence of REX when compared to saline only controls (Figure 6.6A). Densitometric analysis revealed that these decreases in TrkA expression were statistically significant (**$p<0.01$, 2-way ANOVA, *post-hoc* Newmann Keuls). Results are expressed as a percentage of

TrkA expresion/actin expression (mean ± SEM, n=4-5) 27.26 ± 6.8% and 3.650.3 ± 1.43% for saline and kainic acid-treated rats respectively and 4.644 ± 1.4% and 3.536 ± 0.72% for saline/REX and kainic acid/REX treated rats (Figure 6.6B).

6.3.5 The effect of kainic acid administration and the p75NTR inhibitor REX on ERK activity *in vivo*

The effect of kainic acid and the p75NTR inhibitor REX on ERK activity expression was assessed by gel electrophoresis and immunoblotting. ERK activity expression was decreased in kainic acid-treated rats both in the presence and absence of REX and in saline-treated animals in the presence of REX when compared to saline only controls (Figure 6.7A). Densitometric analysis revealed that these decreases in TrkA expression were statistically significant (*$p<0.05$, 2-way ANOVA, *post-hoc* Newmann Keuls). Results are expressed as a percentage of TrkA expresion/actin expression (mean ± SEM, n=4-6) 95.90 ± 6.782% and 69.86 ± 7.361% for saline and kainic acid-treated rats respectively and 66.73 ± 3.264% and 76.32 ± 5.542% for saline/REX and kainic acid/REX treated rats (Figure 6.7B).

6.3.6 The effect of kainic acid administration and inhibition of the p75NTR on proBDNF and BDNF *in vivo*

The levels of proBDNF and BDNF in the hippocampus of the rat following kainic acid (100μM) administration and inhibition of the p75NTR by REX (~10mg/ml) were assessed by western immunoblotting. Densitometric analysis revealed that kainic acid administration had no significant effect on proBDNF expression levels when compared with saline controls (17.98 ± 2.02 and 44.27 ± 14.21 for saline and kainic acid-treated rats respectively (Figure 6.8B). Treatment with the p75NTR inhibitor REX also had no effect on proBDNF levels (19.14 ± 4.9 and 34.87 ± 8.830 for saline/REX and kainic acid/REX treated animals respectively (Figure 6.8B)). Results are expressed as a percentage of proBDNF/actin expression (mean ± SEM; n=4-6) and were analysed by 2-way ANOVA. Mature BDNF (Figure 6.8D) expression levels were unaffected by either kainic acid administration or p75NTR inhibition (23.03 ± 4.9 and 28.96 ± 7.6 for saline and kainic acid-treated rats respectively and 23.50 ± 3.1, 25.43 ± 2.2 for saline/REX and kainic acid/REX treated animals respectively (Figure 6.8D). Results are expressed as a percentage of BDNF/actin expression levels (mean ± SEM; n=5-6). Analysis of the ratio of

proBDNF expression to BDNF expression revealed no significant differences between the groups (Figure 6.9; 2-way ANOVA)

6.3.7 The effect of kainic acid administration and inhibition of the p75NTR on proNGF and NGF *in vivo*

ProNGF and mature NGF levels in the hippocampus following kainic acid administration and inhibition of the p75NTR were assessed. ProNGF levels were analysed by western immunoblot using and an anti-ProNGF antibody which recognises amino acid residues 84-104 of rat NGF (precursor). Western blotting revealed that there was a decrease in proNGF expression levels when kainic acid was administered and when the p75NTR was blocked both in control conditions and in the presence of kainic acid. Statistical analysis of densitometric data revealed that kainic acid administration induces decreased levels of proNGF expression when compared with control levels (Figure 6.10B **$p<0.01$; 2-way ANOVA, *post-hoc* Newmann Keuls; 4.994 ± 1.018 and 2.556 ± 0.3065 for saline and kainic acid-treated rats respectively). Results are expressed as a percentage of proNGF/actin expression (mean ± SEM; n=4-5). Inhibition of the p75NTR by REX alone and when co-administered with kainic acid also resulted in a significant decrease in proNGF levels (Figure 6.10B **$p<0.01$; 2-way ANOVA, *post-hoc* Newmann Keuls; 2.490 ± 0.4 and 1.884 ± 0.45 for saline/REX and kainic acid/REX treated rats respectively). Results are expressed as a percentage of proNGF/actin expression (mean ± SEM; n=5). Mature NGF (Figure 6.10D) expression levels were unaffected by either kainic acid administration or p75NTR inhibition (26.28 ± 7.1 and 16.49 ± 4.1 for saline and kainic acid-treated rats respectively and 21.33 ± 1.9, 15.34 ± 2.8 for saline/REX and kainic acid/REX treated animals respectively (Figure 6.10D)). Results are expressed as a percentage of NGF/actin expression levels (mean ± SEM; n=5). Analysis of the ratio of expression of proNGF to mature NGF revealed no significant differences between any of the groups (Figure 6.11; 2-way ANOVA, n=6).

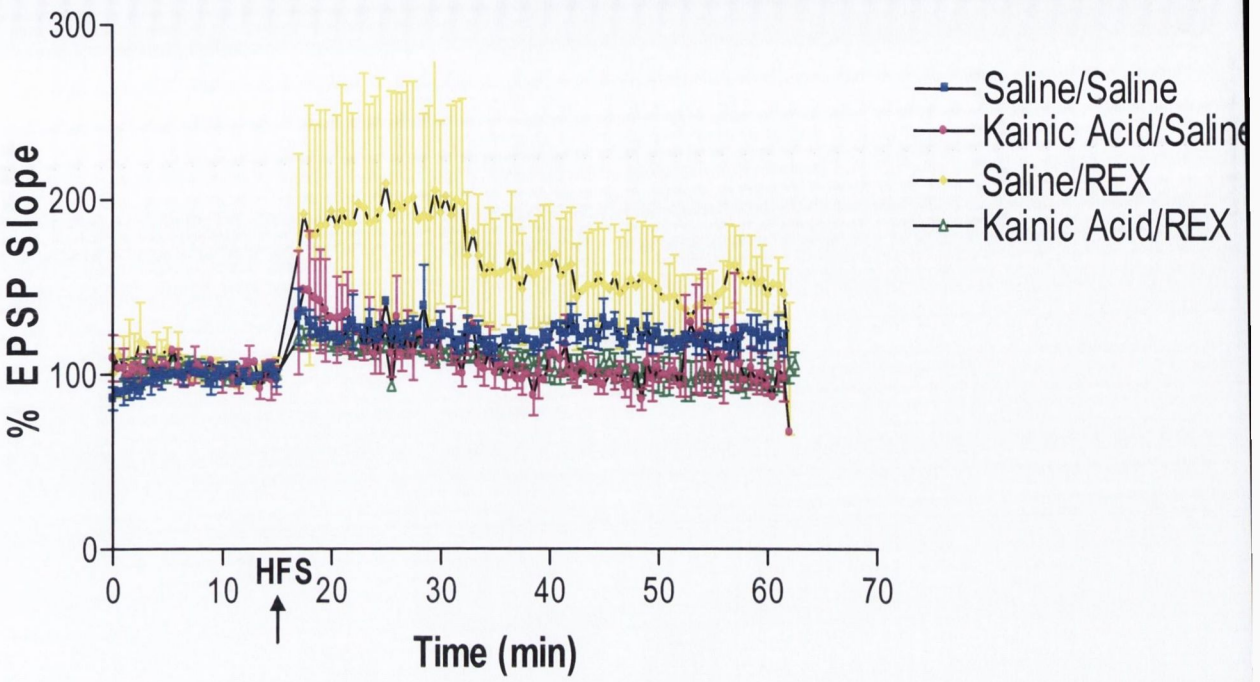
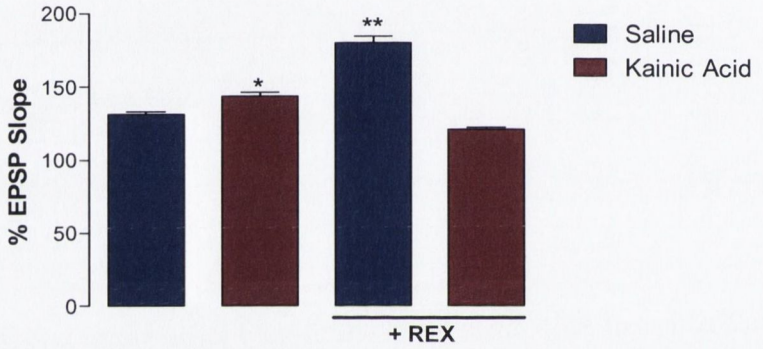


Figure 6.2 Effect of REX on LTP in saline and kainic acid treated rats *in vivo*

Test shocks were given at 30 sec intervals for a 15min period prior to tetanization. High frequency stimulation (HFS; 3 trains of stimuli of 200Hz for 200msec at 20 sec intervals) was delivered to the perforant path. Recording at test shock frequency resumed for 40min following tetanus. LTP was maintained by saline-treated rats (120.2 ± 0.64%); however those treated with kainic acid failed to sustain LTP (107.1 ± 2.654%). Co-administration of the p75NTR inhibitor REX (5μl, ~10mg/ml) with kainic acid failed to reverse the impairment in LTP. Co-administration of the p75NTR inhibitor REX with saline significantly increased the % EPSP slope above control levels (**$p<0.001$, 3-way ANOVA, *post-hoc* Newmann Keuls). Results are expressed as mean EPSP slope normalized with respect to the EPSP slope recorded in the min immediately prior to tetanic stimulation. Results are expressed as means ± SEM of 4-6 independent observations for all treatment groups.

A.

B.



C.

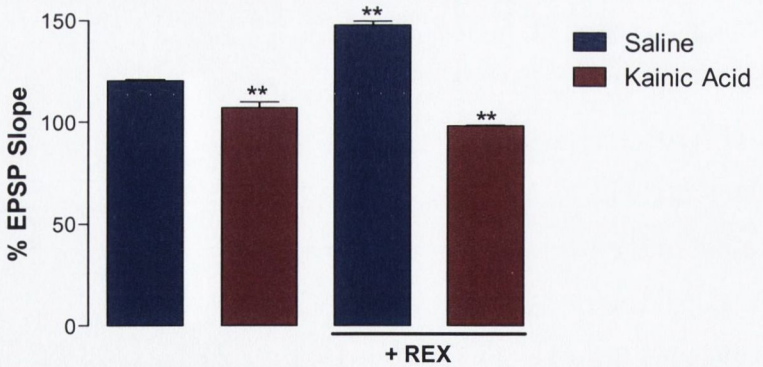
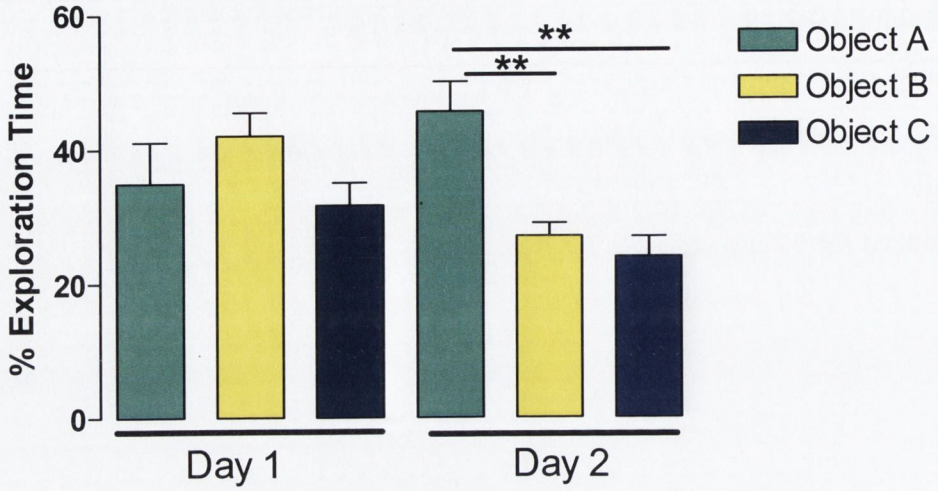


Figure 6.3 Effect of REX on LTP in saline and kainic acid treated rats *in vivo*

Test shocks were given at 30 sec intervals for a 15min period prior to tetanization. High-frequency stimulation (HFS; 3 trains of stimuli of 200Hz for 200msec at 20 sec intervals) was delivered to the perforant path. Recording at test shock frequency resumed 40min following tetanus. There was no significant differences in % epsp slope between any of the groups in the 2 minutes prior to HFS

(**Figure 6.3A**). A significant increase in percentage epsp slope was observed in rats treated with saline, kainic acid and those injected with both saline and REX following HFS (**Figure 6.3B**; 2 minutes post-HFS) LTP was maintained by saline-treated rats; however those treated with kainic acid failed to sustain LTP (**Figure 6.3C;** last 10 minutes of recording). The administration of REX failed to reverse the kainic acid-induced impairment in LTP (**Figure 6.3C**) however it significantly enhanced the % epsp slope in rats treated with saline (**Figure 6.3C**).

A. **Control**



B. **REX**

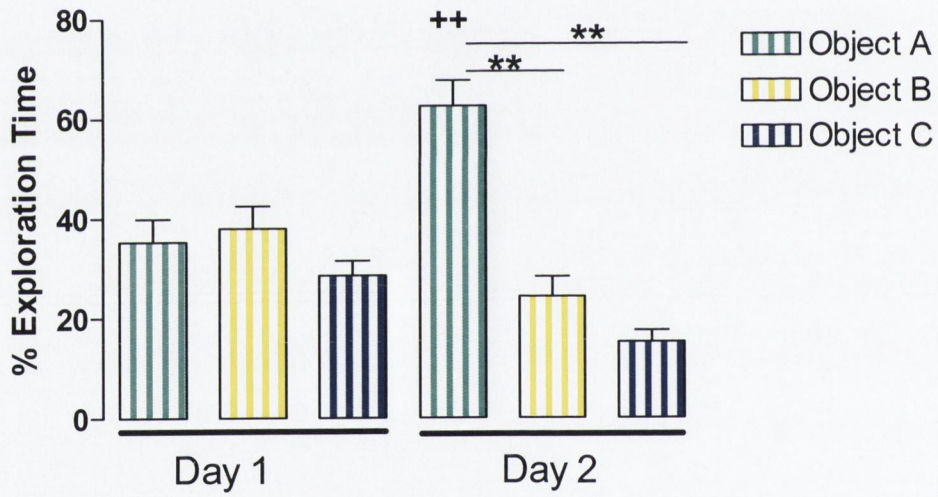
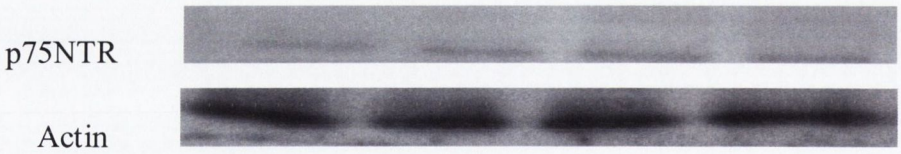


Figure 6.4 Administration of the p75NTR inhibitor REX enhances spatial learning in the rat

The p75NTR inhibitor REX (5μl, ~10mg/ml) or vehicle (5µl) was administered i.c.v. via an implanted cannula one hour prior to training the spatial learning task on day one. Results are expressed as a percentage of the total exploration time (mean ± SEM, n=7-8). Both groups explored objects A, B and C equally on day one. Vehicle treated rats (**A**) successfully recognised that object A had been displaced on day 2 and spent significantly more time actively exploring object A than the non-displaced objects; ** $p<0.001$; 3-way ANOVA, *post hoc* Newmann Keuls. Rats treated with the p75NTR inhibitor REX (**B**) (~10mg/ml) also

successfully recognised the displacement of object A on day 2 and spent significantly more time actively exploring object A than the non-displaced objects; ** $p<0.001$; 2-way ANOVA, *post hoc* Newmann Keuls. Furthermore, rats treated with REX spent significantly more time actively exploring the displaced object when compared to vehicle treated rats; ++ $p<0.001$; 3-way ANOVA, *post hoc* Newmann Keuls.

A.



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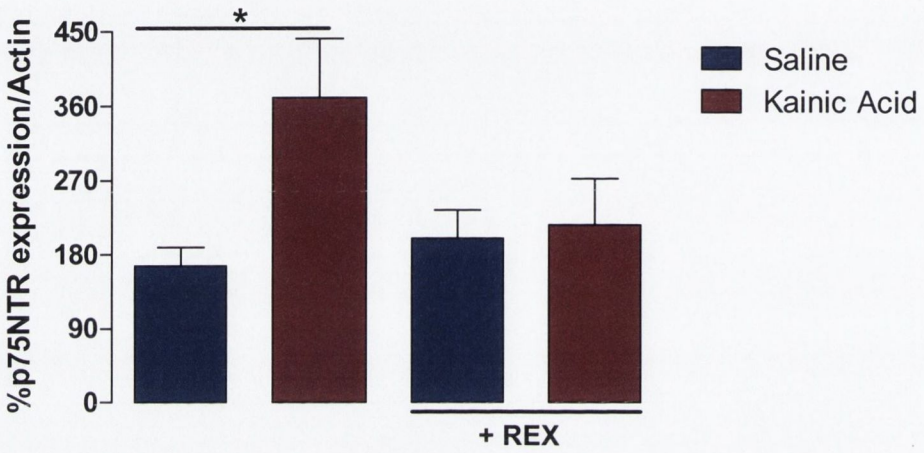


Figure 6.5 Kainic acid administration increases p75NTR expression *in vivo*

A. Sample western immunoblot illustrating increased p75NTR expression in the hippocampus of rats treated with kainic acid

B. The p75NTR inhibitor REX or vehicle was co-administered with either kainic acid (100μM) or saline (0.9% w/v) i.c.v. one hour prior to recording LTP. p75NTR expression in hippocampal homogenate was assessed by western blot analysis. Administration of kainic acid significantly increased p75NTR expression when compared with controls. Results are expressed as a percentage of p75NTR expression/actin expression (mean ± SEM, n=5-6), 2-way ANOVA, *$p<0.05$.

B.

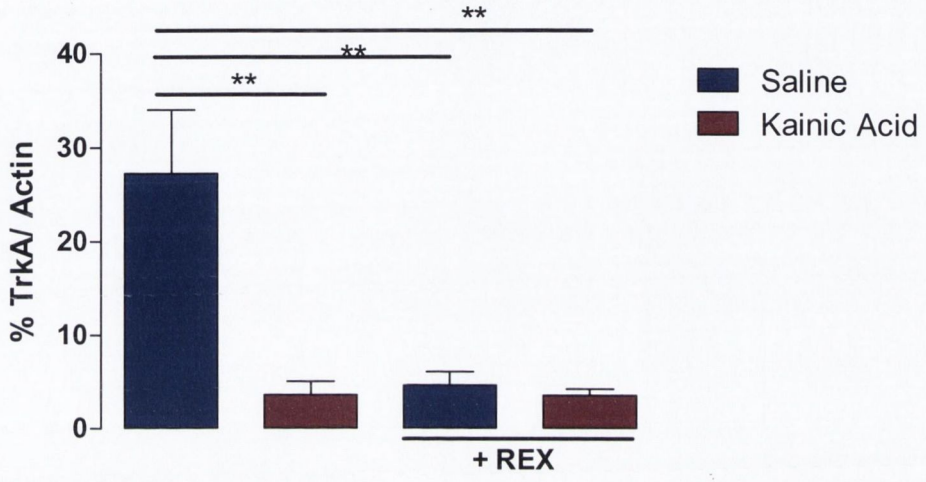
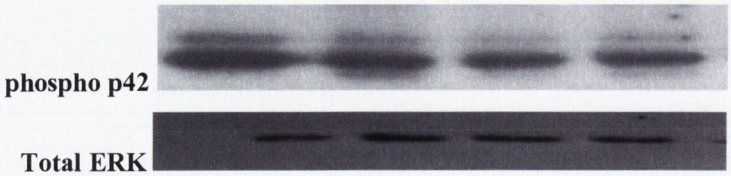


Figure 6.6 Kainic acid administration decreases TrkA expression *in vivo*

A. Sample western immunoblots illustrating increased TrkA expression and actin expression in the hippocampus of rats treated with the p75NTR inhibitor REX in the presence or absence of kainic acid (100μM).

B. The p75NTR inhibitor REX or vehicle was co-administered with either kainic acid (100μM) or saline (0.9% w/v) i.c.v. one hour prior to recording LTP. TrkA expression in hippocampal homogenate was assessed by western blot analysis. Administration of kainic acid significantly increased p75NTR expression when compared to controls. Results are expressed as a percentage of TrkA expression/actin expression (mean ± SEM, n=5-6), 2-way ANOVA, **$p<0.001$.

A.



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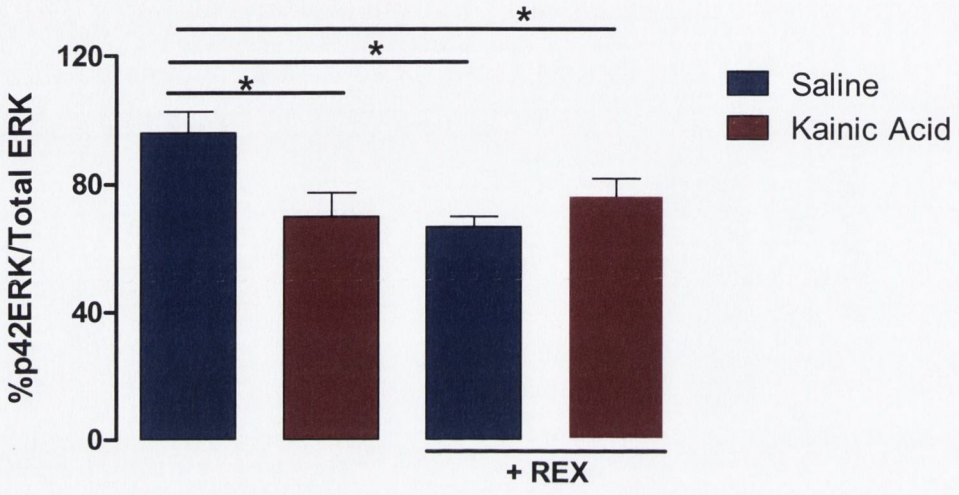
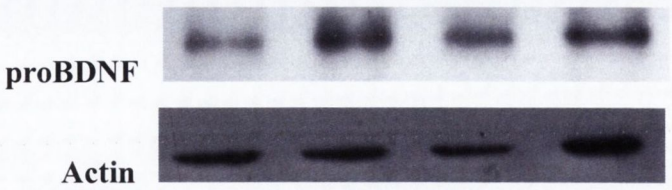


Figure 6.7 The effect of kainic acid administration and inhibition of the p75NTR on ERK activity *in vivo*

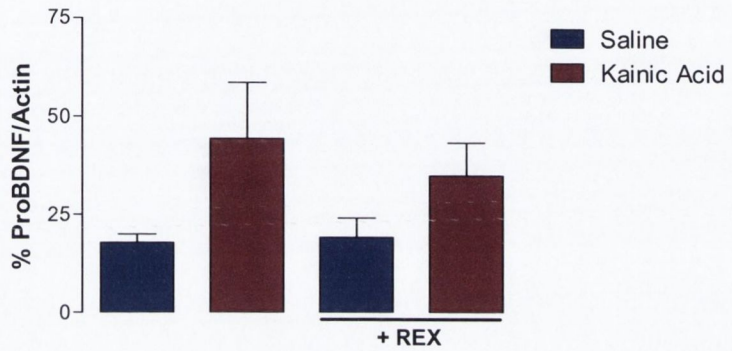
A. Sample western immunoblots illustrating ERK phosphorylation and total ERK expression in the hippocampus of rats treated with saline (0.9%) or kainic acid (100μM) in the presence and absence of the p75NTR inhibitor REX (~10mg/ml).

B. The p75NTR inhibitor REX or vehicle was co-administered with either kainic acid (100μM) or saline (0.9% w/v) i.c.v. one hour prior to recording LTP. ERK activity in hippocampal homogenate was assessed by western blot analysis. Administration of kainic acid significantly decreased ERK activity when compared with saline controls. The p75NTR inhibitor REX also significantly decreased ERK activity. Results are expressed as a percentage of phosphorylated ERK/total ERK expression (mean ± SEM, n=4-5), 2-way ANOVA, *post-hoc* Newmann Keuls, *$p<0.05$.

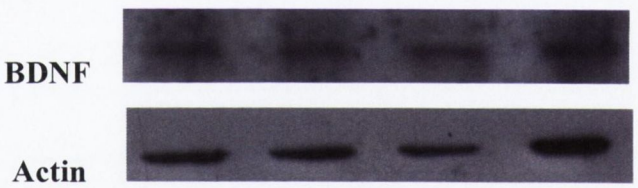
A.



B.



C.



D.

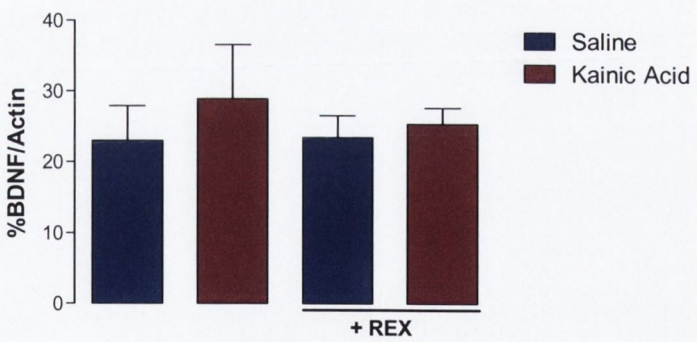


Figure 6.8 The effect of kainic acid administration and inhibition of the p75NTR on proBDNF and BDNF *in vivo*

A. Sample western immunoblot illustrating proBDNF expression levels in the hippocampus of rats treated with saline (0.9%) or kainic acid (100μM) in the presence and absence of the p75NTR inhibitor REX (~10mg/ml)

B. The p75NTR inhibitor REX or vehicle was co-administered with either kainic acid (100μM) or saline (0.9% w/v) i.c.v. one hour prior to recording LTP. ProBDNF expression in hippocampal homogenate was assessed by western blot analysis. Densitometric analysis revealed no significant differences between groups. Results are expressed as a percentage of proBDNF expression/actin expression (mean ± SEM, n=4-5, 2-way ANOVA).

C. Sample western immunoblot illustrating BDNF expression levels in the hippocampus of rats treated with either saline (0.9%) or kainic acid (100μM) and in the presence and absence of the p75NTR inhibitor REX (~10mg/ml)

D. The p75NTR inhibitor REX or vehicle was co-administered with either kainic acid (100μM) or saline (0.9% w/v) i.c.v. one hour prior to recording LTP. BDNF expression in hippocampal homogenate was assessed by western blot analysis. Statistical analysis revealed no significant differences in BDNF expression levels between any of the treatment groups. Results are expressed as a percentage of BDNF expression/actin expression (mean ± SEM, n=5), 2-way ANOVA.

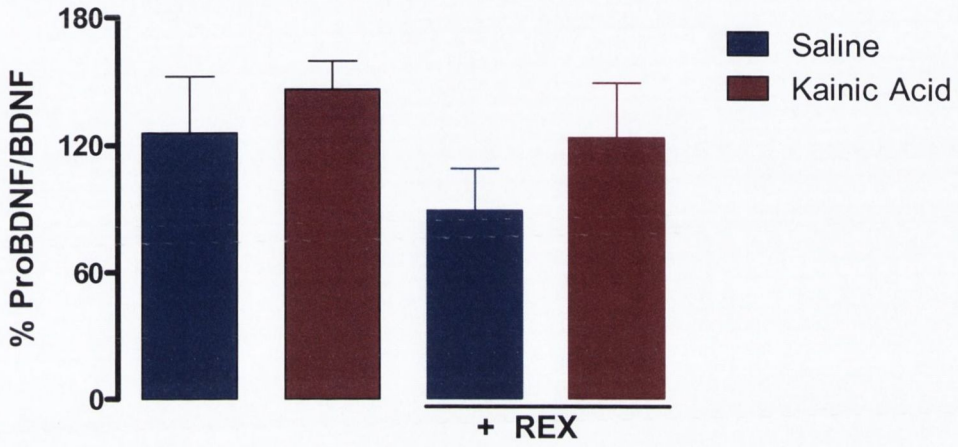
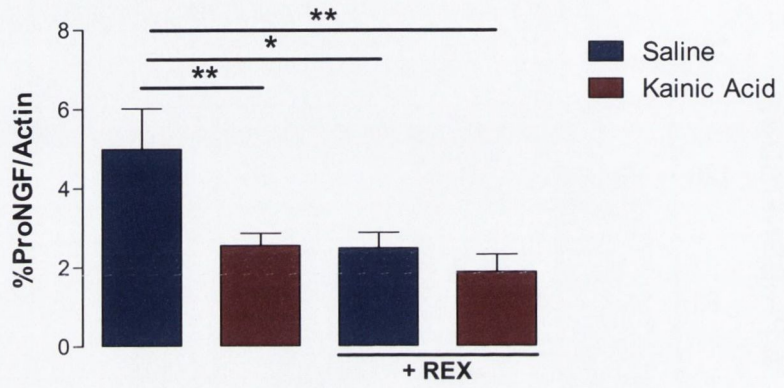


Figure 6.9 Ratio of proBDNF expression to BDNF expression

The p75NTR inhibitor REX or vehicle was co-administered with either kainic acid (100μM) or saline (0.9% w/v) i.c.v. one hour prior to recording LTP. ProBDNF and BDNF expression in hippocampal homogenate was assessed by western blot analysis. Densitometric analysis revealed no significant differences in the ratio of proBDNF to BDNF in any of the groups. Results are expressed as a percentage of proBDNF expression/BDNF expression (mean ± SEM, n=6, 2-way ANOVA).

A

B



C

D

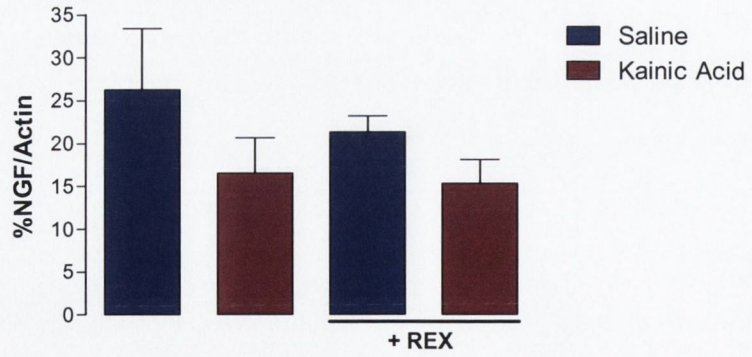


Figure 6.10 Kainic acid administration and inhibition of the p75NTR decreases proNGF expression but not mature NGF expression *in vivo*

A. Sample western immunoblot illustrating decreased proNGF expression levels in the hippocampus of rats treated with kainic acid (100μM) and the p75NTR inhibitor REX (~10mg/ml)

B. The p75NTR inhibitor REX or vehicle was co-administered with either kainic acid (100μM) or saline (0.9% w/v) i.c.v. one hour prior to recording LTP. proNGF expression in hippocampal homogenate was assessed by western blot analysis. Administration of kainic acid significantly decreased proNGF expression when compared with controls. The p75NTR inhibitor REX also significantly decreased proNGF expression levels. Results are expressed as a percentage of proNGF expression/actin expression (mean ± SEM, n=4-5), 2-way ANOVA, *post-hoc* Newmann Keuls, **$p<0.001$.

C. Sample western immunoblot illustrating NGF expression levels in the hippocampus of rats treated with kainic acid (100μM) and in the presence and absence of the p75NTR inhibitor REX (~10mg/ml)

D. The p75NTR inhibitor REX or vehicle was co-administered with either kainic acid (100μM) or saline (0.9% w/v) i.c.v. one hour prior to recording LTP. NGF expression in hippocampal homogenate was assessed by western blot analysis. Statistical analysis revealed no significant differences in NGF expression levels between any of the treatment groups. Results are expressed as a percentage of NGF expression/actin expression (mean ± SEM, n=5), 2-way ANOVA.

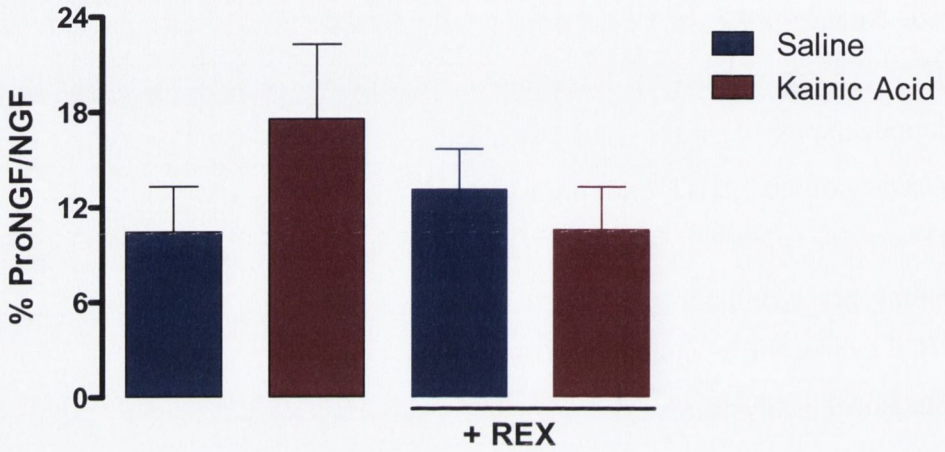


Figure 6.11 Ratio of proNGF expression to NGF expression

The p75NTR inhibitor REX or vehicle was co-administered with either kainic acid (100μM) or saline (0.9% w/v) i.c.v. one hour prior to recording LTP. ProNGF and NGF expression in hippocampal homogenate was assessed by western blot analysis. Densitometric analysis revealed no significant differences in the ratio of proNGF to NGF in any of the groups. Results are expressed as a percentage of proNGF expression/NGF expression (mean ± SEM, n=6, 2-way ANOVA).

6.4 Discussion

The primary aim of this study was to try to establish the role of increased p75NTR expression in the hippocampus following kainic acid administration *in vivo*. This study also aimed to establish the effect of increased p75NTR expression on synaptic plasticity as assessed by LTP.

Results from previous studies indicate that administration of kainic acid increases p75NTR expression (Yi *et al.*, 2003). This kainic acid-induced upregulation in p75NTR expression was once again confirmed in this study. However in this study a decrease in TrkA receptor expression was observed in kainic acid-treated rats. This was surprising as it had not been observed in the previous set of experiments performed. The differential expression of the p75NTR and TrkA receptor is thought to mediate the balance between survival and apoptotic signalling in neuronal populations. Cells presenting a higher expression of p75NTR than TrkA receptors will undergo apoptosis (Barrett and Bartlett, 1994). The profile of neurotrophin receptor expression exhibited in the hippocampal homogenate examined in this study would facilitate apoptotic signalling. Interestingly, analysis of ERK activity, which is a MAPK known to be activated downstream of Trk receptor (Gooney and Lynch, 1998; Loeb et al., 1992) and involved in survival signalling (Anderson and Tolkovsky, 1999) was also downregulated in the same manner as TrkA.

Previous studies have demonstrated that this increase in receptor expression is concomitant with an impairment in LTP. In order to assess the direct impact of increased p75NTR expression on LTP the p75NTR function-inhibiting antibody REX was employed. It was hypothesized that co-administration of REX with kainic acid would ameliorate the kainic acid–induced impairment in LTP. High-frequency stimulation resulted in an increase in percentage epsp slope in all of the groups. Saline-treated animals sustained this increase in percentage epsp slope until the end of recording. Kainic acid-treated rats were unable to sustain this increase and the slope gradually returned to baseline. Inhibition of the p75NTR may not induce a recovery of function in kainic acid-treated animals indicating that the observed impairment in function does not directly involve p75NTR. It would be interesting to repeat the experiment administering REX prior to the administration of kainic acid

as the timing of the increased expression may be a key factor influencing the ability of rats to sustain LTP. Surprisingly, we observed an enhancement in LTP in saline-treated animals that were administered REX. This was an intriguing result; inhibition of the p75NTR in normal animals was augmenting synaptic function. To further investigate this enhancement of synaptic function by inhibition of the p75NTR a hippocampal dependent spatial recognition task was performed over two days. On day one animals treated i.c.v. with either saline or REX placed into an arena containing three objects and a spatial cue. Animals were allowed to actively explore the environment and the objects. 24 hours later one of the objects was displaced and animals were reintroduced to the arena. Analysis showed that both groups recognised that one of the objects had been displaced but the REX-treated animals spent significantly more time than their saline counterparts investigating the displaced object. Blockade of p75NTR, resulted in enhancement in LTP, which is a model for activity dependent memory and is robustly induced in the hippocampus, and an improvement in hippocampal dependent spatial learning.

Studies investigating the effect of p75NTR expression on synaptic plasticity are few and far between. A study by Xu *et al.* (2000) reported that inhibition of the p75NTR using REX had no effect on LTP recording in the CA1 region of the hippocampus *in vitro*. Contrastingly, that same year it was reported p75NTR knockout mice demonstrated improved spatial learning (Greferath *et al.*, 2000). P75NTR was found to mediate a rapid switch in neurotransmitter release in sympathetic neurons acting on cardiac myocytes in culture (Yang *et al.*, 2002). Neurons stimulated with NGF had increased epinephrine release resulting in an excitatory effect leading to an increase in myocyte beat rate. Perfusion with BDNF stimulated the release of acetylcholine which caused an inhibitory effect resulting in a decreased myocyte beat rate. p75NTR knockouts did not release acetylcholine and animals expressing supernumerary p75NTR's had enhanced acetylcholine release. They concluded that p75NTR modulates release of distinct neurotransmitter pools resulting in a functional switch between excitatory and inhibitory neurotransmission in neurons. This apparent functional antagonism of the neurotrophin receptor system caught the attention of Rösch and colleagues. A role for the Trk receptors, in particular, TrkB, in LTP was well documented. As a result they hypothesized that the p75NTR receptor may play a role in LTD. Two strains of p75NTR knockout

mice were used in their experiments (Rösch *et al.*, 2005). They found that LTP was unaffected in hippocampal slices from both strains of mice but that LTD was impaired. In a similar set of experiments by Woo *et al.* (2005) it was elucidated that NMDA receptor-dependent LTD in the CA1 was impaired in p75NTR knockout mice and that application of REX virtually abolished LTD in wildtype mice. Interestingly they reported that activation of the p75NTR by proBDNF enhanced LTD. Studies have shown that BDNF and its receptor are essential for some forms of late-phase LTP (Patterson *et al.*, 2001). It has since been shown that cleavage of proBDNF by plasmin is essential for long-term plasticity (Pang *et al.*, 2004). Though these results did not show any effect of p75NTR inhibition on LTP it is important to note that these experiments were carried out *in vitro* and recorded LTP in the CA1 region of the hippocampus and that there may be region specific differences. These data, together with the data from this study, cumulatively suggest that p75NTR may be exerting some type of tonic inhibition of LTP. Still the question remained as to how the i.c.v. administration of REX in normal rats was resulting in an improvement in LTP.

For many years it was thought that the function of the proneurotrophins was to promote protein folding (Suter *et al.*, 1991) and regulate neurotrophin secretion (Rattenholl *et al.*, 2001). It has since emerged that these peptides are biologically active and can elicit a wide range of biological functions from apoptotic signalling (Lee *et al.*, 2001; Nykjaer *et al.*, 2004) to synaptic plasticity (Rösch *et al.*, 2005; Woo *et al.*, 2005). Combining the results of Woo *et al.* (2005) and Pang *et al.* (2004), the propensity for a synapse to undergo LTD/LTP would appear to depend on the conversion of proBDNF to mature BDNF. Release of proBDNF and its binding to p75NTR facilitates LTD. If the immature neurotrophin is converted to BDNF, by plasmin, furin or the metalloproteinases (MMP's), then its binding to TrkB facilitates late-phase LTP. Analysis of proBDNF revealed an increase in the kainic acid-treated group relative to control. This increase was not significant however it was a definite trend. ProBDNF has been shown to induce apoptosis via its binding to p75NTR/ sortilin complex (Teng *et al.*, 2005). This suggests that the increased availability of ligand and expression of receptor may lead to apoptotic signalling. Analysis of the mature form of BDNF revealed no significant differences between groups. Several studies have shown an increase in BDNF protein following

kainic acid administration (Shetty *et al.*, 2004; Lähteinen *et al.*, 2004; Katoh-Semba *et al.*, 1999). However, these studies looked at BDNF levels after an extended period post-kainic acid injection. One study reported that the kainic acid-induced elevation in BDNF protein concentration commenced 4 hours post injection (Katoh-Semba *et al.*, 1999). The time period in this experiment from i.c.v. injection to sacrifice was approximately two hours which according to the findings of Katoh-Semba and colleagues would be too short to observe an increase in BDNF concentration.

Interestingly, when we looked at the ratio of proBDNF expression to BDNF expression we observed that there was a definitive trend towards a decrease in the ratio of expression in the saline/REX group. This indicated that there was a higher conversion of proBDNF to mature BDNF in this group. If like Woo *et al.* (2005) and Rösch *et al.* (2005) suggest, there is a functional antagonism of the neurotrophin system that is dependent on the conversion of proneurotrophins to mature neurotrophins this would explain the improvement in LTP observed in the saline/REX treated animals.

It has been reported that administration of kainic acid leads to loss of TrkB mRNA expression in the hippocampus (Revuelta *et al.*, 2005). TrkB has been shown to play an integral role in LTP (Kang *et al.*, 1997; Gooney and Lynch, 2001). Therefore the kainic acid-induced impairment in LTP observed may be due to decreased expression of TrkB receptors. This would also explain the inability of REX to ameliorate the kainic acid-induced impairment in LTP. TrkB expression was not analysed in this study but an investigation into its expression following kainic acid administration would be interesting.

Like proBDNF, proNGF has been shown to induce apoptosis via p75NTR/sortilin complex (Nykjaer *et al.*, 2005). ProNGF induces apoptosis in spinal cord lysates *in vitro* (Beattie *et al.*, 2002). ProNGF is secreted following lesion of corticospinal neurons and induces apoptosis via activation of the p75NTR (Harrington *et al.*, 2004). Increased proNGF levels have been observed in the brain of Alzheimer's disease patients (Fahnestock *et al.*, 2001) and have been correlated with the disease associated cognitive decline (Peng *et al.*, 2004). Similarly,

increased levels of proNGF have been associated with age related sympathetic neuron atrophy (Bierl and Isaacson, 2005). These data implicate proNGF as a mediator of neurodegenerative processes. Analysis of proNGF protein expression in this study revealed a significant decrease in all groups compared with control levels. This was not expected for the kainic acid groups as it has been reported that administration of kainic acid increased proNGF levels. Volosin *et al.* (2006) observed increased immunostaining of proNGF in glial fibrillary acidic protein (GFAP; a marker for astrocytes) positive cells of the basal forebrain one day after kainic acid administration. The significant decrease in proNGF could be as a result of increased proteolytic cleavage of the peptide to the mature form. However, when analysis of NGF was carried out no significant difference between any of the groups was observed. There is a discrepancy in the literature with regard to NGF levels post-kainic acid administration. It has been reported that NGF levels decrease after kainic acid injection (Katoh-Semba *et al.*, 1999). These decreases were observed after a six hour time delay and were still diminished after twelve days. However, Shetty *et al.* (2003) reported an upregulation of NGF four days post-kainic acid administration that persisted for 120 days post injection. Intriguingly, the decreased levels of NGF expression observed in this study followed the same pattern as the decreases observed in TrkA receptor expression.

Analysis of the ratio between proNGF expression levels and NGF expression revealed no significant difference between the groups but there was a trend towards an increase in proNGF activity relative to that of NGF. Further work needs to be carried out in order to fully elucidate the fate of proNGF and NGF post-kainic acid administration.

The objective of this study was to establish the precise role of p75NTR in kainic acid-induced impairment in LTP. This study failed to demonstrate a direct link between kainic acid-induced impairment of synaptic function and p75NTR. This may have been due to inappropriate timing of the application of the p75NTR inhibitor REX and needs to be reassessed. This study did establish a role for p75NTR in normal LTP. It appears that there is functional antagonism between the neurotrophins and p75NTR may be tonically suppressing LTP. This is an exciting

new facet to the pleiotropic functions of the p75NTR and requires a great deal more investigation.

Chapter 7

General Discussion

7.1 General discussion

It has been over fifty years since the discovery of the prototypic neurotrophin, NGF, by Rita Levi-Montalcini and Viktor Hamburger. Since its initial discovery, NGF and the other members of the neurotrophin family have been the focus of rigorous research. The simple entry of the term "neurotrophin" into PubMed retrieves over five thousand publications with almost five hundred of these being published in the last year. A brief scan of these publications illustrates the diverse functions of the neurotrophins with everything from the developmental growth of the nervous system, synaptic plasticity in the adult CNS and neurotrophin involvement in neuronal cell death being discussed.

The p75NTR was the first NGF receptor to be discovered, but initial research efforts were concentrated on Trk receptors. The biological functions of the p75NTR were not fully elucidated until the early 1990's. It emerged that the p75NTR was a fully functioning receptor capable of initiating autonomic signalling cascades and was not merely playing an ancillary role to Trk receptors. p75NTR can modulate Trk receptor signalling (Mamidipudi and Wooten, 2002; Bamji *et al.*, 1998; Yoon *et al.*, 1998). It can initiate autonomous signalling cascades that can regulate both cell survival and apoptosis earning its title as a biological paradox. The receptor can interact with a number of co-receptors including sortilin and Nogo resulting in apoptotic signalling (Nykjaer *et al.*, 2004; Wang *et al.*, 2002) and it has been shown to promote Schwann cell migration (Anton *et al.*, 1994). More recently it has been shown to facilitate synaptic plasticity (Rösch *et al.*, 2005; Woo *et al.*, 2005). P75TR expression has also been implicated in the pathogenesis of neurodegenerative conditions (Perini *et al.*, 2002; Greferath *et al.*, 2001; Casha *et al.*, 2001; Roux *et al.*, 1999 Yaar *et al.*, 1997). Therefore it is vital to understand the mechanisms of action of this receptor and the conditions that govern its various functions.

The primary objective of this study was to investigate the effect of neurodegenerative models on p75NTR and Trk receptor expression and signalling. Secondary to this, I wanted to assess the impact of these neurodegenerative models of synaptic function.

The first series of experiments used LPS as a model of neurodegeneration. LPS is a bacterial endotoxin that has profound immunostimulatory and inflammatory properties (See Alexander and Rietschel, 2001; Rankine *et al.*, 2006; Lynch *et al.*, 2004). LPS injection i.p. was shown to manipulate neurotrophin receptor expression *in vivo.* Peripheral injection of LPS increased p75NTR expression in the dentate gyrus and decreased TrkB expression in the CA1 and CA3 *in vivo.* The differential neurotrophin receptor expression induced by LPS administration was accompanied by an impairment in synaptic function as evidenced by the inability of LPS treated rats to sustain LTP *in vivo.* LPS administration increased neuronal cell death in the dentate gyrus *in vivo.* Interestingly, this was the region in which increased p75NTR expression was observed suggesting that p75NTR expression may be involved in LPS mediated cell death. JNK activity, which is associated with apoptotic signalling and is known to be activated downstream of the p75NTR, was increased in LPS treated animals when compared to saline controls. Overall the evidence presented in this study implicates a role for differential neurotrophin receptor expression and signalling in the neurodegeneration observed following LPS administration.

The second study looked at kainic acid as a model of neurodegeneration. Kainic acid is an analogue of the excitatory amino acid glutamate. It is used experimentally to simulate temporal lobe epilepsy and to induce excitotoxicity ((Tremblay and Ben-Ari, 1984; Tasker *et al.*, 2002). Kainic acid selectively induces the neurodegeneration of specific neuronal populations in the hippocampus, the region of the brain studied in these experiments (Oh *et al.*, 2000; Shetty *et al.*, 2003; Yi *et al.*, 2003). Kainic acid increased p75NTR expression both *in vitro* and *in vivo.* This increase in p75NTR was accompanied by a severe impairment in synaptic function as evidenced by the inability of kainic acid-treated rats to induce LTP *in vivo.* There was an increase in JNK activity both *in vivo* and *in vitro* indicating increased cell death signalling which was concomitant with decreased ERK activity *in vivo.* The *in vivo* evidence suggested that the injury-induced expression of the p75NTR was having a neurodegenerative effect in the hippocampus. Inhibition of Trk receptor signalling and incubation with NGF was proposed to stimulate p75NTR signalling. However, JNK activity was abrogated in kainic acid-treated

slices by incubating with NGF and the Trk inhibitor tyrphostin AG879 when compared with kainic acid-treated controls. This suggested that p75NTR might be apoptotically signalling via one of its other pathways such as that involving ceramide.

It has been postulated that the balance between cell survival and cell death is dependent on the ratio of expression between TrkA and p75NTR. The previous studies had demonstrated that p75NTR expression could be upregulated following the administration of either LPS or kainic acid. It was fortunate that a group of genetically hypertensive (GH) rats that had previously been demonstrated to be deficient in NGF and TrkA were kindly donated by Professor Bell. The use of these rats facilitated the isolation of p75NTR signalling *in vivo*. The hypothesis was that LPS administration to the GH rats would increase p75NTR expression as per my previous experiment and that the absence of Trk receptors would facilitate p75NTR apoptotic signalling.

Previous studies had shown that GH rats are impaired in their ability to sustain LTP (Kelly *et al.*, 1998). This study assessed cognitive function by means of a novel object preference task. N control rats successfully identified the presence of the novel object. Their ability to successfully complete the task was revoked by LPS administration. This LPS-induced impairment in cognitive function was concomitant with increased expression of p75NTR and decreased expression of TrkA confirming my previous finding that LPS can manipulate neurotrophin receptor expression in the rat denate gyrus. GH rats are cognitively impaired as evidenced by their inability to identify a novel object. This impairment was further abrogated by the administration of LPS and was associated with decreased activity of the p44ERK isoform. These data demonstrate that impairments in cognitive function are concomitant with changes in neurotrophin receptor expression, though it is apparent that they occur by two distinct mechanisms.

Evidence thus far collated implicated the p75NTR in the impairment of synaptic function and neurodegenerative processes. In a final attempt to definitively link the p75NTR to these processes I employed the use of the p75NTR function inhibitor REX. Consistent with my earlier study, kainic acid-induced a profound

impairment in LTP. Co-administration of the p75NTR inhibitor REX with kainic acid failed to ameliorate any of the kainic acid induced impairment in LTP. This indicated that the p75NTR was not directly mediating the impairment in synaptic function observed. This may have been due to inappropriate timing of the application of the p75NTR inhibitor REX and needs to be reassessed. However, this study did establish a role for p75NTR in normal LTP. Inhibition of the p75NTR enhances LTP. This suggests that there may be a functional antagonism between the neurotrophins and that the p75NTR may be tonically suppressing LTP. This is an exciting new facet to the pleiotropic functions of the p75NTR and requires a great deal more investigation.

Consistently these data show an alteration in the profile of neurotrophin receptor expression following neuronal insult. A review of the literature implicates the expression of the neurotrophins and their receptors in a number of pathological conditions. P75NTR expression has been shown to induce neuronal death experimentally in ischemia (Greferath *et al.*, 2002), pilocarpine and kainic acid induced seizure (Roux *et al.*, 1999; Volosin *et al.*, 2006), nerve axotomy (Cheema *et al.*, 1996; Ferri *et al.*, 1998; Lowry *et al.*, 2001; Jiang *et al.*, 2005). P75NTR expression is highly expressed in the hippocampus and basal forebrain of aged rats and in Alzheimer's patients (Kerwin *et al.*, 1992; Wiley *et al.*, 1995). It has been demonstrated that β amyloid peptide is capable of binding to the p75NTR (Yaar *et al.*, 1997). Ginsberg *et al.* (2006) reported that the downregulation of Trk but not p75NTR gene expression in the basal forebrain of patients suffering from Alzheimer's disease. This alteration in the profile of neurotrophin receptor expression marked the progression of Alzheimer's disease. Increased levels of proNGF in the brain have been reported in Alzheimer's disease (Fahnestock *et al.*, 2001). This Alzheimer's disease-related increase in proNGF has been correlated with loss of cognitive function (Peng *et al.*, 2004). When isolated from the human brain affected by Alzheimer's disease and introduced to neurons in culture, proNGF-induced apoptosis was mediated by the p75NTR (Pedraza *et al.*, 2005). TrkA expression has been implicated in the pathogenesis of asthma (Nassenstein *et al.*, 2006), and progression of prostate cancer (Weeraratna *et al.*, 2001) while the downregulation of BDNF and TrkB expression has been associated with depression (Karege *et al.*, 2002; Saarelainen *et al.*, 2003).

The inextricable association of the neurotrophins and their receptors in the pathogenesis of disease means that they are attractive therapeutic targets. Clinical trials have been undertaken using NGF protein as a possible treatment for Alzheimer's disease. Patients in Sweden were administered NGF i.c.v. continuously for up to three months (Eriksdotter Jonhagen *et al.*, 1998). Generally speaking there were some cognitive improvements in patients; however, these positive results were marred by negative side effects including loss of appetite and pain. Inhibition of the p75NTR *in vitro* has been shown to prevent zinc-induced apoptosis (Park *et al.*, 2000), antisense knockdown of the p75NTR delays motor neuron disease (Turner *et al.*, 2003), while inhibition of neurotrophin binding to the p75NTR prevents apoptosis in hippocampal neurons (Friedman *et al.*, 2000). The paradoxical functions of the neurotrophins make their use as therapeutic agents difficult. However, a greater understanding of the neurotrophic actions and the creation of mimetic peptides may provide treatment to a number of diseases.

The most exciting result in this study came from the realisation that p75NTR was in some way supressing LTP expression and spatial learning in the hippocampus of the normal rat. Yang *et al.* (2002) reported a functional antagonism between neurotrophins. This concept was further investigated by Rösch and colleagues. A role for the Trk receptors in particular, TrkB, in LTP was well documented and as a result they hypothesized that the p75NTR receptor may play a role in LTD. They determined that p75TR was playing a role in LTD but not in LTP *in vitro* (Rösch *et al.*, 2005). Interestingly it has been reported that BDNF/TrkB activation prevents the induction of LTD by low frequency stimulation in the rat visual cortex (Jiang *et al.*, 2003). This further supports the hypothesis that a functional antagonism exists between the neurotrophins.

The data presented in this study support the hypothesis that differential neurotrophin receptor expression is induced by neuronal insult and is associated with neurodegeneration and impairment of synaptic function.

7.2 Future Studies

The data presented in these studies strongly implicate differential neurotrophin receptor expression in neurodegeneration and impairment in synaptic plasticity. However, further work is required.

- The co-localisation of the Trk receptors and the p75NTR following neuronal insult needs to be assessed.
- An investigation into the expression of the p75NTR on apoptotic cells is also required.
- It needs to be ascertained whether the increase p75NTR expression following neuronal insult is neuronal or glial.
- An investigation into the expression of p75NTR's known co-receptors Nogo and sortilin following neuronal insult is also required.
- The downstream consequences of p75NTR activation need to be further explored.
- The role of TrkB expression and activation following REX administration needs to be ascertained.
- A more detailed examination into the role of the proneurotrophins in neurodegeneration and synaptic plasticity is required.

VII Bibliography

Abraham WC, Logan B, Greenwood JM & Dragunow M. (2002). Induction and experience-dependent consolidation of stable long-term potentiation lasting months in the hippocampus. *The Journal of Neuroscience* **22,** 9626-9634.

Alexander C & Rietschel ET. (2001). Bacterial lipopolysaccharides and innate immunity. *Journal of endotoxin research* **7,** 167-202.

Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR & Miller FD. (1998). p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. *The Journal of cell biology* **143,** 1691-1703.

Anderson CN & Tolkovsky AM. (1999). A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. *The Journal of Neuroscience* **19,** 664-673.

Aniksztejn L, Roisin MP, Amsellem R & Ben-Ari Y. (1989). Long-term potentiation in the hippocampus of the anaesthetized rat is not associated with a sustained enhanced release of endogenous excitatory amino acids. *Neuroscience* **28,** 387-392.

Anton ES, Weskamp G, Reichardt LF & Matthew WD. (1994). Nerve growth factor and its low-affinity receptor promote Schwann cell migration. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 2795-2799.

Astur RS, Taylor LB, Mamelak AN, Philpott L & Sutherland RJ. (2002). Humans with hippocampus damage display severe spatial memory impairments in a virtual Morris water task. *Behavioural brain research* **132,** 77-84.

Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG & Miller FD. (1998). The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. *The Journal of cell biology* **140,** 911-923.

Barde YA, Edgar D & Thoenen H. (1982). Purification of a new neurotrophic factor from mammalian brain. *The EMBO journal* **1,** 549-553.

Barouch R, Appel E, Kazimirsky G & Brodie C. (2001). Macrophages express neurotrophins and neurotrophin receptors. Regulation of nitric oxide production by NT-3. *Journal of neuroimmunology* **112,** 72-77.

Barouch R, Kazimirsky G, Appel E & Brodie C. (2001). Nerve growth factor regulates TNF-alpha production in mouse macrophages via MAP kinase activation. *Journal of leukocyte biology* **69,** 1019-1026.

Barrett GL & Bartlett PF. (1994). The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 6501-6505.

Barry CE, Nolan Y, Clarke RM, Lynch A & Lynch MA. (2005). Activation of c-Jun-N-terminal kinase is critical in mediating lipopolysaccharide-induced changes in the rat hippocampus. *Journal of neurochemistry* **93,** 221-231.

Beason-Held, L.L., Rosene, D.L., Killiany, R.J. and Moss, M.B. (1999). Hippocampal formation lesions produce memory impairment in the rhesus monkey. *Hippocampus* **9**, pp. 562–574.

Beattie MS, Harrington AW, Lee R, Kim JY, Boyce SL, Longo FM, Bresnahan JC, Hempstead BL & Yoon SO. (2002). ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. *Neuron* **36,** 375-386.

Bierl MA & Isaacson LG. (2005). Increased NGF proforms in aged sympathetic

neurons and their targets. *Neurobiol Aging.*

Bilderback TR, Grigsby RJ & Dobrowsky RT. (1997). Association of p75(NTR) with caveolin and localization of neurotrophin-induced sphingomyelin hydrolysis to caveolae. *The Journal of biological chemistry* **272,** 10922-10927.

Blanquet PR & Lamour Y. (1997). Brain-derived neurotrophic factor increases Ca2+/calmodulin-dependent protein kinase 2 activity in hippocampus. *The Journal of biological chemistry* **272,** 24133-24136.

Blasing H, Hendrix S & Paus R. (2005). Pro-inflammatory cytokines upregulate the skin immunoreactivity for NGF, NT-3, NT-4 and their receptor, p75NTR in vivo: a preliminary report. *Archives of dermatological research* **296,** 580-584.

Bliss TV, Douglas RM, Errington ML & Lynch MA. (1986). Correlation between long-term potentiation and release of endogenous amino acids from dentate gyrus of anaesthetized rats. *The Journal of physiology* **377,** 391-408.

Bliss TV & Lomo T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *The Journal of physiology* **232,** 331-356.

Bono F, Lamarche I, Bornia J, Savi P, Della Valle G & Herbert JM. (1999). Nerve growth factor (NGF) exerts its pro-apoptotic effect via the P75NTR receptor in a cell cycle-dependent manner. *FEBS letters* **457,** 93-97.

Botchkarev VA, Yaar M, Gilchrest BA & Paus R. (2003). p75 Neurotrophin receptor antagonist retards apoptosis-driven hair follicle involution (catagen). *The Journal of investigative dermatology* **120,** 168-169.

Boyle K, Azari MF, Cheema SS & Petratos S. (2005). TNFalpha mediates Schwann cell death by upregulating p75NTR expression without sustained activation

of NFkappaB. *Neurobiology of disease* **20,** 412-427.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* **72,** 248-254.

Brann AB, Tcherpakov M, Williams IM, Futerman AH & Fainzilber M. (2002). Nerve growth factor-induced p75-mediated death of cultured hippocampal neurons is age-dependent and transduced through ceramide generated by neutral sphingomyelinase. *The Journal of biological chemistry* **277,** 9812-9818.

Caroleo MC, Costa N, Bracci-Laudiero L & Aloe L. (2001). Human monocyte/macrophages activate by exposure to LPS overexpress NGF and NGF receptors. *Journal of neuroimmunology* **113,** 193-201.

Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhauser N, Bohm-Matthaei R, Baeuerle PA & Barde YA. (1996). Selective activation of NF-kappa B by nerve growth factor through the neurotrophin receptor p75. *Science* **272,** 542-545.

Casaccia-Bonnefil P, Carter BD, Dobrowsky RT & Chao MV. (1996). Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Nature* **383,** 716-719.

Casademunt E, Carter BD, Benzel I, Frade JM, Dechant G & Barde YA. (1999). The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. *The EMBO journal* **18,** 6050-6061.

Casha S, Yu WR & Fehlings MG. (2001). Oligodendroglial apoptosis occurs along degenerating axons and is associated with FAS and p75 expression following spinal cord injury in the rat. *Neuroscience* **103,** 203-218.

Castillo PE, Malenka RC & Nicoll RA. (1997). Kainate receptors mediate a slow postsynaptic current in hippocampal CA3 neurons. *Nature* **388,** 182-186.

Castren E, Pitkanen M, Sirvio J, Parsadanian A, Lindholm D, Thoenen H & Riekkinen PJ. (1993). The induction of LTP increases BDNF and NGF mRNA but decreases NT-3 mRNA in the dentate gyrus. *Neuroreport* **4,** 895-898.

Cave, C.B. and Squire, L.R., 1991. Equivalent impairment of spatial and nonspatial memory following damage to the human hippocampus. *Hippocampus* **1**, pp. 329–340

Cheema SS, Barrett GL & Bartlett PF. (1996). Reducing p75 nerve growth factor receptor levels using antisense oligonucleotides prevents the loss of axotomized sensory neurons in the dorsal root ganglia of newborn rats. *Journal of neuroscience research* **46,** 239-245.

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

Cheng B & Mattson MP. (1991). NGF and bFGF protect rat hippocampal and human cortical neurons against hypoglycemic damage by stabilizing calcium homeostasis. *Neuron* **7,** 1031-1041.

Choi DW. (1985). Glutamate neurotoxicity in cortical cell culture is calcium dependent. *Neuroscience letters* **58,** 293-297.

Coan EJ & Collingridge GL. (1985). Magnesium ions block an N-methyl-D-aspartate receptor-mediated component of synaptic transmission in rat hippocampus. *Neuroscience letters* **53,** 21-26.

Coan EJ & Collingridge GL. (1987). Characterization of an N-methyl-D-aspartate receptor component of synaptic transmission in rat hippocampal slices.

Neuroscience **22,** 1-8.

Collingridge GL, Herron CE & Lester RA. (1988). Synaptic activation of N-methyl-D-aspartate receptors in the Schaffer collateral-commissural pathway of rat hippocampus. *The Journal of physiology* **399,** 283-300.

Collingridge GL, Kehl SJ & McLennan H. (1983). Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *The Journal of physiology* **334,** 33-46.

Coogan AN, O'Leary DM & O'Connor JJ. (1999). P42/44 MAP kinase inhibitor PD98059 attenuates multiple forms of synaptic plasticity in rat dentate gyrus in vitro. *Journal of neurophysiology* **81,** 103-110.

Coulson EJ, Reid K, Baca M, Shipham KA, Hulett SM, Kilpatrick TJ & Bartlett PF. (2000). Chopper, a new death domain of the p75 neurotrophin receptor that mediates rapid neuronal cell death. *The Journal of biological chemistry* **275,** 30537-30545.

Creedon DJ, Johnson EM & Lawrence JC. (1996). Mitogen-activated protein kinase-independent pathways mediate the effects of nerve growth factor and cAMP on neuronal survival. *The Journal of biological chemistry* **271,** 20713-20718.

Davies CH, Starkey SJ, Pozza MF & Collingridge GL. (1991). GABA autoreceptors regulate the induction of LTP. *Nature* **349,** 609-611.

De Koninck P & Schulman H. (1998). Sensitivity of CaM kinase II to the frequency of Ca2+ oscillations. *Science* **279,** 227-230.

De Rosa R, Garcia AA, Braschi C, Capsoni S, Maffei L, Berardi N & Cattaneo A. (2005). Intranasal administration of nerve growth factor (NGF) rescues recognition memory deficits in AD11 anti-NGF transgenic mice. *Proceedings of the National Academy of Sciences of the United States of*

America **102,** 3811-3816.

Della-Bianca V, Rossi F, Armato U, Dal-Pra I, Costantini C, Perini G, Politi V & Della Valle G. (2001). Neurotrophin p75 receptor is involved in neuronal damage by prion peptide-(106-126). *The Journal of biological chemistry* **276,** 38929-38933.

Dobrowsky RT, Werner MH, Castellino AM, Chao MV & Hannun YA. (1994). Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. *Science* **265,** 1596-1599.

Dolphin AC, Errington ML & Bliss TV. (1982). Long-term potentiation of the perforant path in vivo is associated with increased glutamate release. *Nature* **297,** 496-498.

Dowling P, Ming X, Raval S, Husar W, Casaccia-Bonnefil P, Chao M, Cook S & Blumberg B. (1999). Up-regulated p75NTR neurotrophin receptor on glial cells in MS plaques. *Neurology* **53,** 1676-1682.

Duffy C, Teyler TJ & Shashoua VE. (1981). Long-term potentiation in the hippocampal slice: evidence for stimulated secretion of newly synthesized proteins. *Science* **212,** 1148-1151.

Encinas M, Iglesias M, Llecha N & Comella JX. (1999). Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. *Journal of neurochemistry* **73,** 1409-1421.

English JD & Sweatt JD. (1997). A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. *The Journal of biological chemistry* **272,** 19103-19106.

Ennaceur A & Delacour J. (1988). A new one-trial test for neurobiological studies

of memory in rats. 1: Behavioral data. *Behavioural brain research* **31,** 47-59.

Epa WR, Markovska K & Barrett GL. (2004). The p75 neurotrophin receptor enhances TrkA signalling by binding to Shc and augmenting its phosphorylation. *Journal of neurochemistry* **89,** 344-353.

Eriksdotter Jonhagen M, Nordberg A, Amberla K, Backman L, Ebendal T, Meyerson B, Olson L, Seiger, Shigeta M, Theodorsson E, Viitanen M, Winblad B & Wahlund LO. (1998). Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. *Dementia and geriatric cognitive disorders* **9,** 246-257.

Ernfors P, Henschen A, Olson L & Persson H. (1989). Expression of nerve growth factor receptor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motoneurons. *Neuron* **2,** 1605-1613.

Ernfors P, Ibanez CF, Ebendal T, Olson L & Persson H. (1990). Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. *Proceedings of the National Academy of Sciences of the United States of America* **87,** 5454-5458.

Errington ML, Lynch MA & Bliss TV. (1987). Long-term potentiation in the dentate gyrus: induction and increased glutamate release are blocked by D(-)aminophosphonovalerate. *Neuroscience* **20,** 279-284.

Evans RH, Francis AA & Watkins JC. (1977). Selective antagonism by Mg2+ of amino acid-induced depolarization of spinal neurones. *Experientia* **33,** 489-491.

Fahnestock M, Yu G, Michalski B, Mathew S, Colquhoun A, Ross GM & Coughlin MD. (2004). The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor.

Journal of neurochemistry **89,** 581-592.

Fazeli MS, Breen K, Errington ML & Bliss TV. (1994). Increase in extracellular NCAM and amyloid precursor protein following induction of long-term potentiation in the dentate gyrus of anaesthetized rats. *Neuroscience letters* **169,** 77-80.

Ferri CC, Moore FA & Bisby MA. (1998). Effects of facial nerve injury on mouse motoneurons lacking the p75 low-affinity neurotrophin receptor. *Journal of neurobiology* **34,** 1-9.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T & Lu B. (1996). Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. *Nature* **381,** 706-709.

Frade JM, Bovolenta P, Martinez-Morales JR, Arribas A, Barbas JA & Rodriguez-Tebar A. (1997). Control of early cell death by BDNF in the chick retina. *Development (Cambridge, England)* **124,** 3313-3320.

Frade JM, Rodriguez-Tebar A & Barde YA. (1996). Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature* **383,** 166-168.

Francia N, Cirulli F, Chiarotti F, Antonelli A, Aloe L & Alleva E. (2006). Spatial memory deficits in middle-aged mice correlate with lower exploratory activity and a subordinate status: role of hippocampal neurotrophins. *The European journal of neuroscience* **23,** 711-728.

Frankland PW, O'Brien C, Ohno M, Kirkwood A & Silva AJ. (2001). Alpha-CaMKII-dependent plasticity in the cortex is required for permanent memory. *Nature* **411,** 309-313.

Friedman WJ. (2000). Neurotrophins induce death of hippocampal neurons via the p75 receptor. *The Journal of Neuroscience* **20,** 6340-6346.

Gadient RA, Cron KC & Otten U. (1990). Interleukin-1 beta and tumor necrosis factor-alpha synergistically stimulate nerve growth factor (NGF) release from cultured rat astrocytes. *Neuroscience letters* **117,** 335-340.

Gentry JJ, Casaccia-Bonnefil P & Carter BD. (2000). Nerve growth factor activation of nuclear factor kappaB through its p75 receptor is an anti-apoptotic signal in RN22 schwannoma cells. *The Journal of biological chemistry* **275,** 7558-7565.

Gentry JJ, Rutkoski NJ, Burke TL & Carter BD. (2004). A functional interaction between the p75 neurotrophin receptor interacting factors, TRAF6 and NRIF. *The Journal of biological chemistry* **279,** 16646-16656.

Giese KP, Fedorov NB, Filipkowski RK & Silva AJ. (1998). Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. *Science* **279,** 870-873.

Ginsberg SD, Che S, Wuu J, Counts SE & Mufson EJ. (2006). Down regulation of trk but not p75NTR gene expression in single cholinergic basal forebrain neurons mark the progression of Alzheimer's disease. *Journal of neurochemistry* **97,** 475-487.

Gobbo OL & O'Mara SM. (2004). Post-treatment, but not pre-treatment, with the selective cyclooxygenase-2 inhibitor celecoxib markedly enhances functional recovery from kainic acid-induced neurodegeneration. *Neuroscience* **125,** 317-327.

Gobbo OL & O'Mara SM. (2005). Exercise, but not environmental enrichment, improves learning after kainic acid-induced hippocampal neurodegeneration in association with an increase in brain-derived neurotrophic factor. *Behavioural brain research* **159,** 21-26.

Gooney M & Lynch MA. (2001). Long-term potentiation in the dentate gyrus of the

rat hippocampus is accompanied by brain-derived neurotrophic factor-induced activation of TrkB. *Journal of neurochemistry* **77,** 1198-1207.

Gorski JA, Balogh SA, Wehner JM & Jones KR. (2003). Learning deficits in forebrain-restricted brain-derived neurotrophic factor mutant mice. *Neuroscience* **121,** 341-354.

Greferath U, Bennie A, Kourakis A & Barrett GL. (2000). Impaired spatial learning in aged rats is associated with loss of p75-positive neurons in the basal forebrain. *Neuroscience* **100,** 363-373.

Greferath U, Bennie A, Kourakis A, Bartlett PF, Murphy M & Barrett GL. (2000). Enlarged cholinergic forebrain neurons and improved spatial learning in p75 knockout mice. *The European journal of neuroscience* **12,** 885-893.

Greferath U, Mallard C, Roufail E, Rees SM, Barrett GL & Bartlett PF. (2002). Expression of the p75 neurotrophin receptor by striatal cholinergic neurons following global ischemia in rats is associated with neuronal degeneration. *Neuroscience letters* **332,** 57-60.

Grover LM & Teyler TJ. (1990). Differential effects of NMDA receptor antagonist APV on tetanic stimulation induced and calcium induced potentiation. *Neuroscience letters* **113,** 309-314.

Guan Z & Fang J. (2006). Peripheral immune activation by lipopolysaccharide decreases neurotrophins in the cortex and hippocampus in rats. *Brain, behavior, and immunity* **20,** 64-71.

Hamanoue M, Middleton G, Wyatt S, Jaffray E, Hay RT & Davies AM. (1999). p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. *Molecular and cellular neurosciences* **14,** 28-40.

Hantzopoulos PA, Suri C, Glass DJ, Goldfarb MP & Yancopoulos GD. (1994). The

low affinity NGF receptor, p75, can collaborate with each of the Trks to potentiate functional responses to the neurotrophins. *Neuron* **13,** 187-201.

Harrington AW, Kim JY & Yoon SO. (2002). Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. *The Journal of Neuroscience* **22,** 156-166.

Harrington AW, Leiner B, Blechschmitt C, Arevalo JC, Lee R, Morl K, Meyer M, Hempstead BL, Yoon SO & Giehl KM. (2004). Secreted proNGF is a pathophysiological death-inducing ligand after adult CNS injury. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 6226-6230.

Hauss-Wegrzyniak B, Lynch MA, Vraniak PD & Wenk GL. (2002). Chronic brain inflammation results in cell loss in the entorhinal cortex and impaired LTP in perforant path-granule cell synapses. *Experimental neurology* **176,** 336-341.

Hauss-Wegrzyniak B, Vannucchi MG & Wenk GL. (2000). Behavioral and ultrastructural changes induced by chronic neuroinflammation in young rats. *Brain research* **859,** 157-166.

Hauss-Wegrzyniak B, Vraniak PD & Wenk GL. (2000). LPS-induced neuroinflammatory effects do not recover with time. *Neuroreport* **11,** 1759-1763.

Hebb CO & Konzett H. (1949). The effect of certain analgesic drugs on synaptic transmission as observed in the perfused superior cervical ganglion of the cat. *Quarterly journal of experimental physiology and cognate medical sciences* **35,** 213-217.

Heinrich G & Lum T. (2000). Fish neurotrophins and Trk receptors. *Int J Dev Neurosci* **18,** 1-27.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF & Chao MV. (1991). High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature* **350,** 678-683.

Herron CE, Lester RA, Coan EJ & Collingridge GL. (1985). Intracellular demonstration of an N-methyl-D-aspartate receptor mediated component of synaptic transmission in the rat hippocampus. *Neuroscience letters* **60,** 19-23.

Hetman M, Kanning K, Cavanaugh JE & Xia Z. (1999). Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *The Journal of biological chemistry* **274,** 22569-22580.

Hollmann M & Heinemann S. (1994). Cloned glutamate receptors. *Annual review of neuroscience* **17,** 31-108.

Holmes GL, Yang Y, Liu Z, Cermak JM, Sarkisian MR, Stafstrom CE, Neill JC & Blusztajn JK. (2002). Seizure-induced memory impairment is reduced by choline supplementation before or after status epilepticus. *Epilepsy research* **48,** 3-13.

Huettner JE. (2003). Kainate receptors and synaptic transmission. *Progress in neurobiology* **70,** 387-407.

Isaac JT, Nicoll RA & Malenka RC. (1995). Evidence for silent synapses: implications for the expression of LTP. *Neuron* **15,** 427-434.

Jia Z, Lu Y, Henderson J, Taverna F, Romano C, Abramow-Newerly W, Wojtowicz JM & Roder J. (1998). Selective abolition of the NMDA component of long-term potentiation in mice lacking mGluR5. *Learning & memory (Cold Spring Harbor, NY* **5,** 331-343.

Jiang B, Akaneya Y, Hata Y & Tsumoto T. (2003). Long-term depression is not

induced by low-frequency stimulation in rat visual cortex in vivo: a possible preventing role of endogenous brain-derived neurotrophic factor. *The Journal of Neuroscience* **23,** 3761-3770.

Jiang Y, Zhang JS & Jakobsen J. (2005). Differential effect of p75 neurotrophin receptor on expression of pro-apoptotic proteins c-jun, p38 and caspase-3 in dorsal root ganglion cells after axotomy in experimental diabetes. *Neuroscience* **132,** 1083-1092.

Johnson H, Hokfelt T & Ulfhake B. (1999). Expression of p75(NTR), trkB and trkC in nonmanipulated and axotomized motoneurons of aged rats. *Brain Res Mol Brain Res* **69,** 21-34.

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

Kang H, Welcher AA, Shelton D & Schuman EM. (1997). Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. *Neuron* **19,** 653-664.

Kang HJ & Schuman EM. (1995). Neurotrophin-induced modulation of synaptic transmission in the adult hippocampus. *Journal of physiology, Paris* **89,** 11-22.

Karege F, Perret G, Bondolfi G, Schwald M, Bertschy G & Aubry JM. (2002). Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psychiatry research* **109,** 143-148.

Katoh-Semba R, Takeuchi IK, Inaguma Y, Ito H & Kato K. (1999). Brain-derived neurotrophic factor, nerve growth and neurotrophin-3 selected regions of the rat brain following kainic acid-induced seizure activity. *Neuroscience research* **35,** 19-29.

Kauer JA, Malenka RC & Nicoll RA. (1988). A persistent postsynaptic

modification mediates long-term potentiation in the hippocampus. *Neuron* **1,** 911-917.

Kelly A, Conroy S & Lynch MA. (1998). Evidence that nerve growth factor plays a role in long-term potentiation in the rat dentate gyrus. *Neuropharmacology* **37,** 561-570.

Kelly A, Vereker E, Nolan Y, Brady M, Barry C, Loscher CE, Mills KH & Lynch MA. (2003). Activation of p38 plays a pivotal role in the inhibitory effect of lipopolysaccharide and interleukin-1 beta on long term potentiation in rat dentate gyrus. *The Journal of biological chemistry* **278,** 19453-19462.

Kerwin JM, Morris CM, Perry RH & Perry EK. (1992). Hippocampal nerve growth factor receptor immunoreactivity in patients with Alzheimer's and Parkinson's disease. *Neuroscience letters* **143,** 101-104.

Khursigara G, Bertin J, Yano H, Moffett H, DiStefano PS & Chao MV. (2001). A prosurvival function for the p75 receptor death domain mediated via the caspase recruitment domain receptor-interacting protein 2. *The Journal of Neuroscience* **21,** 5854-5863.

Kong H, Boulter J, Weber JL, Lai C & Chao MV. (2001). An evolutionarily conserved transmembrane protein that is a novel downstream target of neurotrophin and ephrin receptors. *The Journal of Neuroscience* **21,** 176-185.

Korte M, Staiger V, Griesbeck O, Thoenen H & Bonhoeffer T. (1996). The involvement of brain-derived neurotrophic factor in hippocampal long-term potentiation revealed by gene targeting experiments. *Journal of physiology, Paris* **90,** 157-164.

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

Kume T, Nishikawa H, Tomioka H, Katsuki H, Akaike A, Kaneko S, Maeda T, Kihara T & Shimohama S. (2000). p75-mediated neuroprotection by NGF against glutamate cytotoxicity in cortical cultures. *Brain research* **852,** 279-289.

Lahteinen S, Pitkanen A, Knuuttila J, Toronen P & Castren E. (2004). Brain-derived neurotrophic factor signaling modifies hippocampal gene expression during epileptogenesis in transgenic mice. *The European journal of neuroscience* **19,** 3245-3254.

Lapchak PA, Araujo DM & Hefti F. (1993). Systemic interleukin-1 beta decreases brain-derived neurotrophic factor messenger RNA expression in the rat hippocampal formation. *Neuroscience* **53,** 297-301.

Lee R, Kermani P, Teng KK & Hempstead BL. (2001). Regulation of cell survival by secreted proneurotrophins. *Science* **294,** 1945-1948.

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

Levi-Montalcini R & Hamburger V. (1951). Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *The Journal of experimental zoology* **116,** 321-361.

Levi-Montalcini R, Meyer H & Hamburger V. (1954). In vitro experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. *Cancer research* **14,** 49-57.

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

Liao D, Scannevin RH & Huganir R. (2001). Activation of silent synapses by rapid activity-dependent synaptic recruitment of AMPA receptors. *The Journal of Neuroscience* **21,** 6008-6017.

Linggi MS, Burke TL, Williams BB, Harrington A, Kraemer R, Hempstead BL, Yoon SO & Carter BD. (2005). Neurotrophin receptor interacting factor (NRIF) is an essential mediator of apoptotic signaling by the p75 neurotrophin receptor. *The Journal of biological chemistry* **280,** 13801-13808.

Linnarsson S, Bjorklund A & Ernfors P. (1997). Learning deficit in BDNF mutant mice. *The European journal of neuroscience* **9,** 2581-2587.

Lledo PM, Hjelmstad GO, Mukherji S, Soderling TR, Malenka RC & Nicoll RA. (1995). Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism. *Proceedings of the National Academy of Sciences of the United States of America* **92,** 11175-11179.

Loeb DM, Tsao H, Cobb MH & Greene LA. (1992). NGF and other growth factors induce an association between ERK1 and the NGF receptor, gp140prototrk. *Neuron* **9,** 1053-1065.

Lohof AM, Ip NY & Poo MM. (1993). Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. *Nature* **363,** 350-353.

Lowry KS, Murray SS, Coulson EJ, Epa R, Bartlett PF, Barrett G & Cheema SS. (2001). Systemic administration of antisense p75(NTR) oligodeoxynucleotides rescues axotomised spinal motor neurons. *Journal of neuroscience research* **64,** 11-17.

Lu W, Man H, Ju W, Trimble WS, MacDonald JF & Wang YT. (2001). Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron* **29,** 243-254.

Luscher C, Xia H, Beattie EC, Carroll RC, von Zastrow M, Malenka RC & Nicoll RA. (1999). Role of AMPA receptor cycling in synaptic transmission and plasticity. *Neuron* **24,** 649-658.

Lynch AM, Walsh C, Delaney A, Nolan Y, Campbell VA & Lynch MA. (2004). Lipopolysaccharide-induced increase in signalling in hippocampus is abrogated by IL-10--a role for IL-1 beta? *Journal of neurochemistry* **88,** 635-646.

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

MacDermott AB, Mayer ML, Westbrook GL, Smith SJ & Barker JL. (1986). NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. *Nature* **321,** 519-522.

MacPhee IJ & Barker PA. (1997). Brain-derived neurotrophic factor binding to the p75 neurotrophin receptor reduces TrkA signaling while increasing serine phosphorylation in the TrkA intracellular domain. *The Journal of biological chemistry* **272,** 23547-23551.

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

Mahadeo D, Kaplan L, Chao MV & Hempstead BL. (1994). High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. *The Journal of biological chemistry* **269,** 6884-6891.

Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA & Waxham MN. (1989). An essential role for postsynaptic calmodulin and protein

kinase activity in long-term potentiation. *Nature* **340,** 554-557.

Malenka RC, Kauer JA, Zucker RS & Nicoll RA. (1988). Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. *Science* **242,** 81-84.

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

Mamidipudi V, Li X & Wooten MW. (2002). Identification of interleukin 1 receptor-associated kinase as a conserved component in the p75-neurotrophin receptor activation of nuclear factor-kappa B. *The Journal of biological chemistry* **277,** 28010-28018.

Mamidipudi V & Wooten MW. (2002). Dual role for p75(NTR) signaling in survival and cell death: can intracellular mediators provide an explanation? *Journal of neuroscience research* **68,** 373-384.

Martin-Zanca D, Oskam R, Mitra G, Copeland T & Barbacid M. (1989). Molecular and biochemical characterization of the human trk proto-oncogene. *Molecular and cellular biology* **9,** 24-33.

Massa SM, Xie Y, Yang T, Harrington AW, Kim ML, Yoon SO, Kraemer R, Moore LA, Hempstead BL & Longo FM. (2006). Small, nonpeptide p75NTR ligands induce survival signaling and inhibit proNGF-induced death. *The Journal of Neuroscience* **26,** 5288-5300.

McGahon B, Maguire C, Kelly A & Lynch MA. (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 & Rowan M. (1991). Trans-ACPD enhances long-term potentiation in the hippocampus. *European journal of pharmacology* **197,**

231-232.

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

Minichiello L, Korte M, Wolfer D, Kuhn R, Unsicker K, Cestari V, Rossi-Arnaud C, Lipp HP, Bonhoeffer T & Klein R. (1999). Essential role for TrkB receptors in hippocampus-mediated learning. *Neuron* **24,** 401-414.

Monaghan DT, McMills MC, Chamberlin AR & Cotman CW. (1983). Synthesis of [3H]2-amino-4-phosphonobutyric acid and characterization of its binding to rat brain membranes: a selective ligand for the chloride/calcium-dependent class of L-glutamate binding sites. *Brain research* **278,** 137-144.

Morris RG, Garrud P, Rawlins JN & O'Keefe J. (1982). Place navigation impaired in rats with hippocampal lesions. *Nature* **297,** 681-683.

Moser KV, Reindl M, Blasig I & Humpel C. (2004). Brain capillary endothelial cells proliferate in response to NGF, express NGF receptors and secrete NGF after inflammation. *Brain research* **1017,** 53-60.

Muller D & Lynch G. (1988). Long-term potentiation differentially affects two components of synaptic responses in hippocampus. *Proceedings of the National Academy of Sciences of the United States of America* **85,** 9346-9350.

Nassenstein C, Dawbarn D, Pollock K, Allen SJ, Erpenbeck VJ, Spies E, Krug N & Braun A. (2006). Pulmonary distribution, regulation, and functional role of Trk receptors in a murine model of asthma. *The Journal of allergy and clinical immunology* **118,** 597-605.

Nguyen PV, Abel T & Kandel ER. (1994). Requirement of a critical period of transcription for induction of a late phase of LTP. *Science* **265,** 1104-1107.

Nishimune A, Isaac JT, Molnar E, Noel J, Nash SR, Tagaya M, Collingridge GL, Nakanishi S & Henley JM. (1998). NSF binding to GluR2 regulates synaptic transmission. *Neuron* **21,** 87-97.

Nolan Y, Martin D, Campbell VA & Lynch MA. (2004). Evidence of a protective effect of phosphatidylserine-containing liposomes on lipopolysaccharide-induced impairment of long-term potentiation in the rat hippocampus. *Journal of neuroimmunology* **151,** 12-23.

Nowak L, Bregestovski P, Ascher P, Herbet A & Prochiantz A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature* **307,** 462-465.

Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL & Petersen CM. (2004). Sortilin is essential for proNGF-induced neuronal cell death. *Nature* **427,** 843-848.

Oh JD, Chartisathian K, Chase TN & Butcher LL. (2000). Overexpression of neurotrophin receptor p75 contributes to the excitotoxin-induced cholinergic neuronal death in rat basal forebrain. *Brain research* **853,** 174-185.

Okuno S, Saito A, Hayashi T & Chan PH. (2004). The c-Jun N-terminal protein kinase signaling pathway mediates Bax activation and subsequent neuronal apoptosis through interaction with Bim after transient focal cerebral ischemia. *The Journal of Neuroscience* **24,** 7879-7887.

Olney JW, Rhee V & Ho OL. (1974). Kainic acid: a powerful neurotoxic analogue of glutamate. *Brain research* **77,** 507-512.

Otani S, Roisin-Lallemand MP & Ben-Ari Y. (1992). Enhancement of extracellular protein concentrations during long-term potentiation in the rat hippocampal slice. *Neuroscience* **47,** 265-272.

Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL & Lu B. (2004). Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science* **306,** 487-491.

Patapoutian A & Reichardt LF. (2001) Trk receptors: mediators of neurotrophin action. *Current Opinion in Neurobiology* **11,** 272-280

Park JA, Lee JY, Sato TA & Koh JY. (2000). Co-induction of p75NTR and p75NTR-associated death executor in neurons after zinc exposure in cortical culture or transient ischemia in the rat. *The Journal of Neuroscience* **20,** 9096-9103.

Patterson SL, Pittenger C, Morozov A, Martin KC, Scanlin H, Drake C & Kandel ER. (2001). Some forms of cAMP-mediated long-lasting potentiation are associated with release of BDNF and nuclear translocation of phospho-MAP kinase. *Neuron* **32,** 123-140.

Pedraza CE, Podlesniy P, Vidal N, Arevalo JC, Lee R, Hempstead B, Ferrer I, Iglesias M & Espinet C. (2005). Pro-NGF isolated from the human brain affected by Alzheimer's disease induces neuronal apoptosis mediated by p75NTR. *The American journal of pathology* **166,** 533-543.

Peng S, Wuu J, Mufson EJ & Fahnestock M. (2004). Increased proNGF levels in subjects with mild cognitive impairment and mild Alzheimer disease. *Journal of neuropathology and experimental neurology* **63,** 641-649.

Perini G, Della-Bianca V, Politi V, Della Valle G, Dal-Pra I, Rossi F & Armato U. (2002). Role of p75 neurotrophin receptor in the neurotoxicity by beta-amyloid peptides and synergistic effect of inflammatory cytokines. *The Journal of experimental medicine* **195,** 907-918.

Poltorak A, Smirnova I, He X, Liu MY, Van Huffel C, McNally O, Birdwell D, Alejos E, Silva M, Du X, Thompson P, Chan EK, Ledesma J, Roe B, Clifton S, Vogel SN & Beutler B. (1998). Genetic and physical mapping of the Lps

locus: identification of the toll-4 receptor as a candidate gene in the critical region. *Blood cells, molecules & diseases* **24,** 340-355.

Poucet B, Chapuis, N., Dump, M. & Thinus-Blanc, C. (1986). A study of exploratory behavior as an index of spatial knowledge in hamsters. *Learning & Behavior* **14,** 93-100.

Poucet B. (1989). Object Exploration, Habituation, and Response to a Spatial Change in Rats Following Septal or Medial Frontal Cortical Damage. *Behavioral Neuroscience* **103**.

Purves D., Augustine G.J., Fitzpatrick D, Katz, L.C., LaMantia A. & McNamara J.O. (2001) Neuroscience, 2nd Edition, Sunderland (MA)

Rabizadeh S, Oh J, Zhong LT, Yang J, Bitler CM, Butcher LL & Bredesen DE. (1993). Induction of apoptosis by the low-affinity NGF receptor. *Science* **261,** 345-348.

Rankin SL, Guy CS & Mearow KM. (2005). TrkA NGF receptor plays a role in the modulation of p75NTR expression. *Neuroscience letters* **383,** 305-310.

Rankine EL, Hughes PM, Botham MS, Perry VH & Felton LM. (2006). Brain cytokine synthesis induced by an intraparenchymal injection of LPS is reduced in MCP-1-deficient mice prior to leucocyte recruitment. *The European journal of neuroscience* **24,** 77-86.

Rattenholl A, Ruoppolo M, Flagiello A, Monti M, Vinci F, Marino G, Lilie H, Schwarz E & Rudolph R. (2001). Pro-sequence assisted folding and disulfide bond formation of human nerve growth factor. *Journal of molecular biology* **305,** 523-533.

Regehr WG & Tank DW. (1990). Postsynaptic NMDA receptor-mediated calcium accumulation in hippocampal CA1 pyramidal cell dendrites. *Nature* **345,** 807-810.

Revuelta M, Castano A, Machado A, Cano J & Venero JL. (2005). Kainate-induced zinc translocation from presynaptic terminals causes neuronal and astroglial cell death and mRNA loss of BDNF receptors in the hippocampal formation and amygdala. *Journal of neuroscience research* **82,** 184-195.

Rodriguez-Tebar A, Dechant G & Barde YA. (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron* **4,** 487-492.

Rosch H, Schweigreiter R, Bonhoeffer T, Barde YA & Korte M. (2005). The neurotrophin receptor p75NTR modulates long-term depression and regulates the expression of AMPA receptor subunits in the hippocampus. *Proceedings of the National Academy of Sciences of the United States of America* **102,** 7362-7367.

Rosen LB, Ginty DD, Weber MJ & Greenberg ME. (1994). Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron* **12,** 1207-1221.

Roux PP, & Barker PA. (2002). Neurotrophin signaling through the p75 neurotrophin receptor. *Progress in Neurobiology* 67, 203-233.

Roux PP, Bhakar AL, Kennedy TE & Barker PA. (2001). The p75 neurotrophin receptor activates Akt (protein kinase B) through a phosphatidylinositol 3-kinase-dependent pathway. *The Journal of biological chemistry* **276,** 23097-23104.

Roux PP, Colicos MA, Barker PA & Kennedy TE. (1999). p75 neurotrophin receptor expression is induced in apoptotic neurons after seizure. *The Journal of Neuroscience* **19,** 6887-6896.

Saarelainen T, Hendolin P, Lucas G, Koponen E, Sairanen M, MacDonald E, Agerman K, Haapasalo A, Nawa H, Aloyz R, Ernfors P & Castren E. (2003). Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral

effects. *The Journal of Neuroscience* **23,** 349-357.

Scali C, Casamenti F, Pazzagli M, Bartolini L & Pepeu G. (1994). Nerve growth factor increases extracellular acetylcholine levels in the parietal cortex and hippocampus of aged rats and restores object recognition. *Neuroscience letters* **170,** 117-120.

Schweigreiter R, Walmsley AR, Niederost B, Zimmermann DR, Oertle T, Casademunt E, Frentzel S, Dechant G, Mir A & Bandtlow CE. (2004). Versican V2 and the central inhibitory domain of Nogo-A inhibit neurite growth via p75NTR/NgR-independent pathways that converge at RhoA. *Molecular and cellular neurosciences* **27,** 163-174.

Scoville WB & Milner B. (2000). Loss of recent memory after bilateral hippocampal lesions. 1957. *The Journal of neuropsychiatry and clinical neurosciences* **12,** 103-113.

Shaw KN, Commins S & O'Mara SM. (2001). Lipopolysaccharide causes deficits in spatial learning in the watermaze but not in BDNF expression in the rat dentate gyrus. *Behavioural brain research* **124,** 47-54.

Shaw KN, Commins S & O'Mara SM. (2005). Cyclooxygenase inhibition attenuates endotoxin-induced spatial learning deficits, but not an endotoxin-induced blockade of long-term potentiation. *Brain research* **1038,** 231-237.

Shetty AK, Rao MS, Hattiangady B, Zaman V & Shetty GA. (2004). Hippocampal neurotrophin levels after injury: Relationship to the age of the hippocampus at the time of injury. *Journal of neuroscience research* **78,** 520-532.

Shetty AK, Zaman V & Shetty GA. (2003). Hippocampal neurotrophin levels in a kainate model of temporal lobe epilepsy: a lack of correlation between brain-derived neurotrophic factor content and progression of aberrant dentate mossy fiber sprouting. *Journal of neurochemistry* **87,** 147-159.

Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K & Kimoto M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *The Journal of experimental medicine* **189,** 1777-1782.

Song I, Kamboj S, Xia J, Dong H, Liao D & Huganir RL. (1998). Interaction of the N-ethylmaleimide-sensitive factor with AMPA receptors. *Neuron* **21,** 393-400.

Suter U, Heymach JV, Jr. & Shooter EM. (1991). Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. *The EMBO journal* **10,** 2395-2400.

Sutter A, Riopelle RJ, Harris-Warrick RM & Shooter EM. (1979). The heterogeneity of nerve growth factor receptors. *Progress in clinical and biological research* **31,** 659-667.

Sutter A, Riopelle RJ, Harris-Warrick RM & Shooter EM. (1979). Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. *The Journal of biological chemistry* **254,** 5972-5982.

Tanaka S, Ide M, Shibutani T, Ohtaki H, Numazawa S, Shioda S & Yoshida T. (2006). Lipopolysaccharide-induced microglial activation induces learning and memory deficits without neuronal cell death in rats. *Journal of neuroscience research* **83,** 557-566.

Tasker RA, Bernard PB, Doucette TA, Kerr DS, Zabidin Y, Alvarez-Fernandez L, Fernandez-Maroto B, Fernandez-Sanchez MT & Novelli A. (2002). Comparison of the in vitro and in vivo neurotoxicity of three new sources of kainic acid. *Amino acids* **23,** 45-54.

Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen ZY, Lee FS, Kraemer RT, Nykjaer A & Hempstead BL. (2005). ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. *The Journal of Neuroscience* **25,** 5455-5463.

Thinus-Blanc B, L., Chaix, K., Chapuis, N. & Durup, M. (1987). A Study of Spatial Parameters Encoded During Exploration in Hamsters. *Journal of Experimental Psychology: Animal Behavior Processes* **13,** 418-427.

Tobias PS, Soldau K & Ulevitch RJ. (1986). Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *The Journal of experimental medicine* **164,** 777-793.

Torcia M, Bracci-Laudiero L, Lucibello M, Nencioni L, Labardi D, Rubartelli A, Cozzolino F, Aloe L & Garaci E. (1996). Nerve growth factor is an autocrine survival factor for memory B lymphocytes. *Cell* **85,** 345-356.

Tremblay E & Ben-Ari Y. (1984). Usefulness of parenteral kainic acid as a model of temporal lobe epilepsy. *Revue d'electroencephalographie et de neurophysiologie clinique* **14,** 241-246.

Troy CM, Friedman JE & Friedman WJ. (2002). Mechanisms of p75-mediated death of hippocampal neurons. Role of caspases. *The Journal of biological chemistry* **277,** 34295-34302.

Tuffereau C, Benejean J, Blondel D, Kieffer B & Flamand A. (1998). Low-affinity nerve-growth factor receptor (P75NTR) can serve as a receptor for rabies virus. *The EMBO journal* **17,** 7250-7259.

Turner BJ, Cheah IK, Macfarlane KJ, Lopes EC, Petratos S, Langford SJ & Cheema SS. (2003). Antisense peptide nucleic acid-mediated knockdown of the p75 neurotrophin receptor delays motor neuron disease in mutant SOD1 transgenic mice. *Journal of neurochemistry* **87,** 752-763.

Vereker E, Campbell V, Roche E, McEntee E & Lynch MA. (2000). Lipopolysaccharide inhibits long term potentiation in the rat dentate gyrus by activating caspase-1. *The Journal of biological chemistry* **275,** 26252-26258.

Vignes M, Bleakman D, Lodge D & Collingridge GL. (1997). The synaptic activation of the GluR5 subtype of kainate receptor in area CA3 of the rat hippocampus. *Neuropharmacology* **36,** 1477-1481.

Vignes M & Collingridge GL. (1997). The synaptic activation of kainate receptors. *Nature* **388,** 179-182.

Volosin M, Song W, Almeida RD, Kaplan DR, Hempstead BL & Friedman WJ. (2006). Interaction of survival and death signaling in basal forebrain neurons: roles of neurotrophins and proneurotrophins. *The Journal of Neuroscience* **26,** 7756-7766.

Wang KC, Kim JA, Sivasankaran R, Segal R & He Z. (2002). P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature* **420,** 74-78.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL & He Z. (2002). Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* **417,** 941-944.

Watt JA & Paden CM. (2001). Upregulation of the p75 low-affinity neurotrophin receptor by phagocytically active perivascular active cells in the rat neural lobe. *Cell and tissue research* **303,** 81-91.

Weeraratna AT, Dalrymple SL, Lamb JC, Denmeade SR, Miknyoczki S, Dionne CA & Isaacs JT. (2001). Pan-trk inhibition decreases metastasis and enhances host survival in experimental models as a result of its selective induction of apoptosis of prostate cancer cells. *Clin Cancer Res* **7,** 2237-2245.

Wiley RG, Berbos TG, Deckwerth TL, Johnson EM, Jr. & Lappi DA. (1995). Destruction of the cholinergic basal forebrain using immunotoxin to rat NGF receptor: modeling the cholinergic degeneration of Alzheimer's disease.

Journal of the neurological sciences **128,** 157-166.

Woo NH, Teng HK, Siao CJ, Chiaruttini C, Pang PT, Milner TA, Hempstead BL & Lu B. (2005). Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. *Nature neuroscience* **8,** 1069-1077.

Wright JW, Alt JA, Turner GD & Krueger JM. (2004). Differences in spatial learning comparing transgenic p75 knockout, New Zealand Black, C57BL/6, and Swiss Webster mice. *Behavioural brain research* **153,** 453-458.

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ & Mathison JC. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249,** 1431-1433.

Xia Z, Dickens M, Raingeaud J, Davis RJ & Greenberg ME. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270,** 1326-1331.

Xie CW, Sayah D, Chen QS, Wei WZ, Smith D & Liu X. (2000). Deficient long-term memory and long-lasting long-term potentiation in mice with a targeted deletion of neurotrophin-4 gene. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 8116-8121.

Xu B, Gottschalk W, Chow A, Wilson RI, Schnell E, Zang K, Wang D, Nicoll RA, Lu B & Reichardt LF. (2000). The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB. *The Journal of Neuroscience* **20,** 6888-6897.

Yaar M, Zhai S, Fine RE, Eisenhauer PB, Arble BL, Stewart KB & Gilchrest BA. (2002). Amyloid beta binds trimers as well as monomers of the 75-kDa neurotrophin receptor and activates receptor signaling. *The Journal of*

biological chemistry **277,** 7720-7725.

Yaar M, Zhai S, Pilch PF, Doyle SM, Eisenhauer PB, Fine RE & Gilchrest BA. (1997). Binding of beta-amyloid to the p75 neurotrophin receptor induces apoptosis. A possible mechanism for Alzheimer's disease. *The Journal of clinical investigation* **100,** 2333-2340.

Yan H & Chao MV. (1991). Disruption of cysteine-rich repeats of the p75 nerve growth factor receptor leads to loss of ligand binding. *The Journal of biological chemistry* **266,** 12099-12104.

Yang B, Slonimsky JD & Birren SJ. (2002). A rapid switch in sympathetic neurotransmitter release properties mediated by the p75 receptor. *Nature neuroscience* **5,** 539-545.

Yang DD, Kuan CY, Whitmarsh AJ, Rincon M, Zheng TS, Davis RJ, Rakic P & Flavell RA. (1997). Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature* **389,** 865-870.

Yi JS, Lee SK, Sato TA & Koh JY. (2003). Co-induction of p75(NTR) and the associated death executor NADE in degenerating hippocampal neurons after kainate-induced seizures in the rat. *Neuroscience letters* **347,** 126-130.

Yoon SO, Casaccia-Bonnefil P, Carter B & Chao MV. (1998). Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. *The Journal of Neuroscience* **18,** 3273-3281.

Zafra F, Castren E, Thoenen H & Lindholm D. (1991). Interplay between glutamate and gamma-aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 10037-10041.

Zafra F, Lindholm D, Castren E, Hartikka J & Thoenen H. (1992). Regulation of

brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *The Journal of Neuroscience* **12,** 4793-4799.

VIII Appendix I Suppliers Addresses

Alomone Labs Ltd.
PO Box 4287,
Jerusalem 91042,
Israel.

Alpha Technologies,
The Leinster Technology Centre,
Blessington Industrial Estate,
Co. Wicklow,
Ireland.

Amersham Biosciences UK Ltd.,
Amersham Place,
Little Chalfont,
Buckinghamshire HP7 9NA,
United Kingdom.

Bilaney Consultants Ltd
St Julians,
Sevenoaks,
Kent TN15 0RX,
United Kingdom.

Bio-Rad Laboratories Ltd.,
Bio-Rad House,
Maylands Avenue,
Hemel Hempstead,
Hertfordshire HP2 7TD,
United Kingdom.

Calbiochem-Novabiochem Corp.,
10394 Pacific Center Court,
San Diego,
CA 92121,
USA.

Chemicon International Inc.,
28820 Single Oak Drive,
Temecula,
CA 92590,
USA.

Lennox Laboratory Supplies,
John F. Kennedy Drive,
Naas Road,
Dublin 12,
Ireland.

Pierce Biotechnology Inc.,
3747 N. Meridian Road,
P.O. Box 117,
Rockford,
IL 61105,
USA.

Prestige Dental Products Ltd
7 Oxford Place,
Bradford,
West Yorkshire BD3 0EF,
United Kingdom.

Promed,
Kilorglin,
Co. Kerry,
Ireland.

Santa Cruz Biotechnology Inc.,
2161 Delaware Avenue,
Santa Cruz,
CA 95060,
USA.

Sigma-Aldrich Company Ltd.,
Fancy Road,
Poole,
Dorset BH12 4QH,
United Kingdom.

Upstate Cell Signaling,
290 Concord Road,
Billerica,
MA 01821,
USA

Vector Laboratories Inc.,
30 Ingold Road,
Burlingame,
CA 94010,
USA.

Whatman Plc.

Whatman House,

St. Leonard Road,

20/20 Maidstone,

Kent ME16 0LS,

United Kingdom.

IX Solutions Used

Krebs solution

NaCl	136mM
KCl	2.5mM
KH_2PO_4	1.18mM
$MgSO_4.7H2O$	1.18mM
$NaHCO_3$	16mM
Glucose	10mM
Containing $CaCl_2$	2mM

Phosphate buffered saline (PBS) pH7.4

Na_2HPO_4	80mM
NaH_2PO_4	20mM
NaCl	100mM

Tris buffered saline (TBS) pH7.4

Tris-HCL	20mM
NaCl	150mM

Separating Gel

Bisacrylamide	33% w/v
Tris-HCL	1.5M, pH8.8
SDS	1% w/v
Ammonium persulphate	0.5% w/v
Distilled water	
TEMED	0.1% w/v

Stacking gel

Bisacrylamide	6.5% w/v
Tris-HCL	0.5M, pH6.8
SDS	1% w/v
Ammonium persulphate	0.5% w/v
Distilled water	
TEMED	0.1% w/v

Electrode running buffer

Tris Base	25mM
Glycine	200mM
SDS	17mM

Transfer buffer pH8.3

Tris Base	25mM
Glycine	192mM
Methanol	20% v/v
SDS	0.05% w/v
Distilled water	

Sample buffer

Tris-HCL	0.5M pH6.8
Glycerol	10% v/v
SDS	0.05% w/v
B-mercaptoethanol	5% w/v
Bromophenol blue	0.05% w/v