An investigation of the role of neurotrophins in recognition memory in the rat

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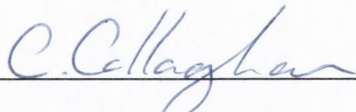
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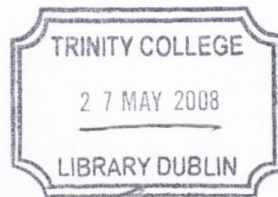
I Declaration

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Charlotte Callaghan

II Abstract

Acquisition and consolidation of newly-acquired information is essential for formation of long-lasting memories, and both these processes may share common signaling pathways. The neurotrophins NGF, BDNF, NT3 and NT4 have diverse functions in the adult brain, including learning and memory. Their biological effects are elicited by binding to Trk receptors and activation of the ERK/MAPK pathway or the PI3K/AKT pathway which is believed to mediate protein synthesis, which has previously been identified as an essential process in memory formation (Kelly *et al.*, 2003). In this study we investigated the roles of neurotrophins in both acquisition and consolidation of recognition memory by using neutralising antibodies against each neurotrophin isoform.

An object recognition task was used to test acquisition and consolidation of memory; rats were tested 10min and 24hr after training to discriminate experimentally between acquisition and consolidation respectively. Rats treated with the pan-Trk antagonist tyrphostin AG879 were unable to discriminate between the novel and the familiar object at both 10min and 24hr when compared with controls, suggesting Trk receptor activation is required for both acquisition and consolidation. In contrast, rats treated with anti-BDNF, anti-NGF and anti-NT4/5 displayed comparable learning to control rats at the 10min timepoint, but displayed learning impairments 24hr later. Treatment with anti-NT3 had no effect on learning at either timepoint.

Next we investigated the role of neurotrophins and their activation of the Trk receptors during memory consolidation and up to 24 hours after consolidation. Here the animals were only trained in day one of the task and were killed at 4 time points following training, immediately (0min), 2hr, 6hr and 24hr post training (n = 6 for each group). The dentate gyrus, hippocampus, perirhinal cortex and entorhinal cortex were removed for analysis. Trk receptor mRNA was analyzed by PCR, Trk receptor protein was assessed by western Immunoblotting and neurotrophin expression was analyzed by ELISA. Analysis of neurotrophin expression revealed the greatest changes at 0min and 2hr post training in the dentate gyrus and the perirhinal cortex.

Having established a role for neurotrophins in recognition memory we examined the role of BDNF release, the ERK/MAPK pathway and the PI3K/AKT signalling

cascade in recognition memory. With use of the object recognition task we found increased ERK phosphorylation immediately after training; this was concomitant with increased release of BDNF in the dentate gyrus and increased PI3K activation in the perirhinal cortex which was accompanied by BDNF release. It was also found that inhibition of Trk receptor activation blocked this increase in ERK and PI3K activation and BDNF release following training in the object recognition task.

The data are consistent with the hypothesis that binding of NGF, BDNF or NT4/5 to their preferred Trk receptor subtype is necessary for consolidation of recognition memory, but that such redundancy does not exist for acquisition, meaning that in the absence of activity of any one of these neurotrophins, learning can still occur. These results further suggest that MAPK/ERK signalling and PI3K/AKT signalling in the medial temporal lobe are involved in consolidation of recognition memory, and that the observed ERK and PI3K activation may be mediated by neurotrophin signalling. Taken together the data strongly suggest a role for the neurotrophin family of proteins in acquisition and consolidation of recognition memory in the rat.

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A special thank you to all who shared room 3.09, what a laugh and I would like to take this opportunity to thank Eoin for being a great friend through our degree, masters and PhDs.

Finally I would like to thank my family for putting up with me over the past few weeks, their support and encouragement has been priceless, Mammy, Daddy, David, Fidelma, Ronan, Darina and Damien, this thesis is dedicated to them.

V Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ANOVA	Analysis of Varience
Arc	Activity-regulated cytoskeleton-associated protein
ATP	Ammonium persulfate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CamK	Calmodulin-dependent protein kinase
CNS	Central nervous system
CREB	c-AMP response element-binding protein
DAB	Diaminobenidine
DAG	Diacylglycerol
DMSO	Dimethyl sulphoxide
DNA	De-oxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
EPSP	Excitatory post-synaptic potential
ERK	Extracellular signal regulated kinase
IkB	Inbititory kappa-B
JNK	C-Jun N-terminal kinase
GABA	Gamma-aminobutyric acid
HRP	Horseradish perioxidase conjugate
KCl	Potassium chloride
LTP	Long-term potentiation
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloprotease
mRNA	messenger ribonucleic acid
NF-kappaB	Nuclear factor-kappa B

NGF	Nerve growth factor
NMDA	N-methyl-D-asparate
NT	Neurotrophin
NTR	Neurotrophin receptor
NT3	Neurotrophin 3
NT4/5	Neurotrophin 4/5
p75	p75 low affinity neurotrophin receptor
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3 kinase
PKC	Protein kinase C
PLC-γ	Phospholipase C-γ
RIP2	Receptor interacting protein 2
proNGF	proneurotrophin NGF
proBDNF	proneurotrophin BDNF
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SDS	Sodium dodecylsulphate
SEM	Standard error of the mean
SOS	Son of sevenless
TRAF	TNF-Receptor-Associated factor
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline-Tween
TMB	3,3' , 5,5' –Tetramethyl-benzidine
Trk	Receptor tyrosine kinase
TrkA	Tyrosine kinase A
TrkB	Tyrosine kinase B
TrkC	Tyrosine kinase C

Chapter 1

General Introduction

1.1 Memory

It is our ability to store and recall information accurately as humans which renders us the most successful animal species, not our size or strength but our ability to learn and remember. Consolidation of long-term memory is a multi-step process comprised of a complex molecular cascade leading to a robust and effective inter-neuronal communication. Storage of information is dependent upon memory, of which there are different stages, the first of which is acquisition. Acquisition of a memory results in the formation of a labile memory trace that is later consolidated. The hypothesis of memory consolidation proposes that these unstable memory traces are converted into stable long-term memory gradually over time (McGaugh, 1966), via molecular and /or structural modifications (Kandel, 2001). Adding to this hypothesis is the theory that memories reconsolidate; this idea was first proposed almost 40 years ago. The recall of a memory seems to place the memory trace back into an active and labile state, from which it is reconsolidated back into an inactive and stable state. It is also at this point where memories can become disrupted by intra-cellular signalling. Molecular cascades triggered by new experiences induce synaptic and cell-wide alterations, among them learning-induced synthesis of new proteins, modulation of gene expression and morphological synaptic remodeling which are ascribed to play a pivitol role in the consolidation process (Flood *et al.*, 1973, Goelet *et al.*, 1986). Research into the underlying signaling mechanisms which are responsible for memory formation has lead to the discovery of the role that calcium movements, NMDA receptors and neurotrophins may play in these processes. Although so far memory is discussed collectively there are many different types of memory including emotional, fear, contextual, taste, odour, recognition, episodic and spatial memory to name but a few. It is possible that for each different type of memory, different proteins are involved in different stages of memory formation.

1.2 Object Recognition Memory

Object recognition can be described as the ability to discriminate the familiarity of previously encountered objects. In 1950, Berlyn 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. Although there are many different types of assays designed to test an animal's ability to learn and remember, such as the radial arm maze, the Morris water maze and fear conditioning tasks, it is the object recognition task developed from the work of Berlyn by Ennaceur and Delacour in 1988 which is a suitable task to test acquisition and consolidation of memory. The object recognition task relies on a rat's innate propensity to explore the novel. When a rat exhibits a preference to explore a new object rather that one it has previously encountered, it can be inferred that the rat has a memory of the familiar and now less-interesting object (Clarke *et al*, 2000). It is not known whether rats recollect or have access to the contextual information, as humans do, and the appropriate paradigm to distinguish between these two kinds of memory does not exist in the rat (Morris *et al*, 2004). The main benefit of the object recognition task is that it can be considered as 'pure' memory paradigm, as there are no incentives for the rat to explore, as the usual positive or negative reinforcers (such as food rewards and foot shocks) are absent from the test, it relies completely on the animal's propensity to explore its surrounding environment. Recognition memory is believed to be differentially processed by areas of the medial temporal lobe such as the hippocampal formation and parahippocampal areas including the perirhinal and entorhinal cortex (Suchan *et al*., 2007, Yonelinas *et al*., 2007, Meunier *et al*., 2003, Cave and Squire, 1991).

1.3 The medial temporal lobe and memory processing

In 1953 William Scoville preformed a surgical procedure on a 27 year old patient who suffered from epilepsy, referred to as H.M., to relieve his symptoms. The procedure, described as "bilateral medial temporal lobe resection was carried out, extending posteriorly for a distance of 8cm from the midpoints of the tips of the temporal lobes, with the temporal horns constituting the lateral edges of resection". In short, Scoville removed two thirds of the hippocampal formation, parahippocampal gyrus and the amygdala with the entire entorhinal cortex being destroyed. Directly following surgery, H.M. lost the ability to retain new memories of facts, to remember individuals he met after his operation and also his sense of hunger and satiety had diminished. HM has been

important not only for the knowledge he has provided about memory impairment and amnesia, but also because his exact brain surgery has allowed a good understanding of how particular areas of the brain may be linked to specific processes hypothesized to occur in memory formation. In this way he has provided vital information about brain pathology, and the results of his surgery has helped form theories of normal memory formation. Specifically this surgery identified the essential role of the hippocampus and sparked a new interest in ascribing functions to distinct areas of the brain. Investigation of the medial temporal lobe soon lead to discoveries such as that by Horel and Misantone in 1976, who found that disruption of the connection between the anterior temporal lobe with dorsal and medial brain regions resulted in monkeys being unable to complete a visual discrimination task. Similarly a study in humans with unilateral frontal or temporal lobe excisions were tested on four self ordered tasks (two verbal and two nonverbal) requiring the organization of a sequence of pointing responses found that patients with left frontal lobe excisions showed impairments in all tasks, those with right frontal lobe excisions showed deficits only on the nonverbal tasks and those with medial temporal lobe excisions showed varied levels of impairments depending on the severity of lesions to the hippocampus (Petrides and Milner, 1982). This information supported the hypothesis that different brain regions perform different functions and with the development of better scientific techniques it became possible to look even closer at the distinct regions of the medial temporal lobe, for instance the hippocampus, entorhinal cortex and the perirhinal cortex and the role they perform in processing memory.
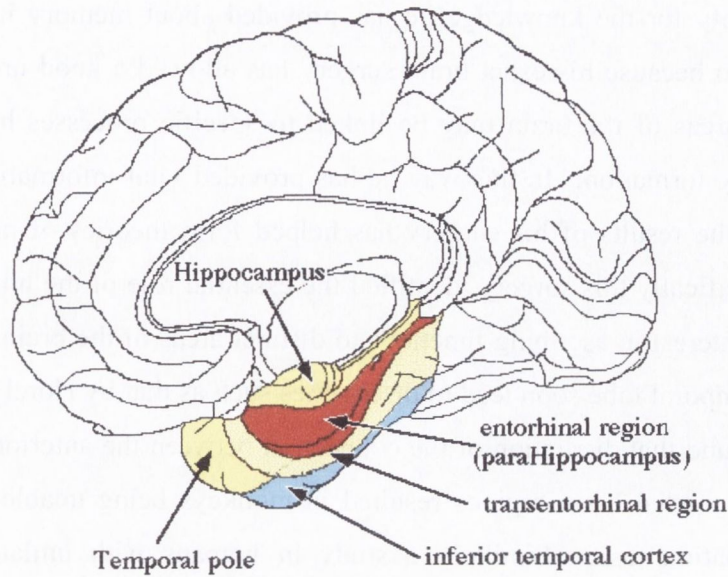
Figure 1.1 Structural layout of the human brain, identifying the temporal lobe, the hippocampus and its associated cortical regions, which are central regions to synaptic plasticity and memory processing.

1.4 The hippocampus and dentate gyrus in memory formation

The hippocampus is a structure found in the subcortical brain and forms part of the limbic system. The anatomist Giulio Cesare Anazari 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 cortex and the entorhinal cortex.

Since the case of HM a new interest in the hippocampus was sparked and scientists began conducting experiments to understand the role this structure plays in memory and learning. One of the first of these studies was by Shapiro and collegues (1965) where the effect of hippocampal ablation on acquisition, retention, and learning-set formation in a simple auditory discrimination task was studied in rhesus monkeys. Results displayed that hippocampal ablation interfered with the retention of discriminations, those learned postsurgically being more affected than those learned presurgically. Successive postsurgical discrimination reversals resulted in the formation

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of discrimination reversal learning sets. It was concluded that while the hippocampus may subserve a facilitatory function in retention, its presence is not an essential condition for retention or for new learning in the rhesus monkey. Interestingly more recent studies in monkeys have shown that destruction of the hippocampus does not produce nearly as great a learning deficit in monkeys as it does in humans, suggesting the hippocampus is more critical for humans than lesser primates (Belcher *et al*, 2006). Since the 1950's numerous reports have implicated the hippocampus in different types of memory such as spatial, contextual, recognition and episodic (Jun *et al*, 1998; Smith and Mizumri, 2006; Dash *et al*, 2002). Neuropsychological experiments in humans have shown that lesions of the hippocampus impair the ability to acquire declarative memories such as word and face recognition (Zola-Morgan *et al*, 1986). Other studies in rats have reported that the hippocampus is also important in fear conditioning tasks, where lesions of the hippocampus have impaired the conditioned fear responses to contextual stimuli (Kim and Fanselow, 1992). What does appear to be certain from previous reports is that damage to the hippocampus indicates that long-term memories are not stored in the hippocampus and therefore its role is both acquisition of new memories and transferrance of these memories to the cerebral cortex for long term storage. However the importance of the hippocampus for non-spatial tasks, including tasks of object recognition memory is not clear (Broadbent *et al*, 2004). It is thought that the dentate gyrus, a sub region of the hippocampus, may be more important in recognition memory (Kelly *et al*, 2003).

The dentate gyrus was the area in which long term potentiation (LTP) was first recorded, the perforant path fibres to the dentate gyrus were stimulated repetitively with extra-cellular micro electrodes in rabbits producing long lasting potentiation (Bliss and Lomo, 1973). This study is at the heart of every experiment investigating the molecular mechanisms and functions of LTP. Recent studies have concentrated on the role of the dentate gyrus in active memory in adults. These studies have found that neurogenesis, producing new neurons which are continuously integrated in neural circuits in the adult dentate gyrus of the mammalian brain and there is accumulating evidence indicating that these new neurons are involved in learning and memory (Kempermann *et al*., 1997, Tashiro *et al*., 2006, Ehninger and Kempermann, 2007). Having established the dentate gyrus as a central organ in memory processing other scientists have concentrated research

on identifying the proteins responsible for enabling neuron-neuron signaling which is believed to form memories (Cho *et al.*, 2007, Mc Hugh *et al.*, 2007).

1.5 The perirhinal cortex in memory processing

Investigation of the perirhinal cortex began in the early eighties with one of the first studies carried out on the hedgehog. It was found that lesions of the perirhinal cortex impaired spatial learning in the hedgehog (Skeen and Masterton, 1982). However it wasn't until the late eighties that Insausti (1987) identified the perirhinal cortex and the parahippocampal cortex as the major source of cortical input to the entorhinal cortex of the hippocampus. Insausti used a retrograde tracer, wheat germ agglutinin, conjugated to horseradish peroxidase and administered it into the associated cortical projections from the entorhinal cortex of the Macaca fascicularis monkey to discover the perirhinal and parahippocampal cortex as the principle route through which information is exchanged between the neocortex and the hippocampal formation (Insausti *et al.*, 1987). This was also found to be the case in humans (Insausti *et al.*, 1995). Based on this theory Zola-Morgan carried out experiments on primates, creating lesions of the perirhinal cortex and analyzing the animals performance in memory tasks similar to those carried out on HM by Scoville and Milner (1953). Zola-Morgan found that the monkeys were as impaired or more so when compared to an earlier studied he carried out with lesions to the hippocampus and amygdala of the primates (Zola-Morgan *et al.*, 1989a, Zola-Morgan *et al.*, 1989b).

In more recent studies the perirhinal cortex has been associated in different types of memory processing including recognition, spatial, verbal and visual (Gilbert *et al* 2003, Winters *et al.*, 2007, Bachevalier and Nemanic., 2007, Lillywhite *et al.*, 2007, Murray *et al.*, 2007). Of particular interest to this thesis, is the debate over the precise role of the perirhinal cortex, some suggest it has a more important function in spatial memory (Bussey *et al*, 1999) where others suggest it is more important than the hippocampus in recognition memory (Brown *et al.*, 1999). Lesions of the perirhinal cortex in both primates and rats disrupt performance on familiarity discrimination tasks with objects (Aggleton and Brown, 1999). The perirhinal cortex also appears to be activated at different stages of recognition memory including encoding, retrieval and

consolidation of object memory. One study showed that blockade of the AMPA receptor in the perirhinal cortex disrupted encoding for short- and long-term memory, as well as retrieval and consolidation. Transient NMDA receptor blockade during encoding also affected only long-term object recognition memory demonstrating the importance of the perirhinal cortex in several stages of memory processing (Winters and Bussey, 2005). A strong role for the perirhinal cortex in synaptic plasticity has been established with both LTP and LTD recorded from this region (Massey *et al.*, 2004). The perirhinal cortex appears to be important in both cognition and synaptic plasticity regardless of the origin of the stimulus.

1.6 The entorhinal cortex in memory processing

The entorhinal cortex, a subregion of the hippocampus has been associated with memory processing since the sixties when Gambetti found that administration of the protein synthesis inhibitor, puromycin, into the entorhinal cortex blocked the ability of mice to perform maze learning tasks for up to 3 months. This study not only identified the entorhinal cortex as a central region involved in memory processing but developed the idea that maintenance of the basic memory trace requires preservation of mRNA formed as a result of a learning experience (Gambetti *et al.*, 1968). A study conducted to assess the influence of ablation of the entorhinal cortex on conditioned reflexes, to present and trace stimuli, found that ablation resulted in the conditioned reflex to time being delayed by five or six times compared with intact animals. This experiment was carried out on both rats and dogs (Mering and Butenko, 1982). Similar to the perirhinal cortex the entorhinal cortex has been shown to express both LTP and LTD (Craig and Commins, 2006), however research in this area does not seem to be focused on types of memory dependent on this region like in the perirhinal cortex.

Research on the entorhinal cortex appears to be focused on the neuroanatomy of the structure and its current expressions linking it to the hippocampus. For example one study by Aoki and collegues concentrates on the chemical and morphological alterations of neuronal spines preceding memory loss following the onset of Alzheimer's disease. Another study focuses on current type emmiting from the entorhinal cortex following stimulation, whether it is calcium dependent or potassium dependent (Khawaja *et al.*,

2007).   Although a direct role of the entorhinal cortex in a specific type of memory has not been identified, it is possible that at any time of memory expression the entorhinal cortex is involved in input and output of the hippocampus (Insausti *et al.*, 1995, Izquierdo and Medina, 1993) which renders it an important structure in memory processing.

1.7 Signaling interaction between brain regions

It is important to understand how these areas of the brain signal to each other, passing and storing information as they are activated. The hippocampus, which is part of the archicortex, forms 2 interlocking C-shapes of cell bodies composed of 4 smaller regions namely CA1, CA2, CA3 and the dentate gyrus, these regions are closely linked to the entorhinal and perirhinal cortices. Neuronal signaling takes place in the synapse between two neurons, synapses can be chemical or electrical. Chemical synapses signal in one direction only from a presynaptic membrane to a postsynaptic membrane (or vice versa) in the form of a neurotransmitter while electrical synapses signal by ion channels which run directly from one synapse into the adjoining cell. The main form of synapse in the brain is chemical therefore signaling is mainly by neurotransmitter release. It is thought that initial visual signals are received by the cerebellum and passed to the perirhinal cortex which then signals to the entorhinal cortex, which signals to the dentate gyrus, the dentate then signals to the CA3 region onto the CA1 region and then back to the entorhinal cortex. There is also several feedback loops from the dentate gyrus to the perirhinal cortex and other rhinal cortices. 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 synapse with pyramidal cells of the CA3 region. Finally, the CA3 pyramidal cells synapse with the CA1 neurons to form the Schaffer collateral pathway.
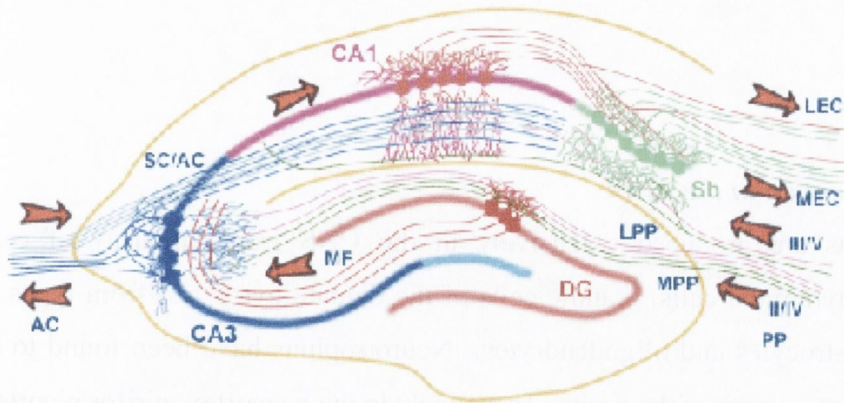
Figure 1.2 A schematic diagram of signaling network of the hippocampus and associated cortical regions. The entorhinal cortex (EC) receives input from the parahippocampal cortex through layers II and IV, which forwards this information to the hippocampus via connections with the dentate gyrus (DG) and the CA3 pyramidal neurons via the perforant path (PP- split into lateral and medial). The CA3 neurons also receive input from the DG via the mossy fibres (MF). They send axons to CA1 pyramidal cells via the Schaffer Collateral Pathway (SC) as well as to CA1 cells in the contralateral hippocampus via the Associational Commissural pathway (AC). CA1 neurons also receive input directly from the PP and send axons to the subiculum (Sb). These neurons in turn send the main hippocampal output back to the EC forming a loop.

1.8 Neurotrophins

The first neurotrophin was discovered over 50 years ago by Rita Levi-Montalcini and Viktor Hamburger, when a mouse sarcoma tumour was implanted close to the spinal cord of a developing chicken in ovo. This secreted a soluble factor that induced hypertrophy and outgrowth of dorsal root ganglion; this soluble factor was subsequently named nerve growth factor (NGF). Brain derived neurotrophic factor (BDNF) was discovered in 1982 when Barde *et al* isolated this neuronal survival factor from pig brain. Over the next twenty years neurotrophin 3 (NT-3; Hohn *et al.*, 1990), neurotrophin 4/5 (NT-4/5; Halbook *et al.*, 1991), neurotrophin 6 (Gotz *et al.*, 1994) and neurotrophin 7 (Lai *et al.*, 1998) were discovered, all of the which are expressed in the mammalian brain except for neurotrophin 6 and 7 which so far have only been identified in fish. Neurotrophins were originally designated the role of typical growth factors in development of the nervous system and maintenance of neurons in the adult brain but in

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more recent years a possible role for neurotrophins in the memory formation process has been postulated.

1.9 Neurotrophin location in the brain

Neurotrophins are produced extensively in the CNS from an array of cells including neurons, pyramidal cells, granule cells of the dentate gyrus and from types of glial cells such as astrocytes and oligodendrytes. Neurotrophins have been found to be expressed in several structures of the brain. These include the neocortex, piriform cortex, amygdaloid complex, hippocampal formation, claustrum, hypothalmus, cerebellum, the substantia nigra and the medulla oblongata (Anderson *et al.*, 1995). Numerous studies investigating neurotrophin expression in the CNS have found the highest concentration of neurotrophins and their receptors in the hippocampus (Phillips *et al.*, 1991, Barbany and Pearson, 1992, Takeda *et al.*, 1992, Rocamora *et al.*, 1992). Neurotrophin expression is upregulated by increases in neuronal activity such as LTP (Lauterborn *et al.*, 2007) and down-regulated in certain disease states, such as in patients with Alzheimer's disease (Phillips *et al.*, 1991).

1.10 Neurotrophin Structure

Neurotrophins are produced as precursor proteins, which are cleaved at dibasic amino acids to form a mature form of 118-120 amino acids (Maisonpierre *et al.*, 1990). The X-ray crystal structure of NGF has been solved and provides a structural model for this family (McDonald *et al.*, 1991). The conservation of structural features indicates that the neurotrophins will adopt similar conformations to that of NGF.

The dimeric NGF structure possesses a novel tertiarty fold that results in a flat asymmetric molecule with dimensions of 60Å by 25Å by 30Å. Each NGF subunit is characterized by two pairs of anti-parallel β-strands that contribute to the molecules flat elongated shape. These β–strands are connected at one end of the neurotrophin by three short loops. These loops are known to be highly flexible and represent the regions in the neurotrophins structure where many amino acid differences exist between the neurotrophins. The three disulfide bridges in each neurotrophin are clustered at the one

end of the molecule and provide rigidity to the structure. The arrangement of the disulfide linkage is unusual as the disulfide bridges and their connecting residues form a ring structure and a tightly packed cystine knot motif (McDonald and Hendrickson, 1993). This cystine knot allows the two pair of β–strands from each neurotrophin to pack against each other, generating an extensive subunit interface. The interface has a largely hydrophobic character comprised primarily of aromatic residues, consistent with the tight association constant, 10^{13}M, measured for NGF.

The structural features of the neurotrophin family, in particular the dimer interface are highly conserved, as evidence in the ability of these members to form dimers in vitro (Arakawa *et al.*, 1994). These heterodimeric proteins give functional activity in many cases, indicating there is overall compatibility of these structures.

1.11 Neurotrophin Synthesis, Storage and release

All neurotrophins are generated as pre-pro-neurotrophin precursors, approximately 240-260 amino acids in length, which are further processed until they are secreted as mature homodimeric proteins into the extracellular space, 118-129 amino acids long. The pre-mRNA sequence which gives rise to the signal peptide of the protein directs the synthesis of the nascent protein to endoplasmic reticulum (ER) attached ribosomes leading to the sequestration of the newly formed peptide chain into the ER. The signal peptide is cleaved off immediately after sequestration into the ER; this pro-neurotrophin can then form non-covalent–linked homodimers in the ER. The pro-neurotrophin in the ER then transits the Golgi apparatus via transport vesicles where they are either cleaved by furin in the trans Golgi or by other pro-convertases in vesicles. The vesicles can then be transported to the plasma membrane where they can fuse and release their contents.

It is well established that the regulation of synthesis of neurotrophins, particularly that of NGF and BDNF occurs in a very rapid manner and is mediated by the interplay of excitatory and inhibitory neurotransmitters (Lindholm *et al.*, 1994). Neurotransmitters are chemical signals released from presynaptic nerve terminals into the synaptic cleft. The subsequent binding of neurotransmitters to specific receptors on postsynaptic neurons transiently changes the electrical properties of the target cells, leading to an enormous

variety of postsynaptic effects, including regulation of neurotrophin release. However unlike neurotrophins, neurotransmitters generate specific patterns and levels of electrical connectivity. Neuronal electrical activity can variously enhance processes which regulated or modulated by neurotransmitters that influence branching morphogenesis involve: selective induction of downstream second messenger signaling cascades, regulated changes in intracellular Ca^{2+} levels, posttranslational protein modifications, and differential gene expression. Neurotransmitter activity is located at the synapse where as neurotrophin activity is dispersed throughout neurons.

This positive regulation of neurotrophins is mediated by the influx of calcium, which then, via activation of calmodulin, results in CREB phosphorylation (Tao *et al.*, 1998), regulating the synthesis of neurotrophins via specific promoter sites (Shieh *et al.*, 1998). The activity-dependent manner in which neurotrophins are secreted has shown unconventional characteristics in that it is independent of extracellular calcium but depends on calcium released from intact intracellular calcium stores (Blochl and Thoenan, 1996). This release is activity-dependent, being stimulated by glutamate receptor activation or high concentrations of potassium, and occurs all along the neuronal processes, particularly dendrites.

1.12 Neurotrophins and Neurogenesis

Proliferation, differentiation and survival of neurons, which is known as neurogenesis is believed to be the extrinsic mechanisms behind strengenthening of synaptic connections and memory storage. Neurogenesis was first observed in songbird brains (Nottebohn, 1989) and later demonstrated in all mammalian species including humans (Gagae, 2000). In adults active generation of new neurons is restricted to two main areas: the subgranular zone of the hippocampus and the subventricular zone of the lateral ventricles. The process of neurogenesis involves multiple, sequential steps including neural stem cells proliferation to give rise to rapidly amplifying neural progenitor cells, these multipotential progenitor cells undergo cell-fate determination and some then differentiate into immature neurons and migrate to their final locations. Ultimately these cells mature into fully functional neurons and are integrated into the existing neural networks (Song et al., 2002). This complex process is tightly regulated at

all steps by several intrinsic and extrinsic factors, one of which is believed to be neurotrophins. Although direct evidence is lacking, substantial experimental data support the view that adult neurogenesis in the dentate gyrus participates in some forms of hippocampus-dependent learning and memory (Gould *et al.*, 1999; Kempermann, 2002). Several lines of evidence indicate that neurotrophins, might be excellent candidates as diffusible factors that regulate neurogenesis in the hippocampus. The specific stages at which neurotrophins may act have not been fully dissected although evidence in the literature supports the hypothesis. The synthesis and release of neurotrophins can be upregulated dynamically through positive feedback signaling triggered either by additional neurotrophin stimulation, or by various forms of neuronal activity (Lindholm *et al.*, 1994). Thus, precise spatiotemporal control of neurotrophin signaling through regulated expression and release may underlie distinct stages of neuronal morphogenesis. The numerous structural changes underlying axonal and dendritic branching morphogenesis ultimately arise through the combined effects of differential gene induction and reorganization of specific cytoskeletal proteins, processes known to be downstream targets of neurotrophins (Tao *et al.*, 1998). For example, actin, microtubules, and numerous microtubule-associated proteins have been reported to be regulated by NT-3 and BDNF at the level of both transcription and post-translational modification (Lee et al., 2002). Despite their ability to exert similar influences on the cytomatrix of axons and dendrites, neurotrophic factors also can produce separate and distinct structural changes for each of these neurite classes during branching morphogenesis. Application of BDNF to neural progenitor cell culture derived from adult brain, significantly increase the number of neurons expressing GABA, acetylcholinesterase, tyrosine hydroxylase and calbindin without effecting the number of new neurons (Takahashi et al., 1999). This only occurs once immature neurons were observed which indicates that neurotrophins may act at a later stage of neurogenesis. However together with other published data suggesting that BDNF regulation of neurogenesis may regulate hippocampal plasticity (Shors et al., 2001) provides a possible role for neurotrophin mediation of neurogenesis in learning and memory.

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1.13 Evidence of a role for NGF in memory processing

Evidence of neurotrophin participation in plastic processes in the brain, including those that accompany memory consolidation has been reported for over 20 years. One of the first of these was a study by Fischer and collegues in 1987 which demonstrated that i.c.v. injections or infusions of NGF in young adult rats have been shown to prevent neuronal cell death and promote behavioural recovery after damage to the septo-hippocampal connections. They also demonstrated that i.c.v. infusion of NGF over a period of four weeks can partly reverse the cholinergic cell body atrophy and improve retention of a spatial memory task in behaviorally impaired aged rats. From this study it was evident that neurotrophins not only promoted cell survival but were also involved in memory processing. NGF as the prototype member of the neurotrophin family is secreted by hippocampal neurons in culture and in slices (Blochl and Thoenan, 1995) and, like other neurotrophins, it delivers its message inside the cell via interaction with cell surface receptors (Segal and Greenberg, 1996). The specific binding of NGF to neurotrophin receptors coupled to tyrosine kinase A leads to dimerization and autophosphorylation of the kinase molecule. The tyrosine-phosphorylated receptor ultimately propagates the NGF signal to the Ras/ MAPK (mitogen activated protein kinases) pathway (Segal and Greenberg, 1996). NGF signaling has been shown to be propagated via two different second messenger systems in cell culture systems (Barker *et al.*, *2002*, Gao *et al.*, *2003*). One signals through the MAPK and ERK phosphorylation, the other second messenger system used by NGF is through lipid signaling products of PI-3K/AKT pathway. One study investigating NGF-mediated spatial memory found that MAPK inhibitors attenuated performance in the morris water maze and both Trk A and phospho-ERK immunoreactivities were diminished in aged rats and phospho-ERK immunoreactivity correlated with aged rat performance in the Morris water maze. In addition NGF has also been suggested to play a role in LTP. Maguire and colleagues (1999) found that NGF increased phosphorylation of Trk A, a neurotrophic receptor, and ERK activation in dentate gyrus in vitro. In addition to this, tyrphostin AG879, a Trk receptor antagonist inhibited the expression of LTP in perforant path-granule cell synapses, suggesting that NGF may be an important protein in production of LTP.

1.14 Evidence of a role for BDNF in memory processing

Studies in the last few years have identified a novel function of neurotrophins: regulation of synaptic function and plasticity (Lo *et al.*, 1995; Thoenen, 1995; Lu and Figurov 1997). Substantial evidence indicates that neurotrophins can influence a wide variety of synapses, not only during development but in the adult nervous system as well. Out of all the neurotrophins the most focus has been on BDNF and its role in memory processing. The function of BDNF in synaptic transmission and plasticity in the hippocampus has been one of the major focuses in recent neurotrophin research. In primary cultures of embryonic hippocampal neurons, acute application of exogenous BDNF rapidly enhances neuronal and synaptic activity and transmitter release (Knipper *et al*, 1994; Levine *et al*, 1995; Takei *et al*, 1997), this data suggests that BDNF is capable of modulating synaptic function in the hippocampus. BDNF also appears to play a role in long term potentiation (LTP) in the hippocampus. Tetanic stimulation enhances the expression of BDNF mRNA in the hippocampus (Patterson *et al.*, 1992; Castern *et al.*, 1993). On further investigation of the role of BDNF in LTP one study analyzed LTP in the dentate gyrus of awake rats which triggered a rapid increase in Trk B and Trk C gene expression and a delayed increase in BDNF and NT-3 gene expression (Bramham *et al*, 1996). While BDNF immunoreactivity is found in several structures in the brain, including the neocortex, piriform cortex, amygdaloid complex, hippocampal formation, claustrum, some thalamic and hypothalamic nuclei, the substantia nigra and some brainstem structures, BDNF mRNA was not located in all these areas (Yan *et al.*, 1997). This suggests that there is anterograde transport of endogenous brain-derived neurotrophic factor in the central nervous system. In the dentate gyrus there is a high expression of BDNF mRNA located in the granule cell soma with little detection of BDNF IR, suggesting the dentate gyrus may be transporting BDNF to other areas of the brain where BDNF is required for synaptic plasticity (Carter *et al*, 2002). The importance of BDNF activity in short term memory (STM) and long term memory (LTM) has also been investigated and one study analyzing hippocampal BDNF demonstrated that it is required for both STM and LTM formation in an inhibitory avoidance test (Alonso *et al*, 2002). Transgenic mice overexpressing BDNF showed

deficits in LTM (Croll *et al.*, 1999). In contrast, BDNF mutant mice exhibited learning, but not memory retention, deficits of a multi-trial spatial task (Linnarsson *et al.*, 1997). In addition acute or chronic intracerebral administration of BDNF produced no effects on memory retention (Cirulli *et al.*, 2000; Johnston *et al.*, 1999). The BDNF/Trk B system in the hippocampus has been shown to play a crucial role not only in the acquisition, but also in the retention and recall of spatial memory (Yamada *et al.*, 2003). The role of BDNF is still not fully understood given the results of transgenic studies and BDNF administration studies.

1.15 Evidence of a role for NT3 in memory processing

NT3 unlike the other neurotrophins does not appear from the literature to play a central role in memory processing. Numerous studies have reported little or no changes in NT3 expression following LTP. One study investigating neurotrophin mRNA expression following induction of LTP in the hippocampus found that BDNF and NGF mRNA were increased and observed no changes in NT3 mRNA expression (Kang and Schuman, 1995). A similar study by Bramham and colleagues measured Trk B and Trk C gene expression and BDNF and NT3 gene expression following induction of LTP in the dentate gyrus of awake rats. They reported increased gene expression of Trk B and Trk C within 2 hours following LTP and a delayed increase in BDNF and NT3 gene expression between 6 hours and 24 hours. In addition to this they found that Trk B and NT3 increases were restricted to the granule cells of the dentate gyrus and BDNF and Trk C elevations occurred in the granule cells of the dentate gyrus also and the hippocampal pyramidal cells (Bramham *et al.*, 1996). In a more recent study, the involvement of neurotrophins in LTP was examined using two highly specific, function-blocking monoclonal antibodies against BDNF and NT3, as well as a Trk B-IgG fusion protein. The results show that NT3 antibodies did not have an effect on LTP. However, both TrkB-IgG fusion proteins and BDNF antibody similarly reduced LTP, suggesting that NT3 is not as important in synaptic plasticity as the other neurotrophins (Chen *et al.*, 1999). It is possible that NT3 may serve a more important function in maintaining neuronal tissue; a role which is of increasing importance as the brain ages, this theory is supported by evidence from a study investigating age impaired memory deficits in rats.

It has been reported that infusions of NT3 over a four week period improved acquisition and retention of spatial memory in aged rats along with a significant reduction in cholinergic neuron atrophy in the septum, nucleus basalis and striatum (Fischer *et al.*, 1994).

1.16 Evidence of a role for NT4/5 in memory processing

NT4/5 is one of the more recently discovered neurotrophins so there are very few studies investigating its role in memory processing as with most studies concentrating on BDNF. The diverse functions of neurotrophins are mediated by their specific interaction with the Trk receptors, however both NT4/5 and BDNF activate the same Trk B receptor which renders the problem of differentiating the effects of BDNF from those of NT4/5. One way of attempting this is to use mutant mice lacking neurotrophins. BDNF, NGF or NT3 $^{-/-}$ mice die during early postnatal stages with severe neuronal deficits and behavioural symptoms, where as NT4/5 $^{-/-}$ mice are viable and fertile with only a mild sensory deficit (Ibanez, 1996). This study suggests that NT4/5 is the neurotrophin of lesser importance, although there is some very conflicting data on this subject. To address this matter one study replaced the BDNF coding sequence with the NT4/5 sequence in mice, essentially meaning that the mice were expressing NT4/5 genes instead of BDNF, these mice proved viable and NT4/5 supported many BDNF dependent functions in vivo (Fan *et al.*, 2000). This study also suggests that the Trk B receptor is capable of distinguishing between the binding of NT4/5 or BDNF, and NT4/5 once bound to the Trk B receptor, may elicit a different signaling pattern than BDNF. To support this theory one study investigating the effects of BDNF, NT3 and NT4/5 in promoting the survival and/or regulation of expression of phenotypic markers of dopaminergic and GABAergic neurons found that seven days treatment with either BDNF or NT-3 resulted in dose-dependent increases in the number of tyrosine hydroxylase-positive neurons, with maximal increases of 3-fold and 2.3-fold, respectively. Dopamine uptake activity and dopamine content showed similar increases. NT4/5 treatment elicited the greatest increase in tyrosine hydroxylase-positive neurons, being 7fold but had no effect on dopamine uptake capacity. The study also assessed high-affinity GABA uptake, glutamic acid decarboxylase (GAD) activity, and

endogenous GABA content which were used to detect GABAergic neurons. The study found that GAD activity was increased by BDNF (1.8-fold) and NT-3 (3 fold) treatment, but not by NT-4/5, whereas GABA uptake and GABA content was increased to a similar extent by all three neurotrophins. Although BDNF and NT-4/5 act through the same high-affinity receptor, Trk B, it is evident that these two neurotrophins have distinct as well as overlapping actions toward neuronal regulation which may help to explain the reported differences of their functions in memory processing.

1.17 Neurotrophin receptor classification

The biological functions of the neurotrophins are mediated via two types of receptor: the Trk receptors and the p75 receptor. Signaling by the Trk receptors is believed to induce neuronal survival and proliferation (Zhang *et al*, 2000) whereas signaling by the p75 receptor is believed to induce apoptosis (Dechant et Barde; 1997) although there is much debate surrounding the true function of this receptor. All the neurotrophins can bind the p75 receptor (Rodriguez *et al.*, 1991), but the Trk receptors shown much greater ligand specificity. Trk A is primarily a receptor for NGF, although it can also be activated by NT3. Trk B is the receptor for both BDNF and NT4/5 and can also be activated by NT3. Trk C is a receptor for NT3, with little activation by other ligands.

1.18 Receptor Location

Trk receptor expression is found in an array of regions in the CNS as those previously mentioned for neurotrophin location, and similarly these receptors are found in the highest concentrations in the hippocampus (Phillips *et al.*, 1991, Barbany and Pearson, 1992, Takeda *et al.*, 1992, Rocamora *et al.*, 1992). It is the location of the Trk receptors which has given rise to the most questions. A large controversy has grown around the question of whether neurotrophins at synaptic sites bind to receptors on the pre- or postsynaptic cell (Altar & DiStefano 1998, Conner *et al.* 1998, Tonra 1999). Abundant data now indicate that receptors are present both on presynaptic axon terminals and on postsynaptic dendrites (Aloyz *et al.* 1999, Carter *et al.* 2002, Gottschalk *et al.* 1998, Levine *et al.* 1995, Schinder *et al.* 2000, von Bartheld *et al.* 1996, Wu *et al.* 1996).

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Thus, neurotrophins not only function as target-derived factors to promote the survival of developing afferent neurons but also act in an anterograde fashion, released by presynaptic cells to regulate the postsynaptic targets. This data indicates that the nature of the response of the Trk receptor can differ depending on whether the receptors are on the axons of presynaptic cells, or on the dendrites of postsynaptic targets.

Similarly the p75 receptor is expressed in a wide variety of cells in the CNS. Its expression appears in several neuronal populations as they differentiate, including spinal motor neurons and brain stem motor nuclei, medial terminal nucleus, amygdala, thalamic nucleus and cerebellum. Postnatal expression of the p75 receptor is significantly reduced in most tissue and is confined to the basal cholinergic neurons, motor neurons and the cerebeller Purkinjie neurons (Roux and Barker, 2002).

1.19 Trk Receptor Structure

Trk was initially discovered from a colon cancer-derived oncogene in which tropomyosin was fused to a novel tyrosine kinase domain (Martin-Zanca et al., 1986). The normal cellular counterpart of Trk is a single-pass transmembrane molecule that is highly expressed in the developing nervous system. The extracellular domain of Trk, which is not present in the oncogene, encodes a leucine rich motif flanked by two cysine rich domains and an immunoglobulin-like domain which is required for ligand binding (Ultsch et al., 1999, Wiesman et al., 1999). The intracellular domain of Trk is much smaller than that of many other receptor tyrosine kinases and contains only 70 amino acids before and 15 amino acids after the tyrosine kinase domain.

Before Trk A was even identified as the receptor for NGF, a related kinase, termed Trk B was purified and shown to be highly expressed in the nervous system (Klein et al., 1990), shortly thereafter another receptor, termed Trk C was identified (Lamballe et al 1991). Trk receptors are similar mostly in the kinase domain where there is 88% conservation, the extracellular domain has the same overall structure but is only 57% conserved. Investigators soon identified that Trk A preferentially binds NGF (Kaplan et al., 1991a), Trk B binds BDNF and NT-4/5 (Klein et al., 1991b) and NT-3 binds Trk C (Lamballe et al., 1991). These receptors are expressed in a wide variety of neuronal and non-neuronal

tissue including, the hippocampus, cerebellum, neurons of the peripheral nervous system, arteries and submaxillary gland.
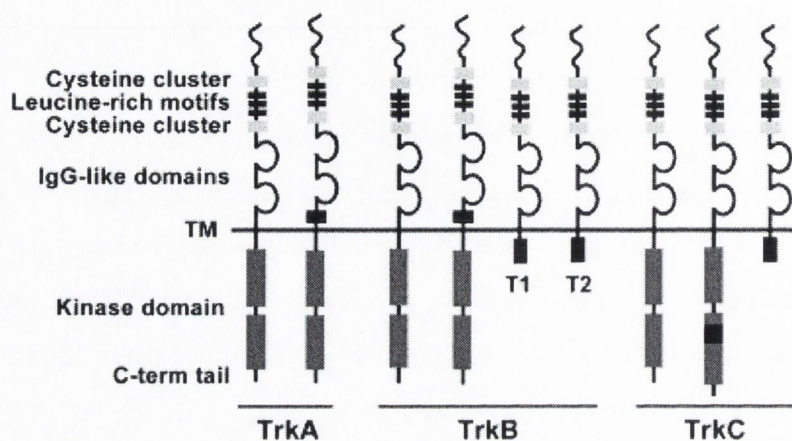
Figure 1.2 Schematic representation of the Trk receptor isoforms. The three Trk genes (Trk*A*, Trk*B*, and Trk*C*) encode a full-length receptor, and multiple alternatively spliced isoforms. Splicing leads to TrkA and TrkB receptors that lack a short amino acid sequence in their extracellular domains, TrkB and TrkC receptors that lack the intracellular kinase domain, as well as TrkC receptor with an insert in the kinase domain.

1.20 Receptor internalisation and retrograde transport; 2 possible mechanisms for NT signaling

As discussed previously Trk receptors have been identified on both presynaptic axon terminals and on postsynaptic dendrites (Aloyz *et al.* 1999, Carter *et al.* 2002, Gottschalk *et al.* 1998, Levine *et al.* 1995, Schinder *et al.* 2000, von Bartheld *et al.* 1996, Wu *et al.* 1996) and as a result may act in an anterograde fashion, being released from presynaptic cells to regulate postsynaptic targets. There is a proposed signaling endosome hypothesis to explain the specialized mechanisms for retrograde signaling which is initiated by target-derived neurotrophins that stimulate the presynaptic cells (Beattie *et al.*, 1996). According to this hypothesis, neurotrophins bind Trk receptors at nerve terminals, activate the receptor, and induce receptor internalization. An endocytic vesicle, containing activated Trk receptor together with the neurotrophin ligand, is then transported from the synaptic terminal to the cell body, and this signaling endosome mediates a biological response. It has been suggested that the retrograde signal therefore

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has a special requirement for vesicle formation and transport. In support of this model, it has been shown that Trk receptors are endocytosed into vesicles in response to neurotrophins (Grimes *et al*. 1997, Grimes *et al*. 1996). Furthermore, Trk receptors remain catalytically active within these intracellular vesicles. Small endocytic vesicles containing activated Trk receptors have been isolated from undifferentiated PC12 cells following NGF stimulation, and these vesicles are associated with downstream signaling molecules including Shc, the small G-protein Rap, Erks, and phospholipase C (Howe *et al*. 2001, Wu *et al*. 2001).

1.21 Trk receptor signaling

Trks resemble other receptor tyrosine kinases in that they dimerize (receptors phosphorylate each one another at critical tyrosine residues Y490 and Y785) in response to ligand binding (Jing *et al*. 1992). The dimeric receptors phosphorylate one another, and rapid phosphorylation of the tyrosines within the activation loop of the Trks further enhances catalytic activity of the kinases (Cunningham & Greene 1998). The active receptor tyrosine kinase phosphorylates its partner at two additional tyrosine residues: the NPXY motif in the juxtamembrane domain (Stephens *et al*. 1994) and the YLDIG motif in the carboxy terminus (Loeb *et al*., 1994). These additional phosphotyrosines generate attachment sites for Sch and for phospholipase C (PLC) respectively. Once bound to the active receptor, Shc and PLC become phosphorylated by the receptor tyrosine kinase (Kaplan and Miller, 2000). Recruitment and tyrosine phosphorylation of PLC activates this enzyme, which catalyzes the breakdown of lipids to diacyl glycerol and inositol (1,4,5) triphosphate (IP3) (Rhee 2001). Binding of IP3 to specific receptors promotes release of calcium from intracellular stores, while diacyl glycerol allows maximal activation of several protein kinase C isoforms. In addition, the phospholipase pathway can indirectly activate MAP kinases and phosphatidylinositol 3'-kinase (PI3 kinase) by changes in intracellular calcium.

1.22 Signaling through Ras

Ras regulates neuronal differentiation, promoting neuronal survival in many neurons either through PI3K or the MAPK/ERK pathway. Binding of a neurotrophic factor

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results in the phosphorylation of the Y490 residue, initiating recruitment of an adaptor protein, initiating a cascade of signaling events. Shc recruitment and phosphorylation results in recruitment to the membrane of a complex of the adaptor Grb-2 and the Ras exchange factor SOS, thereby stimulating transient activation of Ras. Activation of Ras results in activation of the PI3K, and p38 MAPK. Ras activates in turn c-Raf which phosphorylates MEK-1 and/or MEK-2 and they phosphorylate ERK. ERK1 and ERK 2; targets of ERK are the ribosomal S6 kinase (RSKs). Both RSK and MAPK-activating protein kinase 2 phosphorylate CRE-binding protein (CREB), C-fos and other transcription factors (Xing, 1998). This Trk receptor-mediated stimulation of Ras through Shc and Grb-2/SOS results in transient and not prolonged activation of ERK signaling. Prolonged signaling through ERK appears to be mediated by another signaling pathway involving the adaptor protein Crk, the G protein Rap-1 the tyrosine phosphatase Shp2 and the serine threonine kinase B-Raf. The activation of this pathway depends on the recruitment of Frs2 by phosphorylated Y490 (Roux and Barker, 2002, Patapoutian and Reichardt, 2001).

1.23 Signaling through PI3K

Trk receptors can activate PI3K through at least two distinct pathways, whose importance differs between neuronal subpopulations. In the majority of cases Ras-dependent activation of PI3K is the most important pathway through which neurotrophins promote cell survival. In some cases PI3K can be activated directly through the adaptor proteins Shc, Grb-2, and Gab-1. Phosphorylation of Grb-2 provides a docking site for Gab-1, which in turn is bound by PI3K (Holgado-Madruga *et al*, 1997). Lipid products generated by PI3K recruit many proteins containing pleckstrin-homology domains to the membrane, including Akt kinases and 3-phosphoinositide –dependent kinases (PDKs) (Datta *et al*., 1996, Hemmings., 1997). Akt is activated at the membrane by PDK, after which it phosphorylates several proteins important in controlling cell survival mediating Trk receptors (Yao & Cooper, 1995).

1.24 Signaling through PLC-γ

Phosphorylated Y785 on Trk A and similarly placed residues on other Trk receptors recruit PLC-γ1. The Trk kinase then phosphorylates and activates PLC-γ1, which acts to hydrolyze phosphatidylinositides to generate diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3). IP3 induces the release of Ca^{2+} from internal stores, increasing levels of cytoplasmic Ca^{2+} activating protein kinase C and thereby activating many pathways controlled by Ca^{2+} (Patapoutian & Reichardt, 2001).

1.25 Activation of Transcription factors

Transcription factors are the proteins involved in the regulation of gene expression that bind to the promoter elements upstream of genes to facilitate transcription, through this process they control gene expression. There are a number of transcription factors activated via Trk receptor activation, the most well known are the immediate early genes such as c-fos and *zif*268 or the cAMP response binding element, CREB. These transcription factors have been proven to be activated downstream of the ERK/MARK pathway (Cammarota *et al.*, 1999, Bozon *et al.*, 2002). Studies have shown that LTP induces transcriptional regulation of a variety of genes including transcription factors (Cole *et al.*, 1989, Abraham *et al.*, 1993, Sajikumar *et al.*, 2007) and inhibition of transcription affects the duration of LTP (Nguyen *et al.*, 1994, Smolen *et al.*, 2006).

1.26 P75 Receptor

The P75 receptor has been shown to bind each of the neurotrophins (NGF, BDNF, NT3 and NT4/5) with approximately equal affinity (Sutter *et al.*, 1979, Rodriguez-Tebar *et al.*, 1990, Ernfors *et al.*, 1990). The P75 receptor 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). Despite its discovery almost 30 years ago the biological function of the p75 receptor 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. The p75 receptor has now emerged as a paradox with different studies reporting

its role in cell survival and others its role in apoptosis (Kume *et al.*, 2000, Roux *et al.*, 1999).

1.27 P75 structure

The P75 receptor was the first member of what is now known as the tumour necrosis family (TNF). Like the Trk receptors, it too is a type I transmembrane protein, a 399-amino acid chain. The extracellular domain of the receptor consists of four cystine rich loops, with six cysteines per loop. It is within these loops that the neurotrophin binding domains are contained (Yan and Chao, 1991), each of these cystine rich domains contains three intrachain disulfide bridges that create the receptors elongated structure. The cytoplasmic domain features a death domain which features six 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 p75 receptor death domain has a prosurvival function (Khursigara *et al.*, 2001)

1.28 P75 interaction with Trk receptors

P75 interaction with Trk receptors has been mainly documented in studies with Trk A. These studies showed that co-expression of p75 neurotrophin receptor (p75NTR) with TrkA receptors in transformed cells produced high-affinity NGF binding sites (Hempstead *et al.*, 1991, Rodriguez *et al.*, 1992, Mahadeo *et al.*, 1994), and enhanced NGF-mediated TrkA activation (Barker*et al.*, 1991, Mahadeo *et al*, 1994, Verdi *et al.*, 1994). The high-affinity binding sites generated by p75NTR and Trk A have been well characterized kinetically (Hempstead *et al.*, 1991, Mahadeo *et al.*, 1994, Rodriguez *et al.*, 1992). In the absence of Trk A, p75NTR has a rapid rate of ligand association and dissociation whereas in the absence of p75NTR, Trk shows very slow association and dissociation kinetics. When the two receptors are co-expressed, the rate at which NGF can associate with Trk A increases by about 25-fold (Mahadeo *et al.*, 1994), resulting in the generation of high-affinity binding sites. Consistent with this, Trk activation that occurs in response to low concentrations of neurotrophin is enhanced in the presence of

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p75NTR (Hantzopoulos *et al.*, 1994). The presence of p75NTR, therefore, appears to confer increased responsiveness to low neurotrophin concentrations.

As mentioned previously, NGF is the preferred ligand for Trk A, but high concentrations of NT-3 and NT-4/5 can also activate the receptor. Trk B is readily activated by BDNF and NT-4/5, and can also be activated by high concentrations of NT-3. Several studies have shown that the ligand specificity of Trk receptors is sharply limited by p75NTR. In the presence of p75NTR, NGF readily activates Trk A, but activation by NT-3 and NT-4/5 is greatly attenuated (Lee *et al.*, 1994, Bibel *et al.*, 1999, Mischel *et al.*, 2001). Similarly, BDNF-mediated Trk B activation is not altered in the presence of p75NTR, but Trk B activation mediated by NT-3 and NT-4/5 is reduced by p75NTR co-expression (Bibel *et al.*, 1999)

1.29 P75 signalling

The pleiotrophic functions of the p75 receptors mean that there are several distinct signaling pathways that are activated downstream of the receptor, the first of which is, its suggested mediation of survival signaling. P75 receptor signaling appears to involve the activation of nuclear factor-kappa –B (NF-κB). Activation of NF-κB by the p75 receptor was initially reported in cultured Schwann cells, where NGF binding to p75 increased NF-κB DNA binding activity, and p65 nuclear translocation (Carter *et al.*, 1996). P75 receptor-dependent NF-κB activation has since been shown in several other cell types but the level of induction is modest compared to activities induced by other members of the TNF receptor family. P75-mediated NF-κB activation appears to promote survival of several cell types (Hamanoue *et al.*, 1999, Gentry *et al.*, 2000, Khursigara *et al.*, 2001). Khursigara and collegues identified a role for p75 mediated survival in Schwann cells through the activation of RIP2, an adaptor protein with a serine threonine kinase and a caspase recruitment domain (CARD). They found that NGF activation of p75, induced interaction with RIP2 resulting in enhanced NF-κB activation which blocked apoptosis in Schwann cells. P75 has also been shown to activate AKT via a PI3K/AKT dependent signaling pathway (Roux *et al.*, 2001). This pathway is an important signaling pathway that is frequently activated downstream of the Trk receptors.

P75 signaling has also been reported to activate apoptotic signaling pathway both *in vivo* and *in vitro*. The precise mechanisms by which p75 activates apoptosis remain elusive. Although certain studies have identified some key signaling pathways by which p75 may act, namely JNK activation (Yoon *et al.*, 1998), ceramide induced apoptosis (Casaccia-Bonnefil, 1996) or activation of caspase 2, 3 and 8 for execution of cell death (Coulson *et al.*, 2000).

1.30 ERK and Recognition memory

The ERK MAP kinase cascade has been implicated in memory, long lasting synaptic plasticity, and biochemical information processing at the molecular level (Adams *et al.*, 2002). The first direct evidence that ERK cascade is involved in long term memory (LTM) showed that ERK is required for the expression of LTM induced by fear-conditioning paradigm in the rat. It was found that ERK2 phosphorylation was increased in the rat hippocampus 1hr after training using either a cued or cued plus contextual conditioning paradigm (Atkins *et al.*, 1998). The use of an ERK inhibitor SL327 has also led to a blockade of memory formation. The results showed that the MAPK cascade is required for long term fear memory. The MAPK cascade has also been shown to be involved in spatial memory, another form of hippocampal-dependent LTM (Blum *et al.*, 1999) thereby showing that hippocampal ERK activation occurred during learning. The MAPK pathway has been shown to be critical in the processes of consolidation and reconsolidation of object recognition memory, using the object recognition task and a specific inhibitor of the upstream kinase, MEK. Rats injected with the inhibitor were unable to distinguish between the familiar object, seen 24hrs previously and the novel object. The authors found increased ERK phosphorylation in the entorhinal hippocampal circuitry, implicating the ERK MAPK pathway in the signaling cascade during memory consolidation (Kelly *et al.*, 2003). The initial triggers for ERK activation are unknown, but it may be speculated that binding of neurotrophins to Trks may be involved.

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1.31 PI3 Kinase and Recognition Memory

The Akt/PI3K pathway has been implicated in a number of biological responses, including promotion of cell survival by its anti-apoptopic activity. More recently, a number of studies have implicated PI3K in synaptic plasticity, learning and memory but there is little understanding of its functional role. Studies have shown that activation of PI3K is necessary for the expression of LTP in the dentate gyrus (Kelly & Lynch, 2000) and CA1 region of the hippocampus (Raymond *et al.*, 2002). Others have suggested a role in expression or induction of LTP in CA1 and the amygdala that may involve the MAPK pathway (Lin *et al.*, 2001). In some behavioural studies inhibiting PI3K activity results in impaired spatial learning (Mizuno *et al.* 2003), and consolidation (Lin *et al*, 2001) and retrieval and extinction of fear-associated memories (Chen *et al*, 2005). One study using the PI3K inhibitor LY294002 showed that it impaired long term consolidation of recognition memory but not short term recognition memory or spatial learning and repeated training in the recognition memory task overcame the deficit in consolidation (Horwood *et al.*, 2006). These results suggest that activation of the PI3K/Akt pathway may contribute to the mechanisms of synaptic plasticity and memory consolidation.

1.32 Transcription factors and memory processing.

Transcription factors regulate expression of effector genes which mediate their role in cellular maintance and structural rearrangement, as a result it is almost impossible to designate a transcription factor one precise role. With this in mind numerous transcription factors have been linked to memory processing. One study has investigated c-fos expression in the hippocampus in the hours following acquisition, finding significant increases in c-fos expression immediately following acquisition in both the hippocampus and the entorhinal cortex (Smith *et al.*, 2007). Measurement of c-fos has previously been used to characterize populations of neurons that are undergoing activity-dependent modification and will be used similarly in this study. Another study found increases of c-fos, CREB and Elk-1 in the hippocampus after a one-trial avoidance learning task in rats, and that this increase was blocked by inhibition of the NMDA

receptor, suggesting that NMDA which activates neurotrophin release mediates these intracellular cascades during learning (Cammarota *et al.*, 1999). CREB was identified as being necessary for induction of LTP and recognition memory in the perirhinal cortex (Warburton *et al.*, 2005). In this study the inhibition of CREB in the perirhinal cortex by an adenovirus impaired the preferential exploration of novel over familiar objects at 24hrs but not at a short 15min test, it also impaired LTP in slices of the perirhinal cortex. A study investigating *zif*268 showed that when a consolidated memory for objects is recalled, *zif*268 mutant mice are impaired in further long-term but not short-term recognition memory. This impairment was specific to reactivation with the previously memorized objects in the relevant context, occurs in delayed recall, and does not recover over several days. These findings indicate that IEG-mediated transcriptional regulation in neurons may be a common molecular mechanism for the storage of newly formed and reactivated recognition memories (Bozon *et al.*, 2003).

1.33 Tyrophostin AG879

Tyrophostin AG879 is a member of a family of synthetic tyrphostins, antineoplastic agents and protein tyrosine kinases antagonists and inhibitors. Tyrphostin AG 879 inhibits the neurotrophin-dependant pp140-Trk tyrosine phosphorylation. In doing so it also blocks receptor associated proteins such as p38, PIC-γ1, Pi3K and MAPK (Ohmici *et al*, 1993). Tyrphostin AG879 inhibits the action of Trk receptors by binding to the tyrosine kinase activation site on the intracellular kinase domain of the Trk receptor structure preventing the Trk receptor signaling cascade. Previously tyrphostin AG879 has been proven to block LTP in the perforant path-granule cell synapse and the accompanying increase in transmitter release by inhibition of the Trk A receptor, preventing NGF activation (Maguire *et al.*, 1999). Tyrphostin AG879 has been reported to block LTP and spatial learning in the rat, this effect is believed to be mediated by blocking the Trk B receptor which regulates ERK activation in the dentate gyrus (Gooney *et al.*, 2002). A similar study reported that long-term potentiation was accompanied by increased endogenous glutamate release and increased tyrosine phosphorylation of synaptophysin, but these changes were attenuated when long-term potentiation was inhibited by the tyrosine kinase inhibitor tyrphostin AG879 (Mullany and Lynch, 1998).

Tyrphostin has the ability to block all tyrosine kinases as shown by Dieter and collegues (2001) when they investigated transcription and aromatic hydrocarbon response elements in relation to drug metabolizing agents.

1.34 Objectives

1. The primary aim of this study was to investigate the possible role that individual neurotrophins and their receptors play in acquisition and consolidation of recognition memory in the rat.

2. Having identified that recognition memory is dependent upon neurotrophin action I attempted to investigate the precise role that neurotrophins and the Trk receptors play within the 24hour period following learning in the rat.

3. In addition to the above I investigated the intracellular signaling processes which are believed to mediate neurotrophin signaling.

4. The final part of this thesis will access if the Trk receptor inhibitior, tyrphostin AG879 will affect the intracellular signaling of neurotrophins identified in part 3, also to investigate PI3K as a mediator of neurotrophin action during memory consolidation.

Chapter 2

Materials & Methods

2.1 Materials

Animals

Wistar Rats (3 month old males)	Bioresources, TCD
Laboratory rat diet	Red Mills

ELISA: Plastics and Kits

Rat BDNF ELISA kit	Promega
Rat NGF ELISA kit	R&D Systems
Rat NT 3 ELISA kit	Promega
Rat NT 4/5 ELISA kit	R&D Systems
Substrate Solution	R&D Systems

Experimental Treatments

Anti-BDNF neutralizing antibody	Chemicon
Anti-NGF neutralizing antibody	Chemicon
Anti-NT 3 neutralizing antibody	Chemicon
Anti-NT 4/5 neutralizing antibody	Chemicon
DMSO	Sigma
Normal Goat Serum	Vector
Normal Rabbit Serum	Chemicon
Tyrphostin AG879	Calbiochem

General Laboratory Chemicals

Acrylamide	Sigma
Ammonium persulphate	Sigma
Bio-Rad dye reagent concentrate	Bio-Rad
Bis-acrylamide	Sigma
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
Calcium chloride	Lennox
Diethyl Pyrocarbonate (DEPC)	Sigma

Dimethyl sulphoxide	Sigma
Di-Sodium hydrogen orthophosphate (Na_2HPO_4)	Sigma
DL-Dithiothreitol (DTT)	Sigma
Ethanol	Lennox
Glucose	Lennox
Glycerol	Sigma
Glycine	Sigma
Hydrochloric acid	Lennox
Hydrogen peroxide	Sigma
Hyperfilm	Amersham
Hyperfilm	Pierce
Leupeptin	Sigma
Magnesium sulphate	Sigma
Magnesium Chloride ($MgCl_2$)	Sigma
β-Mercaptoethanol	Sigma
Methanol (MeOH)	Lennox
Nitrocellulose membrane	Amersham
Potassium chloride (KCL)	Sigma
Potassium dihydrogen orthophosphate (KH_2PO_4)	Sigma
Potassium hydroxide	Sigma
Potassium phosphate	Sigma
2-Propanol	Sigma
Sodium carbonate (Na_2CO_3)	Sigma
Sodium bicarbonate($NaHCO_3$)	Sigma
Sodium chloride (NaCl)	Sigma
Sodium dodecylsulphate (SDS)	Sigma
Sodium hydrogen carbonate	Lennox
Sodium hydroxide (NaOH)	Lennox
Sodium phosphate (monobasic)	Sigma
Sodium phosphate (dibasic)	Sigma
Sucrose	Sigma

Standard grade No. 3 filter paper	Whatman
Tetramethyl-diamine (TEMED)	Sigma
Tetramethylbenzidine (TMB)	R&D Systems
Tris-base	Sigma
Tris-HCl	Sigma
Tween-20	Lennox

General Laboratory Products

Microtest 96-well flat bottomed plates	Sarstedt
Pipette Tips	Sarstedt
Microtubes	Sarstedt
Plastic transfer pipettes	Sarstedt
Laboratory Roll	Parafilm

Molecular Reagents

Absolute ethanol	Sigma
Agarose	Condra
Biosphere filter pipette tips	Sarstedt
Ethidium Bromide	Sigma
High Capacity cDNA archive kit	Applied Biosystems
Loading Dye (6X)	Promega
Molecular Grade Water	Sigma
Optical adhesive covers	Applied Biosystems
PCR tubes	Sarstedt
RNA*later*^{TM}	Ambion
RNase away	Invitrogen
RNase-free microtubes	Ambion
RNase Zap wipes	Ambion
Total RNA isolation kit	Macherney-Nagel
Taqman gene expression assays (see table 2.2)	Applied Biosystems
Taqman universal PCR master mix	Applied Biosystems

10 X TBE buffer	Invitrogen
96-well optical reaction plates	Applied Biosystems

Western Blotting Reagents and Antibodies

Anti-mouse IgG HRP	Sigma
Anti-rabbit IgG HRP	Sigma
Anti-Trk A antibody	Santa Cruz
Anti-Trk B antibody	Upstate Cell Signaling
Anti-Trk C antibody	Santa Cruz
Anti c-fos antibody	Calbiochem
ERK2 antibody	Santa Cruz
Hyperfilm	Pierce
Nitrocellulose membrane	Amersham
pERK antibody	Santa Cruz
Pi3K antibody	Santa Cruz
pPi3K antibody	Santa Cruz
PC12 cells (positive control)	Santa Cruz
p75 antibody	Gift from Dr.Phil Barker
Prestained dual band molecular weight standard	Biorad
ReBlot Plus strong antibody stripping solution	Chemicon
SuperSignal®	Pierce

2.2 Animals

Male Wistar rats aged between 2 and 4 months were used in all experiments. They were an inbred strain supplied by the BioResources Unit (BRU) of Trinity College Dublin and weighed between 250 and 350g. Animals were housed in groups of 4. 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.

2.3 Surgical procedure and drug delivery

Rats were anesthetized with ketamine (100μg/kg; Bayer Healthcare, Bioresources, Trinity College Dublin, Ireland) and xylazine (100mg/kg: Rompun®, Bayer Healthcare, Bioresources, Trinity College Dublin, Ireland) and supplemented throughout the surgical procedure as necessary. The procedure takes approximately 1 hour in total. Before starting the surgery the rats head is shaved using as shavers or scissors. Using a sterile scalpel an incision is made over the rats skull, the skin is held back using forceps, the top of the skull is scraped with the scalpel to remove all meninges and to stop any bleeding, this should clearly expose bregma. A single hole was drilled in the skull over the left ventricle (coordinates, bregma, 0.9mm; midline, 1.6mm). At this stage dental glue is applied to the skull and allowed to dry, the cannula (Bilaney Consultants Ltd., Kent, UK) is then lowered slowly into the ventricle to a depth of 2.6mm 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). The rats were closely monitored for the first 24 hours following surgery. Rats were given 7-10 days to fully recover before being tested. The Trk receptor antagonist Tyrphostin AG879 (8μg) (Calbiochem) or the individual neurotrophin antagonists anti-BDNF, anti-NGF, anti-NT3, anti-NT4/5 (5μl) (All Chemicon) or controls (rabbit serum, normal goat serum or DMSO; 5μl) were administered via implanted cannula one hour prior to performing the object recognition task.

2.4 The Object Recognition Task

The apparatus consists of a black circular open field (Figure 2.1, diameter, 0.9m; height, 0.48m) placed in a dimly-lit room, in which were placed objects constructed from Lego. All rats were handled daily for 1 week and habituated to the arena; with 10 min of exploration in absence of objects each day for 3 days before the actual experiment was performed. Objects were fixed to the floor of the arena, 15cm from the walls. Objects were cleaned thoroughly between trials to eliminate any olfactory cues. For the object recognition task, the exploration criteria were based strictly on active exploration. The rats must be seen to have at least touched the object with their nose for it to be deemed as true exploration. The discrimination between familiar and novel objects is measured by time spent exploring and not on the basis of the rat's first choice. Measurement of the time spent actively exploring each object was recorded and expressed as a percentage of total exploration time in seconds.

Consolidation Protocol: On day 1, rats were placed in the arena containing two different objects for three 5 min periods in which to explore the objects with an inter-trial interval of 5 min. During the 5 min interval rats were placed in a holding cage in the room. 24 hr later one object was exchanged for a novel object and rats were reintroduced into the arena for a single 5 min period, and time spent actively exploring each object was recorded. For the neutralizing study, animals were administered the neutralizing antibodies or control one hour prior to commencing the experiment.

Reconsolidation Protocol: On day 1, rats were placed in the arena containing two different objects for three 5 min periods in which to explore the objects with an inter-trial interval of 5 min. During the 5 min interval rats were placed in a holding cage in the room. 24 hrs later rats were placed back in the arena for a single 5 min period with the same objects as day 1 and time spent actively exploring each object was recorded.

Figure 2.1. Picture of Arena used in object recognition tasks consists of a black circular open field (diameter, 0.9m; height, 0.48m) which is placed in a dimly-lit room. Animals were habituated to the arena prior to starting the experiment

Figure 2.2. Picture of arena containing example objects A and B (constructed from lego) used for training in the object recognition task.
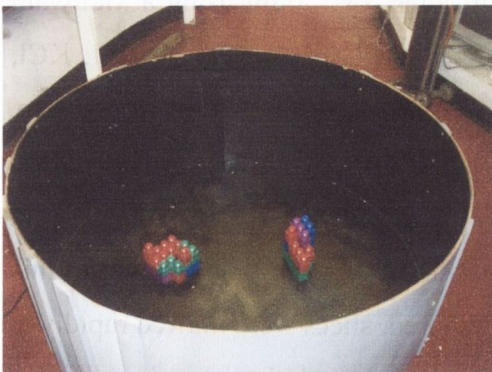

Figure 2.3 Picture of arena containing example objects A and C used for testing in the object recognition task

2.5 Preparation of tissue

Animals were killed by cervical dislocation and decapitation. The brains were quickly removed and placed on ice and the hippocampi, dentate gyrii, perirhinal cortices and entorhinal cortices were dissected free. All discections were performed with the use of Paxinons and Watsons rat brain atlas. To dissect the entorhinal cortex, use a forceps to pinch of the approximate area where the entorhinal cortices are located at the back edge of both hemispheres as per the rat brain atlas. To remove the perirhinal cortices, forceps were used to pinch away the tissue on both sides of the rhinal fissure on both sides of the brain. To remove the hippocampus and the dentate gyrus, one must begin by slicing off the olfactory bulbs and sitting the brain upright on this end, using a scalpel and a forcepts the grey matter from the centre of the brain can be removed, the brain will lie flat on its outer surface, using the forcepts the hippocampus should role off gently from the inside of the remaining tissue, once the hippocampus is separated flip it over onto its front and from the back surface, approximately in the centre there is discrete line indicating the start of the dentate gyrus, again with the forceps gently push along this line and the dentate will come away from the rest of the hippocampal structure. The complete procedure takes approximately 2 min.

2.6 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, hippocampus, perirhinal cortex or entorhinal cortex, was sliced bi-directionally 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).

2.7 Protein quantification using the Bradford assay

Protein concentrations were calculated according to the method of Bradford (1976). Samples, which were analyzed 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 absorbance was 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.

2.8 Preparation of samples for gel electrophoresis

Slices of dentate gyrus, hippocampus, perirhinal cortex or entorhinal cortex were thawed rapidly, washed 3 times in Krebs solution containing 2mM $CaCl_2$ and homogenized (x 10 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 (100µl) were added to sample buffer (100µl; composition: Tris-HCL pH 6.8, 0.5M; SDS, 10% w/v; glycerol, 10% v/v; 2-β-mercaptoethanol, 5% w/v; bromophenol blue, 0.05% w/v). The samples were boiled for 2-3 min.

2.9 Gel electrophoresis

Acrylamide gels, 7.5% or 10% (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) were loaded into the wells and run at 30mA for approximately 40mins.

2.10 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 or for 2hr at room temperature 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 supersignal® West Dura chemiluminescence reagents (Pierce). The membranes were then exposed to photographic (Hyperfilm, Amersham, UK) film and developed using a Fuji Processor.

2.11 Analysis of Trk Receptor Expression

Analysis of Trk receptors was performed using 7.5% SDS Page gels.

Expression of the Trk A receptor was assessed, non-specific binding was blocked by incubation of the nitrocellulose membrane in TBS-T containing 5% BSA for 2hr at room temperature. The membrane was then washed for 10mins, three times in TBS-T and incubated with the primary antibody (rabbit anti-Trk A monoclonal IgG; Santa Cruz; 1:500 in TBS-T containing 2% BSA) overnight at 4°C. The membrane was washed three times for 10min and incubated with the secondary antibody (goat anti-rabbit IgG-HRP; Sigma; 1:2000 in TBS containing 2% BSA) for 1hr at RT. The membrane was washed three times for 10min in TBS-T and one 5min wash with distilled water, before SuperSignal® (Pierce) was added for 5min. The membrane was exposed to the photographic film in the dark and the film was development using a Fuji X-ray processor.

Analysis of Trk B expression began with blocking non-specific binding of the nitrocellulose membrane by incubation with PBS-T containing 5% dried milk for 2hr at room temperature. The membrane was then washed for 10mins, three times in PBS-T and incubated with the primary antibody (rabbit anti-Trk B monoclonal IgG; Upstate; 1:500 in PBS-T containing 2% dried milk) for overnight at 4°C. The membrane was washed once for 5min and incubated with the secondary antibody (goat anti-rabbit IgG-HRP; Sigma; 1:1000 in PBS containing 2% dried milk) for 1 and 1/2 hr at RT. The membrane was washed three times for 10min in TBS-T, SuperSignal® was then added for 5min. The membrane was exposed to the photographic film in the dark and the film was development using a Fuji X-ray processor.

For analysis of Trk C receptor expression, non-specific binding was blocked by incubation of the nitrocellulose membrane in TBS-T containing 5% BSA for 2hr at room temperature. The membrane was then washed for 10mins, three times in TBS-T and incubated with the primary antibody (rabbit anti-Trk C monoclonal IgG; Santa Cruz; 1:500 in TBS-T containing 2% BSA) overnight at 4°C. The membrane was washed three times for 10min and incubated with the secondary antibody (goat anti-rabbit IgG-HRP; Sigma; 1:1000 in TBS containing 2% BSA) for 1hr at RT. The membrane was washed three times for 10min in TBS-T and one 5min wash with distilled water, before SuperSignal® was added for 5min. The membrane was exposed to the photographic film in the dark and the film was development using a Fuji X-ray processor.

2.12 Analysis of P75 Receptor Expression

Expression of the p75 receptor was assessed on 7.5% SDS Page gels. The nitrocellulose membrane was blocked for non-specific binding by incubation with TBS-T containing 5% BSA for 2hr at room temperature. The membrane was then washed for 10mins, three times in TBS-T and incubated with the primary antibody (rabbit anti-p75 monoclonal IgG; gift from Dr. Phil Barker; 1:200 in TBS-T containing 2% BSA) for overnight at 4°C. The membrane was washed three times for 10min and incubated with the secondary antibody (goat anti-rabbit IgG-HRP; Sigma; 1:500 in TBS containing 2%

BSA) for 1hr at RT. The membrane was washed three times for 10min in TBS-T and one 5min wash with distilled water, before SuperSignal® was added for 5min. The membrane was exposed to the photographic film in the dark and the film was development using a Fuji X-ray processor.

2.13 Analysis of the activity of protein kinases

Protein kinase activity was assessed using 10% SDS Page Gels. 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. 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. 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). 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.

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In the case of PI3K 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-T and incubated with the primary antibody (mouse anti-phospho PI3K monoclonal IgG; Santa Cruz; 1:500 in TBS-T containing 2% BSA) overnight at 4°C. The membrane was washed three times for 10min and incubated with the secondary antibody (goat anti-mouse IgG-HRP; Amersham; 1:1000 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® for 5min. The membrane was then exposed to the photographic film for 5-10 seconds, depending on the strength of the signal. The film was processed using a Fuji X-ray processor.

Following Western immunoblotting for PI3K 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 PI3K expression. 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 PI3K (1:500 dilution in TBS containing 2% BSA; Santa Cruz, California, USA). Membranes were incubated overnight at 4°C in the presence of the antibody and washed for 10 min 3 times in TBS-T. The secondary antibody (1:1000 dilution; goat anti-mouse IgG-HRP in TBS-T containing 0.1% 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 and the film was development using a Fuji X-ray processor.

2.14 Analysis of the activity of the transcription factor c-fos

In the case of c-fos expression, 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 (rabbit anti-phospho c-fos monoclonal IgG; Santa Cruz; 1:100 in TBS-T containing 2% BSA) for 2hr at RT. The membrane was washed three times for 10min and incubated with the secondary antibody (goat anti-rabbit IgG-HRP; Sigma; 1:100 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 the film was development using a Fuji X-ray processor.

2.15 Analysis of β-Actin Expression

Following Western immunoblotting for protein of interest, all blots were stripped with an antibody stripping solution (1:10 dilution in dH_2O; ReBlot plus Strong Antibody Stripping Solution; Chemicon) and reprobed for β-actin expression, an endogenous control gene used 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 actin (1:1000 dilution in TBS-T containing 2% BSA; Sigma). 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 (1:2000 dilution; goat anti-mouse IgG-HRP in TBS-T 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 the film was development using a Fuji X-ray processor.

Primary Antibody	Dilution	Secondary Antibody	Dilution
Trk A (Santa Cruz)	1:500	Anti-Rabbit (Sigma)	1:2000
Trk B (Upstate)	1:500	Anti-Rabbit (Sigma)	1:1000
Trk C (Santa Cruz)	1:500	Anti-Rabbit (Sigma)	1:1000
p75 (Gift from Dr.Phil Barker)	1:200	Anti-Rabbit (Sigma)	1:200
pERK (Santa Cruz)	1:3000	Anti-Mouse (Sigma)	1:1000
ERK 2 (Santa Cruz)	1:1000	Anti-Mouse (Sigma)	1:1000
pPI3K (Santa Cruz)	1:500	Anti-Mouse (Sigma)	1:1000
PI3K (Santa Cruz)	1:500	Anti-Mouse (Sigma)	1:1000
c-fos (Santa Cruz)	1:100	Anti-Rabbit (Sigma)	1:100
Actin (Sigma)	1:1000	Anti-Mouse (Sigma)	1:2000

Table 2.1 List of Antibodies used for western Immunoblotting for proteins of interest.

2.16 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).

2.17 Preparation of Samples for ELISA

Slices of dentate gyrus, hippocampus, perirhinal cortex or entorhinal cortex were thawed rapidly, washed 3 times in Krebs solution containing 5mM $CaCl_2$. The slices

were then incubated in Krebs solution (250µl) at 37°C with O_2 for 10 min. Slices were spun down at 500g for 5 min and the supernatant was removed for later assessment of BDNF by ELISA. Following this, the slices were then stimulated with 250µl Krebs containing 50mM KCL and incubated at 37°C with O_2 for 10 min. Tissue was spun down again at 500g for 5 min and the supernatant was removed for later assessment of BDNF by ELISA. Slices were then homogenized in Krebs solution and spun down at 11,000g for 10min and the supernatant removed for later assessment of BDNF by ELISA.

2.18 Analysis of BDNF concentration by ELISA

BDNF expression was analyzed in supernatant prepared from stimulated slices of dentate gyrus, hippocampus, perirhinal cortex and entorhinal cortex or from supernatant prepared from homogenized samples of the same brain regions using ELISA (Enzyme Linked Immunosorbant Assay). 96-well plates (Nunc-Immuno plate with maxiSorp surface) were coated with anti-BDNF antibody pAb (100µl; 10µl in 10ml carbonate coating buffer; Promega) and incubated at room temperature over night. Plates were washed in TBS-Tween (0.05%, pH 7.6), a Block and Sample 1X Buffer was added to the wells (200µl; 42.4ml deionized water with 10.6ml of Block and Sample 5X Buffer; Promega) and the plates were incubated for 1 hr at room temperature without shaking. Plates were washed in TBS-Tween and duplicate (100µl) sample and standards (100µl; 0-500pg/ml; BDNF Standard), were added to plates and incubated for 2 hrs at room temperature with agitation. Subsequent to further washing, Anti-Human BDNF mAb (100µl; 20µl Anti-Human BDNF mAb to 9.98ml of Block and Sample 1X Buffer) was added to each well and incubated at room temperature for 2 hrs with agitation. The plates were washed and detection reagent (100µl; 50µl Anti-IgY HRP conjugated to 9.95ml Block and Sample 1X Buffer) was added to each well and incubated for 1 hr at room temperature with agitation. Plates were again washed with TBS-Tween and room temperature TMB One Solution (100µl) was added and incubated at room temperature for 10min with agitation or until the color developed. After this time the reaction was stopped using 100µl 1N hydrochloric acid. Plates were read at 450nm using a 96 well plate reader (Labsystems, Multiskan RC) and BDNF concentrations were estimated

following extrapolation from the standard curve and results were expressed as pg BDNF/ml.

2.19 Analysis of NGF

NGF expression was analyzed in supernatant prepared from stimulated slices of dentate gyrus, hippocampus, perirhinal cortex and entorhinal cortex or from supernatant prepared from homogenized samples of the same brain regions using ELISA. 96-well plates (Nunc-Immuno plate with maxiSorp surface) were coated with capture antibody (100µl; 0.4µg/ml in PBS; R&D Systems) and incubated at room temperature over night. Plates were washed in PBS-Tween (0.05%, pH 7.2-7.4), a blocking buffer, regent diluent was then added to the wells (300µl; 1% BSA in PBS) and the plates were incubated for 1 hr at room temperature without shaking. Plates were washed in PBS-Tween and duplicate (100µl) sample and standards (100µl; 0-1000pg/ml; NGF Standard; R&D Systems), were added to plates and incubated for 2 hrs at room temperature. Subsequent to further washing, detection antibody was added to the plate (100µl; 100ng/ml in reagent diluent) was added to each well and incubated at room temperature for 2 hrs. The plates were washed and Streptavidin-HRP (100µl; 1:40 dilution in reagent diluent; R&D Systems) was added to each well and incubated for 20min at room temperature away from direct light. Plates were again washed with PBS-Tween and room temperature Substrate Solution (100µl) was added and incubated at room temperature for 20min or until the color developed, also been kept out of direct light. After this time the reaction was stopped using 50µl 2N sulphuric acid. Plates were read at 450nm using a 96 well plate reader (Labsystems, Multiskan RC) and NGF concentrations were estimated following extrapolation from the standard curve and results were expressed as pg NGF/ml.

2.20 Analysis of NT 3

NT3 expression was analyzed in supernatant prepared from stimulated slices of dentate gyrus, hippocampus, perirhinal cortex and entorhinal cortex or from supernatant prepared from homogenized samples of the same brain regions using ELISA. 96-well

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plates (Nunc-Immuno plate with maxiSorp surface) were coated with anti-NT3 antibody (100µl; 10µl in 10ml carbonate coating buffer; Promega) and incubated at 4°C over night. Plates were washed in TBS-Tween (0.05%, pH 7.6), a Block and Sample 1X Buffer was added to the wells (200µl; 42.4ml deionized water with 10.6ml of Block and Sample 5X Buffer; Promega) and the plates were incubated for 1 hr at room temperature without shaking. Plates were washed in TBS-Tween and duplicate (100µl) sample and standards (100µl; 0-300pg/ml; NT3 Standard), were added to plates and incubated for 6 hrs at room temperature with agitation. Subsequent to further washing, Anti-NT3 mAb (100µl; 20µl Anti-NT3 mAb to 9.98ml of Block and Sample 1X Buffer) was added to each well and incubated at 4°C overnight without agitation. The plates were washed and detection reagent (100µl; 50µl Anti-IgG HRP conjugated to 9.95ml Block and Sample 1X Buffer) was added to each well and incubated for 2.5 hr at room temperature with agitation. Plates were again washed with TBS-Tween and room temperature TMB One Solution (100µl) was added and incubated at room temperature for 10min with agitation or until the color developed. After this time the reaction was stopped using 100µl 1N hydrochloric acid. Plates were read at 450nm using a 96 well plate reader (Labsystems, Multiskan RC) and NT3 concentrations were estimated following extrapolation from the standard curve and results were expressed as pg NT3/ml.

2.21 Analysis of NT 4/5

NT4/5 expression was analyzed in supernatant prepared from stimulated slices of dentate gyrus, hippocampus, perirhinal cortex and entorhinal cortex or from supernatant prepared from homogenized samples of the same brain regions using ELISA. 96-well plates (Nunc-Immuno plate with maxiSorp surface) were coated with capture antibody (100µl; 1.0µg/ml in PBS; R&D Systems) and incubated at room temperature over night. Plates were washed in PBS-Tween (0.05%, pH 7.2-7.4), a blocking buffer, regent diluent was then added to the wells (300µl; 1% BSA in PBS) and the plates were incubated for 1 hr at room temperature without shaking. Plates were washed in PBS-Tween and duplicate (100µl) sample and standards (100µl; 0-2000pg/ml; NT4/5 Standard; R&D Systems), were added to plates and incubated for 2 hrs at room temperature. Subsequent to further washing, detection antibody was added to the plate

(100µl; 100ng/ml in reagent diluent) was added to each well and incubated at room temperature for 2 hrs. The plates were washed and Streptavidin-HRP (100µl; 1:40 dilution in reagent diluent; R&D Systems) was added to each well and incubated for 20min at room temperature away from direct light. Plates were again washed with PBS-Tween and room temperature Substrate Solution (100µl) was added and incubated at room temperature for 20min or until the color developed, also been kept out of direct light. After this time the reaction was stopped using 50µl 2N sulphuric acid. Plates were read at 450nm using a 96 well plate reader (Labsystems, Multiskan RC) and NT4/5 concentrations were estimated following extrapolation from the standard curve and results were expressed as pg NT4/5/ml.

2.22 Harvesting tissue for mRNA expression

Samples of dentate gyrus, hippocampus, perirhinal cortex or entorhinal cortex from each rat were placed in RNase-free tubes containing RNA*later*^{TM} and stored for 7 days at 4°C. All samples were then removed from the RNA*later*^{TM} solution, transferred to fresh RNase-free tubes and frozen at -85°C until RNA extraction was performed.

2.23 Preparation of Tissue for real time polymerase chain reaction (PCR)
RNA extraction procedure

A total RNA isolation kit (Macherney-Nagel) was used to extract RNA. Briefly, samples were removed from freezer, placed in 350µl of RA1 buffer and 3.5µl of β–mercaptoethanol and homogenized using a polytron tissue disrupter (kinetatica). Sample homogenate was added to Neucleospin® Filter units and filtered by centrifugation at 13000rpm for 1 minute. 350µl of 70% ethanol was added to each sample lysate and mixed by pipetting up and down approximately 5 times. Each sample mix was placed in Neurleospin® RNA II columns and centrifuged at 13,000 rpm for 30 seconds to bind the RNA to the silica membrane. Following the centrifugation the column was placed in a new collecting tube and 350µl of membrane desalting buffer (supplied) was added. The column was then centrifuged at 13,000 rpm for 1 minute. DNA was digested using rDNase and DNase Reaction Buffer (supplied). rDnase was diluted 1:10 in DNase Reaction Buffer and 95µl of this solution was pipetted directly into the centre of the silica

column. Samples were incubated with DNase mix for 15 minutes at room temperature. 200µl of buffer RA2 was added to the column and centrifuged at 13000 rpm for 30 seconds, following which, the column was placed in a new collecting tube. 600µl of RA3 buffer (50ml of ethanol was added to 25ml of RA3 buffer concentrate) was added to the column and centrifuged at 13000 rpm for 30 seconds. The flow-through was discarded and the collecting tube re-used for the second RA3 wash. 250µl of RA3 buffer was added to the column and centrifuged at 13000 rpm for 2 minutes. Column was placed in fresh RNase –free microtube and RNA eluted by addition of 60µl of H_2O and centrifugation at 13000rpm for 1 minute. Eluted RNA was then frozen and stored at -85°C for qualification, quantification and reverse transcription.

Assessment of RNA quality

RNA was separated on a 1% agarose gel to check integrity of extracted RNA samples. 1.3g of agarose was added to 130ml of TBE (1.0M Tris, 0.9M Boric acid, 0.01M EDTA) and fully dissolved by heating in microwave. The solution was allowed to cool such that it could be hand-held before 1.3µl ethidium bromide was carefully added and swirled to mix. The agarose solution was poured into a sealed agarose gel tray containing a comb and allowed to solidify for approximately 30 minutes. The tray was then transferred to a gel tank (model) and flooded with 1X TBE running buffer. 3µl of RNA sample was mixed with 2µl DEPC H_2O and 1µl of loading dye, 4µl of this mixture loaded onto a gel. RNA was separated using 90 volts for 30 minutes up to 1.5 hrs (or until yellow dye at front reaches the end of the gel). At this point the power supply was disconnected from the gel rig and the gel taken to UV transilluminator and RNA visualized. Only extracted RNA that demonstrated visible 28S and 18S ribosomal RNA bands were used as this indicated that the RNA had not been degraded during the extraction process.

RNA Quantification

Optical density (OD) of RNA was measured using a spectrophotometer (UV/vis Beckman Coulter Du730) to determine RNA concentration and purity. Concentration of RNA can be measured due to its ability to absorb light at 260nm. As an OD reading of

1.0 at 260nm is equivalent to an RNA concentration of 40μg/ml, sample RNA concentration can be quantified using the following equation: RNA = OD_{260} X dilution factor X 40μg/ml.

The purity of RNA may also be established by measuring absorbance at 280nm. A ratio of OD_{260}/OD_{280} of approx. 1.8-2.0 is indicative of pure RNA. All RNA samples used had ratios of >1.5. RNA concentrations were then equalized so that equal concentrations of RNA could be used as a template for cDNA transcription. Samples were aliquoted in equal volumes until reverse-transcribed

Reverse Transcription of RNA

A high capacity cDNA archive kit (Applied Biosystems) was used to reverse transcribe samples to extracted using the total RNA isolation kit (Macherney-Nagel). Briefly, 20-50μl of 0.5-2.5μg of RNA was mixed in a PCR mini-tube with an equal volume of 2X master mix that was made up as follows: 1.5 dilution of 10X Reverse Transcription Buffer , 1:12.5 dilution of 25X dNTPs, 1:5 dilution of Random Primers, 1:10 dilution of MultiScribe Reverse Transcriptase and 1:2.381 dilution H_2O. Samples were then placed in thermal cycler and incubated at 25°C for 10 minutes followed by 2 hr incubation at 37°C. Resultant cDNA was frozen at -20°C until ready for real time polymerase chain reaction (PCR) analysis.

2.24 Real Time PCR

Gene expression of targets (see table for list) was assessed using off the shelf Taqman gene expression assays containing specific target primers, and FAM-labelled MGB target probes. β–actin gene expression was used to normalize gene expression between samples, and was quantified using a β–actin endogenous control gene expression assay containing specific primers, and a VIC-labeled MGB probe for rat β–actin.

Gene Name	Assay Number	NCBI Gene Reference*
Trk A	Rn00572130_m1	NM 021589.1
Trk B	Rn00820626_m1	NM 012731.1
Trk C	Rn00570389_m1	NM 019248.1
P75	Rn00561634_m1	NM 012610.1
BDNF	Rn00560868_m1	-------
NGF	Rn00824646_m1	NM 031523.1
NT 3	Rn00579280_m1	NM 031073.2
NT 4/5	Rn01645105_m1	NM 013184.2

Table 2.2 List of Gene assays used.

* Gene reference as listed on the national centre for biotechnology information (NCBI) Entrez-Nucleotide website:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide

Plate set-up for multi-target (multiplex) QPCR: Briefly. cDNA was diluted 1:4 and 10μl of diluted cDNA was pipetted onto a PCR plate, to which 1.25μl of target primer/probe and 1.25μl β–actin primer/probe and 12.5μl of Taqman master mix was added (25μl reaction volume). Samples were run in duplicate, and electronic (EDP3 20-200μl, 2-20μl, and 10-100μl) were used to ensure pipetting accuracy.

Samples were placed in a real time PCR thermocycler (Applied Biosystems 7300) using the following programme; step 1: 95°C for 10 minutes, step 2: 95°C for 15 seconds

followed by 1 minute at 60°C. Step two was repeated 40 times, and fluorescence read during the annealing and extension phase (60°C) for the duration of the program.

During step two of the PCR reaction, the double stranded coda is denatured at 95°C for 15 seconds. As the temperature begins to fall to 60°C (annealing and extension) the target probe is first to anneal to the single-stranded coda as it has a higher melting temperature that the target primers (Applied Biosystems). This probe contains FAM/VIC dye and a proprietary non-fluorescent quencher (NFQ) dye, this dye prevents the dye from emitting a fluorescence signal by fluorescence resonance energy transfer (FRET) technology (Applied Biosystems). At 60°C the primers anneal and the strand is extended by 5' nuclease activity of Taq polymerase. This displaces the FAM/VIC-labeled probe causing the FRET between the dye and quencher to be broken, and the generation of a fluorescence signal. Due to the specify of the probe and primers for the cDNA sequence, one fluorescence signal is generated for each new cDNA copy and measured during the annealing stage of the PCR cycle (60°C).

Real-time PCR analysis

The ΔΔCT method (Applied Biosystems RQ software, Applied Biosystems, UK) was used to assess gene expression for all real-time PCR analysis. This method is used to assess relative gene expression by comparing gene expression of experimental samples to a normal/control sample, rather than quantifying the exact copy number of the target gene. In this manner the fold-difference (increase-decrease) can be assessed between experimental and control samples. The fold-difference is assessed using the cycle number (CT) difference between samples. Briefly, a threshold for fluorescence is set, against which CT is measured. To accurately assess differences between gene expression the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is 100% efficient. Thus samples with low CT readings demonstrate high fluorescence, indicating greater amplification and hence, greater gene expression. When a PCR is 100% efficient a one-cycle difference between samples means a 2-fold difference in copy number (2^2), similarly a 5-fold cycle difference is a 32-fold difference (2^5).

To measure this fold-difference relative to control, the CT of the endogenous control (β-actin) is subtracted from the CT of the target gene for each sample, thus accounting for any difference in cDNA quantity that may exist. This normalized CT value is called the (ΔCT). The CT difference (ΔCT) of the control is subtracted from itself to give 0, and subtracted from all other samples, this is the $\Delta\Delta$CT value. The $\Delta\Delta$CT (cycle difference corrected for β-actin) is then converted into a fold-difference. As a one-cycle difference corresponds to a two-fold increase or decrease relative to control, 2 to the power of the $-$ $\Delta\Delta$CT (difference in control and sample CT corrected for actin) gives the fold-difference in gene expression between the control and experimental samples. The control sample always has a $\Delta\Delta$CT value of 0, thus 2^{-0} gives a $2^{-\Delta\Delta CT}$ of 1, against which all other samples are referenced, as outlined in Table below.

	Target CT	β-actin CT	ΔCT	$\Delta\Delta$CT	$2^{-\Delta\Delta CT}$ Fold Difference
Control	20	16	20 -16 = 4	4 -4 = 0	2^{-0} = 1
Sample 1	21	16	21 -16 = 5	5 -4 = 1	2^{-1} = 0.5
Sample 2	22	16	22 -16 = 6	6 -4 = 2	2^{-2}= 0.25
Sample 3	19	16	19 -16 = 3	3 -4 = -1	$2^{-(-1)}$ = 2
Sample 4	18	16	18 -16 = 2	2 -4 = -2	$2^{-(-2)}$ = 4
Sample 5	17	16	17 -16 = 1	1 -4 = -3	$2^{-(-3)}$ = 8

2.25 Statistics

Data are expressed as means $\pm$ SEM. In the *in vitro* and object recognition experiments a two-way analysis of variance (ANOVA) was performed to determine whether there were significant differences between groups. When this analysis indicated significant differences between conditions, post hoc Student Newman-Keuls test analysis was used to determine which conditions were significantly different from each other.

Where appropriate the student T-test for independent means was used to establish statistical significance. Statistical analysis was carried out using the statistical package Graphpad Prism 4.0.

Chapter 3

An investigation of the role of discrete neurotrophin subtypes in recognition memory

3.1 Introduction

Acquisition and consolidation of newly-acquired information are essential for formation of long-lasting memories, and both these processes may share common signaling pathways. Consolidation of long-term memory is a multi-step process comprised of complex molecular cascades leading to a robust and effective inter-neuronal communication. Learning-induced synthesis of new proteins is ascribed to play a pivotal role in the consolidation process (Bekinschtein *et al.*, 2007) but detailed information covering specific proteins and timing of their translation is still lacking.

Neurotrophins are classically regarded as the mediators of growth, differentiation and maintance of the developing nervous system. It was not until the early 1990's with the work by Lohof and colleagues that the neurotrophins also emerged as key modulators of the mature nervous system. Since this many studies have been produced linking neurotrophins to synaptic plasticity, learning & memory and LTP (Bekinschtein *et al.*, 2007, Kelly *et al.*, 2003, Patterson *et al.*, 1997). BDNF and NGF and their corresponding receptors Trk B and Trk A have been shown to play a fundamental role in synaptic plasticity (Korte *et al.*, 1996; Kelly *et al* 1998; Gooney and Lynch, 2001; Krause *et al.*, 2007). To a lesser extent the potential role of NT4/5 in LTP has been investigated (Fischer *et al.*, 1994). A role for BDNF in generation of LTP in the dentate gyrus has been suggested by the evidence that BDNF infusion potentiates the synaptic response (Messaoudi *et al.*, 1998) and that LTP induces an increase in BDNF mRNA (Bramham *et al.*, 1996). In 1999, it was reported by Maguire and collegues that NGF and its receptor Trk A played a role in LTP. This was demonstrated with the use of the tyrosine receptor kinase (Trk) inhibitor tyrphostin AG879, which blocked NGF-mediated cellular signalling and hence inhibited the expression of LTP in the dentate gyrus. A role for NT4/5 in plasticity is suggested by the report that NT4/5 mutant mice displayed an impairment in LTP and long term memory, indicating that loss of NT4/5/Trk B signalling may disrupt protein synthesis essential for long term memory storage (Xie *et al.*, 2000). Although NT3 and the Trk C receptor are promenently expressed in the hippocampus (Linden *et al.*, 2000) there is little evidence supporting their possible role in learning and memory. One study found that deletion of Trk B but not Trk C significantly reduces

spine density of CA1 pyramidal neurons thus possibly compromising the efficency of LTP (Halbach *et al.*, 2007).

The object recognition memory task, in which the rat is required to discriminate between a novel and familiar object, is the learning paradigm of choice in this study. It is a suitable task to target both acquisition and consolidation as it relies upon the rats propensity to explore novelty. It allows a series of novel objects to be explored by the rat at timepoints corresponding to phases of acquisition and consolidation. In this study we investigated the roles of neurotrophins in both acquisition and consolidation of memory by using neutralising antibodies against each neurotrophin isoform and by targeting the Trk receptors using the Trk receptor antagonist tyrphostin AG879.

3.2 Materials and methods

Male Wistar rats aged between 2 and 4 months weighing between 250 and 350g were used for these experiments. Rats were anesthetized and surgery was performed to implant cannula as described in section 2.3. Following surgery animals were allowed to recover for 7-10 days before training in the object recognition task (for details see section 2.4). Animals were administered with the Trk receptor antagonist Tyrphostin AG879 (i.c.v.; 5μl, 1.6μg/ml, i.p.; 100μl, 1.6μg/ml; Calbiochem) or the individual neurotrophin antagonists anti-BDNF, anti-NGF, anti-NT3, anti-NT4/5 (5μl, 1mg/ml; all Chemicon) or controls (rabbit serum, normal sheep serum or DMSO; 5μl) via implanted cannula one hour prior to training in the object recognition task. In some experiments animals were injected intraperitoneally (i.p.) with tyrphostin AG879 (100μl; 1.6μg/ml) one hour prior to training in the object recognition task.

3.3 Results

3.3.1 Intraperitioneal administration of the tyrosine kinase receptor Trk antagonist Tyrphostin inhibits recognition memory in the rat

Tyrphostin AG879 (100μl, 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl; 10% DMSO in ddH$_2$O) was administered intraperitioneally (i.p.) one hour prior to training in the object recognition task on day 1. Rats were tested at 24hr to test memory consolidation. Both control and tyrphostin-treated rats explored objects equally during the training phase (Figure 3.1 A and B; Control A; 48.29 ± 5.35%, B; 51.57 ± 5.35%; tyrphostin A: 47.26 ± 2.703%, B; 52.74 ± 2.7% seconds). The time spent exploring each object is expressed as a percentage of the total exploration time. 24hr following training object B was replaced with a novel object C and rats were placed back in the arena and allowed to explore. Vehicle-treated rats successfully recognised the familiar object A, spending significantly greater time exploring the novel object C (***$p<0.0001$; 1-way ANOVA, *post hoc* Newman Keuls, Control A: 24.89 ± 4.65%, C: 75.11 ± 4.65%). In contrast, tyrphostin-treated rats failed to identify the novel object (A: 42.57 ± 5.03%, C: 47.43 ± 5.03% seconds).

3.3.2. Intracerebroventricular administration of the receptor tyrosine kinase receptor Trk antagonist tyrphostin AG879 inhibits recognition memory in the rat

Tyrphostin AG879 (5μl, 1.6μg/ml, Chemicon) or vehicle (DMSO; 5μl; 10% DMSO in ddH$_2$O) was administered intracerebroventricularly (i.c.v.) via an implanted cannula one hour prior to training in the object recognition task on day 1. Rats were tested at 10min following the training phase to assess acquisition and again at 24hr to test memory consolidation. Both control-treated and tyrphostin treated rats explored objects equally during the training phase (Figure 3.2 A and B; Control A; 47.81 ± 2.199%, B; 52.18 ± 2.201%; tyrphostin A; 46.17 ± 15.46%, B; 53.83 ± 15.46% seconds). The time spent exploring each object is expressed as a percentage of the total exploration time. 10 minutes following training object B was replaced with a novel object C and rats were placed back in the arena and allowed to explore. Vehicle treated rats successfully

recognised the familiar object A, spending significantly greater time exploring the novel object C (***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls, Control A; 25.3 ± 13.21%, C; 74.7 ± 13.21%). In contrast, tyrphostin-treated rats failed to identify the novel object (A: 52.56 ± 13.70%, C: 47.44 ± 13.70% seconds). Rats were also tested at 24hours following the training phase; at this time object C was replaced with the novel object D and rats were placed back in the arena and allowed explore. Again, vehicle-treated rats effectively identified the novel object, spending significantly greater time exploring object D (***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls, Control A: 21.96 ± 4.389%, D: 78.03 ± 4.389%) and the tyrphostin-treated rats failed to recognize the familiar object spending equal time exploring both objects (A: 42.57 ± 12.33%, D: 57.43 ± 12.33% seconds).

3.3.3 Administration of anti-BDNF antibody inhibits recognition memory in the rat

Anti-BDNF (5μl; 1mg/ml, Chemicon) or vehicle (sheep serum; 5μl) was administered i.c.v. via an implanted cannula, one hour prior to training in the object recognition task on day 1. The object recognition task was used to assess acquisition and consolidation of recognition memory in the rat. The task takes place over 2 days and assesses the ability of the rat to discriminate between a novel and a familiar object. Rats were tested at 10 min following the training phase to assess acquisition and again at 24hr to test memory consolidation. Both control treated and anti-BDNF treated rats explored objects equally during the training phase (Figure 3.3 A and B; Control A: 51.45 ± 3.939%, B: 48.51 ± 3.923%; anti-BDNF A: 56.78 ± 4.187%, B: 43.25 ± 4.192% seconds). Time spent exploring each object is expressed as a percentage of the total exploration time (mean ± SEM, n=6). 10min following training object B was replaced with a novel object, C, and rats were placed back in the arena and allowed to explore. Both vehicle-treated rats and anti-BDNF treated rats successfully recognised the familiar object A, spending significantly greater time exploring the novel object C (***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls, Control A: 16.23 ± 3.799%, C: 84.26 ± 3.616%; anti-BDNF, A: 24.36 ± 4.91%, C: 75.64 ± 4.91% seconds). Rats were also

tested at 24hours following the training phase; at this time object C was replaced with the novel object, D, and rats were placed back in the arena and allowed explore. Again, vehicle treated rats effectively identified the novel object, spending significantly greater time exploring object D (***$p<0.0001$; 1-way ANOVA, *post hoc* Newman Keuls, Control A: $10.66 \pm 6.616\%$, D: $89.31 \pm 6.596\%$ seconds) and the anti-BDNF rats failed to recognize the familiar object spending equal time exploring both objects (A: $50.49 \pm 3.643\%$, D: $49.51 \pm 3.643\%$ seconds).

### 3.3.4 Administration of anti-NGF antibody inhibits recognition memory in the rat

Anti-NGF (5µl; 1mg/ml, Chemicon) or vehicle (rabbit serum, 5µl) was administered i.c.v. via implanted cannula one hour prior to training in the object recognition task on day 1. Rats are tested at 10 minutes following the training phase to assess acquisition and again at 24hr to test memory consolidation. Both control treated and anti-NGF treated rats explored objects equally during the training phase (Figure 3.4 A and B; Control A: $54.12 \pm 8.922\%$, B: $44.24 \pm 9.231\%$; anti-NGF A: $49.02 \pm 1.905\%$, B: $49.98 \pm 3.984\%$ seconds). Results are expressed as a percentage of the total exploration time (mean $\pm$ SEM, n=6). 10 minutes later, object B was replaced with a novel object, C, and rats were placed back in the arena and allowed to explore. Both vehicle-treated rats and anti-NGF treated rats successfully recognised the familiar object A, spending significantly greater time exploring the novel object C (***$p<0.0001$; 1-way ANOVA, *post hoc* Newman Keuls, Control A: $5.99 \pm 7.074\%$, C: $94.01 \pm 7.074\%$; anti-NGF, A: $26.4 \pm 4.37\%$, C: $73.6 \pm 4.37\%$ seconds). Rats were also tested at 24hr following the training phase; at this time object C was replaced with the novel object D and rats were placed back in the arena and allowed explore. Again, vehicle-treated rats effectively identified the novel object, spending significantly greater time exploring object D (***$p<0.0001$; 1-way ANOVA, *post hoc* Newman Keuls, Control A: $16.87 \pm 16.47\%$, D: $83.13 \pm 16.47\%$). However the anti-NGF-rats failed to recognize the familiar object spending equal time exploring both objects (A: $47.43 \pm 3.74\%$, D: $52.56 \pm 3.74\%$ seconds).
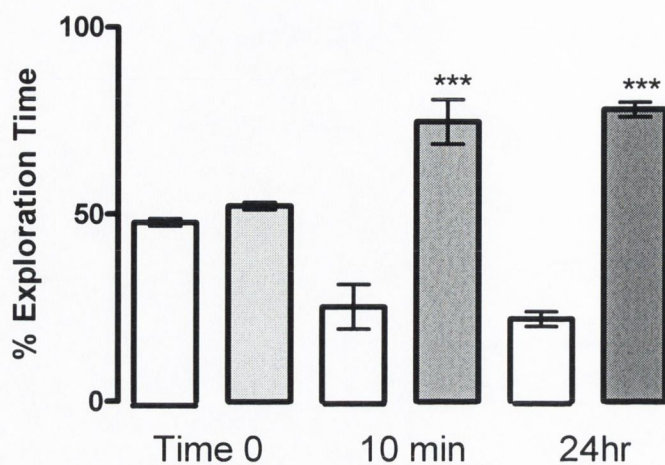
3.3.5 Administration of anti-NT3 antibody inhibits recognition memory in the rat

Anti-NT3 (5μl; 1mg/ml Chemicon) or vehicle (rabbit serum; 5μl) was administered i.c.v. via implanted cannula one hour prior to training in the object recognition task on day 1. Rats were tested at 10 minutes following the training phase to assess acquisition and again at 24hr to test memory consolidation. Both control treated and anti-NT3 treated rats explored objects equally during the training phase (Figure 3.5 A and B; Control A: $54.12 \pm 8.922\%$, B: $44.24 \pm 9.231\%$; anti-NT3 A: $51.49 \pm 7.052\%$, B: $48.51 \pm 7.053\%$ seconds). The time spent exploring each object is expressed as a percentage of the total exploration time (mean $\pm$ SEM, n=6). 10 minutes following training; object B was replaced with a novel object C and rats were placed back in the arena and allowed to explore. Vehicle-treated rats successfully recognised the familiar object A, spending significantly greater time exploring the novel object C, the anti-NT3 treated rats also identify the novel object and spent significantly greater time exploring it (***$p<0.0001$; 1-way ANOVA, *post hoc* Newman Keuls, Control A; $5.99 \pm 7.074\%$, C; $94.01 \pm 7.074\%$; anti-NT3 A; $20.96 \pm 10.05\%$, C; $68.04 \pm 31.23\%$ seconds). Rats were also tested at 24hr following the training phase, at this time object C was replaced with the novel object D and rats were placed back in the arena and allowed explore. Again vehicle treated rats and anti-NT3 treated rats effectively identified the novel object, spending significantly greater time exploring object D (***$p<0.0001$; 1-way ANOVA, *post hoc* Newman Keuls, Control A; $16.87 \pm 16.47\%$, D; $83.13 \pm 16.47\%$; anti-NT3 A; $20.09 \pm 7.892\%$, D; $79.93 \pm 7.892\%$ seconds).

3.3.6 Administration of anti-NT4/5 antibody inhibits recognition memory in the rat

Anti-NT4/5 (5μl; 1mg/ml, Chemicon) or vehicle (sheep serum; 5μl) was administered i.c.v. via implanted cannula one hour prior to training in the object recognition task on day 1. Rats are tested at 10 minutes following the training phase to assess acquisition and again at 24hr to test memory consolidation. Both control treated and anti-NT4/5 treated rats explored objects equally during the training phase (Figure 3.6

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A and B; Control A: 51.45 ± 3.939%, B: 48.51 ± 3.923%; anti-NT4/5 A: 54.04 ± 6.644%, B: 45.82 ± 6.959% seconds). The time spent exploring each object is expressed as a percentage of the total exploration time (mean ± SEM, n=6). 10 minutes following training object B was replaced with a novel object C and rats were placed back in the arena and allowed to explore. Both vehicle-treated rats and NT4/5-treated rats successfully recognised the familiar object A, spending significantly greater time exploring the novel object C (***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls, Control A; 16.23 ± 3.799%, C; 84.26 ± 3.616%; anti-NT4/5, A: 8.73 ± 2.31%, C: 91.23 ± 2.31% seconds). Rats were also tested at 24hr following the training phase; at this time object C was replaced with the novel object D and rats were placed back in the arena and allowed explore. Again vehicle treated rats effectively identified the novel object, spending significantly greater time exploring object D (***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls, Control A; 10.66 ± 6.616%, D; 89.31 ± 6.596%) and the anti-NT4/5-treated rats failed to recognize the familiar object spending equal time exploring both objects (A: 49.27 ± 1.658%, D: 50.73 ± 1.658% seconds).

A. Control
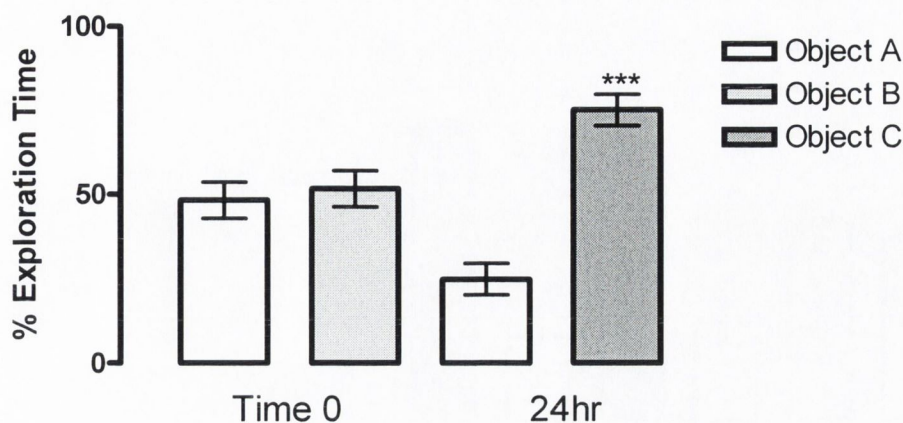
B. Tyrphostin AG879 (i.p. administration)
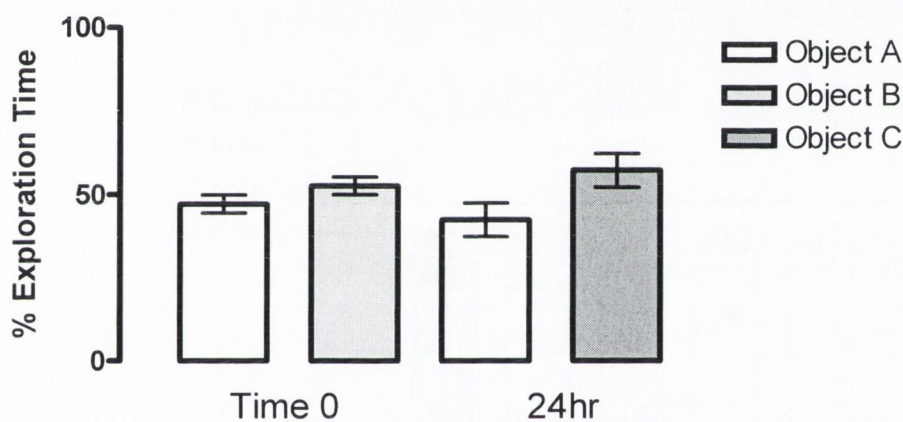
Figure 3.1 I.P. administration of the receptor tyrosine kinase antagonist tyrphostin AG879 inhibits recognition memory in the rat

Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hr prior to training in the object recognition task on day 1. Results are expressed as a percentage of the total exploration time (mean ± SEM, n=6). Both groups explored objects A and B equally during training in the task. Veh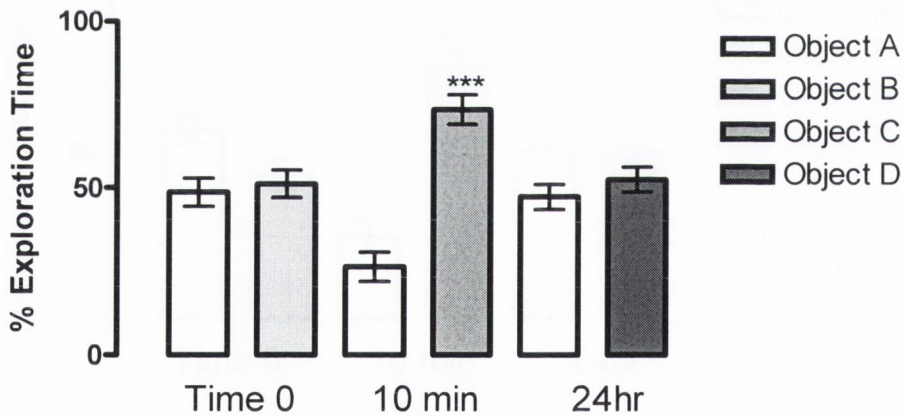icle treated rats (**A**) successfully recognised that object C was the novel object when acquisition was tested at the 24hr timepoint and spent significantly more time actively exploring the novel object; ***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls. Tyrphostin AG879 treated rats (**B**) failed to identify the novel object when tested 24hr after training.

A. Control

B. Tyrphostin AG879 (i.c.v. administration)
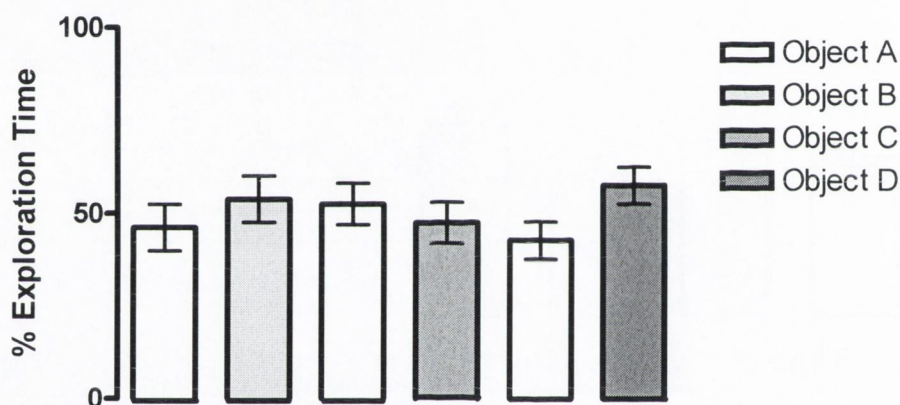
Figure 3.2 I.C.V. administration of the receptor tyrosine kinase antagonist tyrphostin AG879 inhibits recognition memory in the rat

Tyrphostin AG879 (5μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 5μl) was administered i.c.v. via implanted cannula one hour prior to training in the object recognition task on day 1. Results are expressed as a percentage of the total exploration time (mean ± SEM, n=6). Both groups explored objects A and B equally during training in the task. Vehicle treated rats (**A**) successfully recognised that object C was the novel object when acquisition was tested at the 10 min timepoint and spent significantly more time actively exploring the novel object; ***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls. Tyrphostin AG879 treated rats (**B**) failed to identify the novel object

when tested at 10 minutes and spent equal time exploring both objects. Vehicle treated rats (**A**) also successfully identified object D as the novel object when memory consolidation was tested 24 hr after training, animals spent significantly more time actively exploring the novel object D; ***p<0.0001; 1-way ANOVA, *post hoc* Newmans Keuls. However rats treated with tyrphostin AG879 (**B**) failed to recognise the novel object D when testing memory consolidation at the 24 hr timepoint and spent equal time exploring both objects A and D.
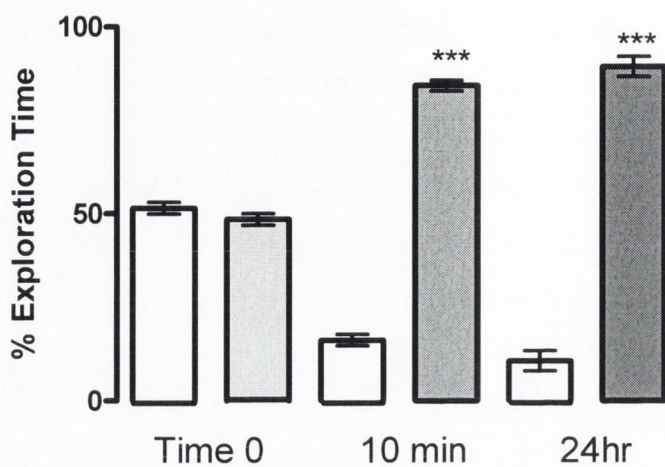
A. Control
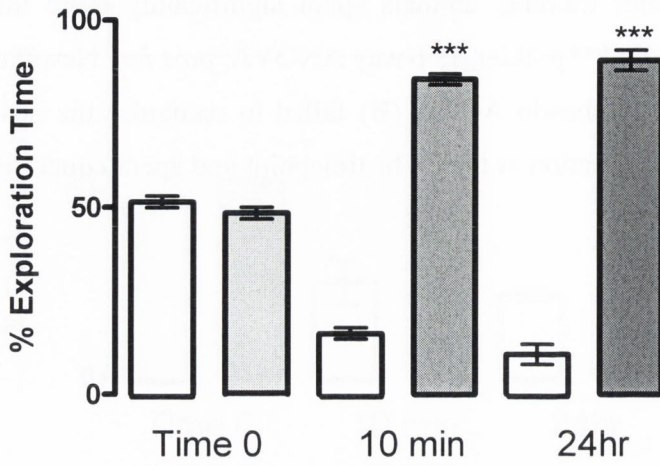
B. Anti-BDNF
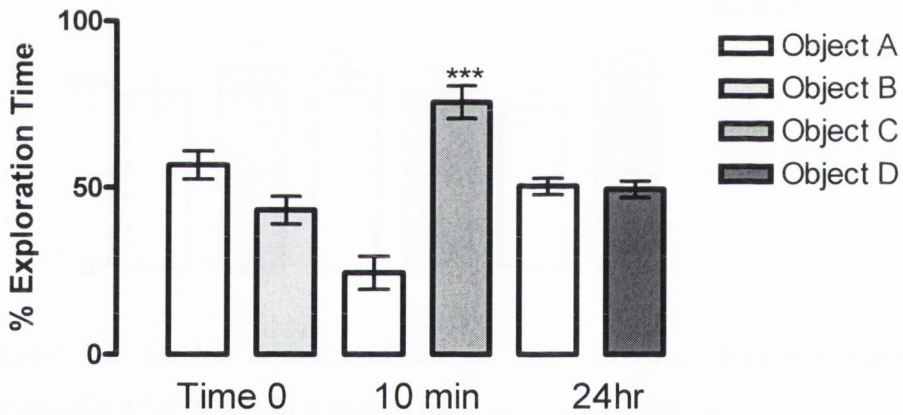
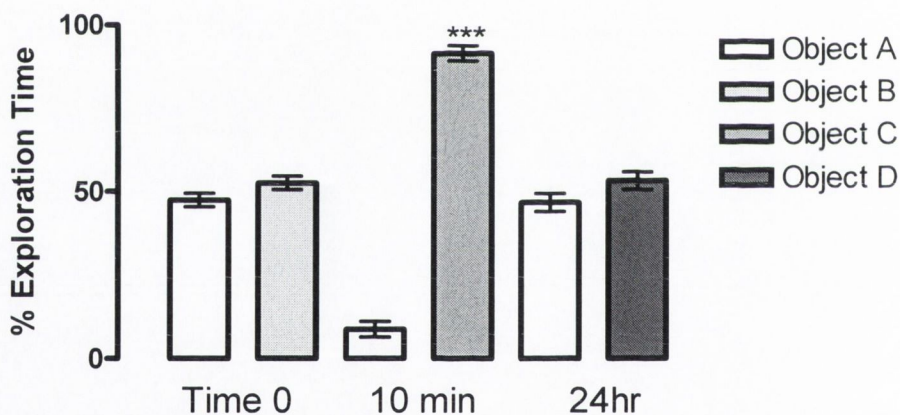**Figure 3.3 Administration of anti-BDNF antibody inhibits recognition memory in the rat**

Anti-BDNF (5μl; 1mg/ml) or vehicle (sheep serum; 5μl) was administered i.c.v. via implanted cannula one hour prior to training in the object recognition task on day 1. Results are expressed as a percentage of the total exploration time (mean ± SEM, n=6). Both groups explored objects A and B equally during training in the task. Both vehicle treated rats (**A**) and anti-BDNF treated rats (**B**) successfully recognised that object C was

the novel object when acquisition was tested at the 10 min timepoint and spent significantly more time actively exploring the novel object; ***p<0.0001 for both; 1-way ANOVA, *post hoc* Newman Keuls. Vehicle treated rats (**A**) also successfully identified object D as the novel object when memory consolidation was tested 24 hr after training. These animals spent significantly more time actively exploring the novel object D; ***p<0.0001; 1-way ANOVA, *post hoc* Newmans Keuls. However rats treated with anti-BDNF (**B**) failed to recognise the novel object D when testing memory consolidation at the 24 hr timepoint and spent equal time exploring both objects A and D.

A. Control
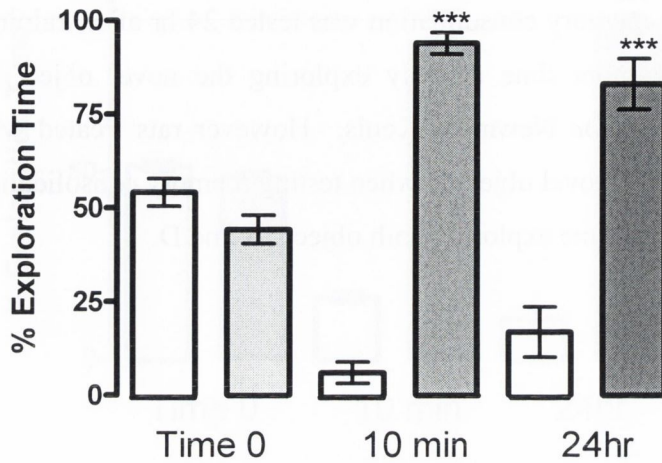
B. Anti-NGF

Figure 3.4 Administration of anti-NGF antibody inhibits recognition memory in the rat

Anti-NGF (5μl; 1mg/ml) or vehicle (rabbit serum; 5μl) was administered i.c.v. via implanted cannula one hour prior to training in the object recognition task on day 1. Results are expressed as a percentage of the total exploration time (mean ± SEM, n=6). Both groups explored objects A and B equally during training in the task. Both vehicle

treated rats (**A**) and anti-NGF treated rats (**B**) successfully recognised that object C was the novel object when acquisition was tested at the 10 min timepoint and spent significantly more time actively exploring the novel object; ***p<0.0001 for both; 1-way ANOVA, *post hoc* Newman Keuls. Vehicle treated rats (**A**) also successfully identified object D as the novel object when memory consolidation was tested 24 hr after training. These animals spent significantly more time actively exploring the novel object D; ***p<0.0001; 1-way ANOVA, *post hoc* Newmans Keuls. However rats treated with anti-NGF (**B**) failed to recognise the novel object D when testing memory consolidation at the 24 hr timepoint and spent equal time exploring both objects A and D.

A. Control
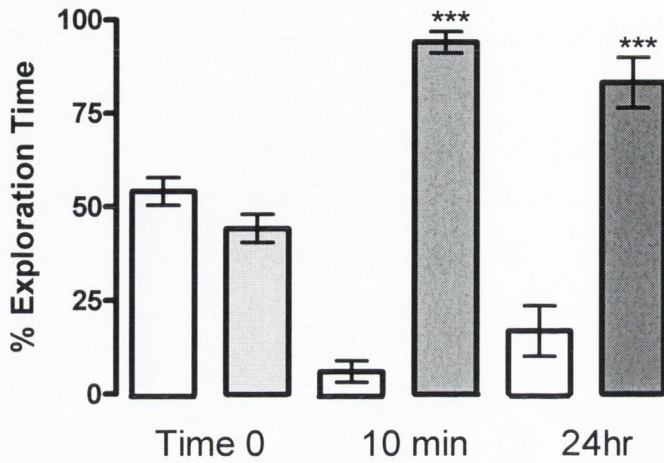
B. Anti-NT3
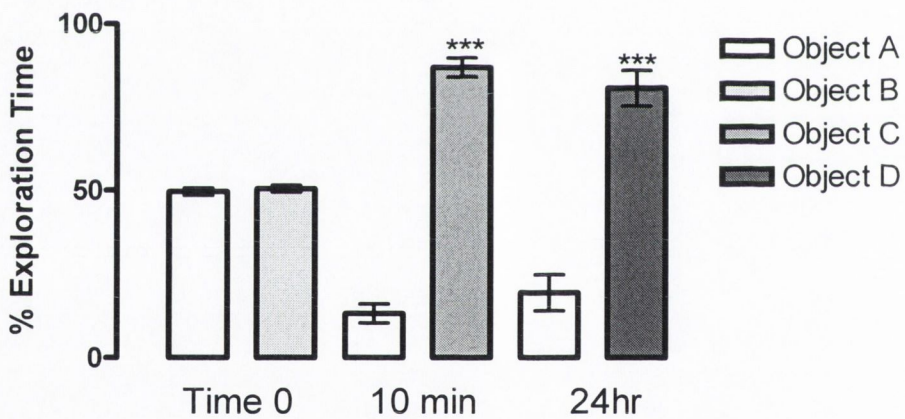
Figure 3.5 Administration of anti-NT3 antibody does not inhibit recognition memory in the rat

Anti-NT3 (5μl; 1mg/ml) or vehicle (rabbit serum; 5μl) was administered i.c.v. via implanted cannula one hour prior to training in the object recognition task on day 1. Results are expressed as a percentage of the total exploration time (mean ± SEM, n=6). Both groups explored objects A and B equally during training in the task.   Both vehicle treated rats (**A**) and anti-NT3 treated rats (**B**) successfully recognised that object C was

the novel object when acquisition was tested at the 10 min timepoint and spent significantly more time actively exploring the novel object; ***p<0.0001 for both; 1-way ANOVA, *post hoc* Newman Keuls. Vehicle treated rats (**A**) also successfully identified object D as the novel object when memory consolidation was tested 24 hr after training, animals spent significantly more time actively exploring the novel object D; ***p<0.0001; 1-way ANOVA, *post hoc* Newmans Keuls. Similarly anti-NT3 treated rats (**B**) recognised the novel object D when testing memory consolidation at the 24 hr timepoint and spent significantly more time exploring the novel object D; ***p<0.0001; 1-way ANOVA, *post hoc* Newmans Keuls.

A. Control

Anti-NT4/5

Figure 3.6 Administration of anti-NT4/5 antibody inhibits recognition memory in the rat

Anti-NT4/5 (5µl; 1mg/ml) or vehicle (5µl) was administered i.c.v. via implanted cannula one hour prior to training in the object recognition task on day 1. Results are expressed as a percentage of the total exploration time (mean ± SEM, n=6). Both groups explored objects A and B equally during training in the task. Both vehicle treated rats (**A**) and anti-NT4/5 treated rats (**B**) successfully recognised that object C was the novel

object when acquisition was tested at the 10 min timepoint and spent significantly more time actively exploring the novel object; ***p<0.0001 for both; 1-way ANOVA, *post hoc* Newman Keuls. Vehicle treated rats (**A**) also successfully identified object D as the novel object when memory consolidation was tested 24 hr after training. These animals spent significantly more time actively exploring the novel object D; ***p<0.0001; 1-way ANOVA, *post hoc* Newmans Keuls. However rats treated with anti-NT4/5 (**B**) failed to recognise the novel object D when testing memory consolidation at the 24 hr timepoint and spent equal time exploring both objects A and D.

3.4 Discussion

The most remarkable feature of the brain is its capacity to acquire information from the environment and store it to produce changes in behavior. Memory processing may be divided into at least four different stages including acquisition, consolidation, storage and retrieval. A mounting body of evidence has emerged during the last decade suggesting that neurotrophins play a crucial role in learning and memory. Consistent with this view the primary objective of this study was to investigate the possible roles of neurotrophins and their receptors in acquisition and consolidation of memory in the rat. Furthermore this study attempted to identify the roles of the neurotrophins individually in both acquisition and consolidation of recognition memory. These data suggest that Trk receptor activation by neurotrophins is essential to acquisition and consolidation of recognition memory, that neurotrophins do not individually regulate acquisition but that all neurotrophins, with the exception of NT3, are required for the processes involved in consolidation of recognition memory.

Here the object recognition task was used to assess learning in the rat. This learning paradigm assesses the ability of animals to recognize the presence of a novel object. It is a suitable task to target both acquisition and consolidation of information as it allows a series of novel objects to be explored by the rat at time points corresponding to phases of acquisition and consolidation. In this case the exploration was timed manually by the experimenter in the preference to using a computerized tracking programme. It has been suggested that these programmes may not differentiate between active exploration of objects and mere proximity of the rat to the objects.

Tyrphostin AG879 has previously been used to block LTP and spatial memory by inhibition of the Trk receptors (Maguire *et al.*, 1999, Gooney *et al.*, 2002). It has never been used before in assessment of acquisition and consolidation of recognition memory as in this study. Trk receptors share extremely similar structural arrangements. Presently there are no pharmacological tools available to block these receptor subtypes individually *in vivo*, therefore we chose to use tyrphostin AG879 and block all the Trk receptors non-

specifically. Tyrphostins have been shown to inhibit autophosphorylation of protein tyrosine kinases (Lyall *et al.*, 1989) and more specifically tyrphostin AG879 has been shown to block Trk A signaling indicating that AG879 competes for the substrate binding site, preventing signaling, which renders tyrphostins useful inhibitors for investigating possible roles and signaling mechanisms of the Trk receptors (Ohmichi *et al.*, 1993, Rende *et al.*, 2000). Here we identified that both i.p. and i.c.v. administration of tyrphostin AG879 blocks both acquisition and consolidation of recognition memory by inactivation of the neurotrophin receptors Trk A, Trk B and Trk C. This data suggests that both acquisition and consolidation of recognition memory is dependent upon Trk receptor activation by neurotrophins, however we cannot discount the possibility that tyrphostin AG879 may be exerting non-specific effects on signaling.

Having established a role for tyrosine kinase activation in recognition memory, we used neutralizing antibodies to target the neurotrophins individually. It was considered that antibody binding would prevent the binding of neurotrophins to their preferred receptors. The antibodies chosen have less that 1% cross-reactivity with the other neurotrophins, according to the manufacturer, lending confidence to the specificity of their effects. This strategy allowed us to discriminate between the actions of BDNF and NT4/5, which are ligands at the same receptor, Trk B.

BDNF is the most extensively-investigated of the neurotrophins and there is ample evidence supporting a role for BDNF in learning and memory (Bramham *et al.*, 1996, Mizuno *et al.*, 2003, Krause *et al.*, 2007). The first evidence that BDNF may be involved in long-term synaptic plasticity came from the demonstration that the expression of BDNF mRNA is enhanced by LTP-inducing tetanic stimulation of CA1 (*Patterson et al.*, 1992) and the dentate gyrus (Castren *et al.*, 1993, Dragunow *et al.*, 1993). BDNF appears to play a particularly important role in the hippocampus, an area associated with a number of types of memory formation and synaptic plasticity; indeed this region has the highest neuroanatomical expression of BDNF and its Trk B receptor in the mammalian brain (Murer *et al.* 2001). BDNF has previously been shown to be involved in short and long term memory formation of a hippocampal-dependent one-trial fear-motivated learning task in rats (Alonso., 2002). Consistent with these data, in this study we identified a role for BDNF in consolidation of recognition memory. We found that anti-

BDNF treated animals were unaffected when tested 10min following training, suggesting that BDNF function is not essential to memory acquisition, but when animals were tested at 24hr we found these animals were unable to identify the novel object suggesting that memory consolidation was interrupted by blocking BDNF. This result is partly consistent with other data showing that inhibition of BDNF can impair both short and long term fear consolidation memory in the rat (Alonso *et al.*, 2005). Although BDNF also binds to the p75 receptor with low affinity, all known synaptic effects of BDNF mediating plasticity are mediated by Trk B activation. Here we found that blockade of the Trk receptors blocked both acquisition and consolidation of recognition memory. In a similar study investigating BDNF and Trk B in spatial memory Mizuno and colleagues (2003) found that inhibition of Trk B by a tyrosine kinase antagonist PP2 delayed memory acquisition in the radial arm maze, suggesting the importance of BDNF/Trk B signaling in spatial memory. Direct evidence for BDNF regulation of hippocampal LTP, the best known model of synaptic plasticity in the brain, comes from studies in which BDNF signaling is inhibited, either by BDNF gene knockout, BDNF antisense oligonucleotides, or BDNF scavengers or antibodies. Here we provide evidence for the direct involvement of neurotrophin/Trk signaling in acquisition and consolidation of recognition memory in the rat.

NGF was the first of the neurotrophins to be discovered and is perhaps, next to BDNF, the most extensively researched in the context of learning and memory. Numerous studies have indicated a role for this neurotrophin in various forms of synaptic plasticity in a similar fashion to BDNF. Kelly and colleagues (1998) displayed a role for NGF in LTP of the perforant path-grannule cell synapse using an inbred strain of rats deficient in NGF, this deficiency was accompanied by an impairment in LTP. In addition, i.c.v. injection of NGF reversed this impairment. Here we support this evidence with our findings that NGF is necessary for consolidation of recognition memory in the rat. We found that anti-NGF treated animals could identify the novel object when tested at 10min following training, suggesting that NGF function is not essential to memory acquisition, but when animals were tested at 24hr we found these animals were unable to identify the novel object suggesting that memory consolidation of the objects was

interrupted by blocking of NGF. In agreement with this, NGF deprivation has been shown to cause spatial learning impairment and cause cholinergic hypofunction in the medial septal area (Van Der Zee *et al.*, 1995). Infusions of NGF have been shown to facilitate spatial memory performance (Janis *et al.*, 1995, Fischer *et al.*, 1996), NGF protein and NGF mRNA levels have been found to increase in the hippocampus after contextual learning (Kang and Schuman, 1995, Woolf *et al.*, 2001). In contrast to this evidence, one study found that NGF infusions decreased object discrimination in the object recognition task in both young and old rats compared to controls. The study also showed evidence that NGF infusions improves spatial memory in aged rats, suggesting that NGF is more important in spatial memory than recognition memory (Niewiadomska *et al.*, 2006). With the exception of the work of Niewiadomska and colleagues, the majority of evidence in the literature supports the findings of this study for a positive role of NGF and the Trk A receptor in recognition memory.

In contrast to the other neurotrophins, there is little or no evidence supporting a role for NT3 in memory formation. Most studies have seen insignificant changes in NT3 or Trk C expression following learning (Kang and Schuman, 1995, Bramham *et al.*, 1996, Chen *et al.*, 1999). In broad agreement with this we report that inhibition of NT3 had no effect on acquisition or consolidation of recognition memory, with anti-NT3 treated animals successfully identifying the novel objects when tested at both 10min and 24hr. For this reason NT3 appears to be of lesser importance than the other neurotrophins in mediation of synaptic plasticity. This result is perhaps surprising given that NT3 activates the Trk C receptor in a similar fashion to BDNF activation of Trk B, eliciting similar intracellular signaling cascades activating transcription. Although it has been reported that BDNF activation of Trk receptor elicits a much more potent response than NT3, it has also been reported that BDNF can evoke action potentials in neurons of the CNS as can NT3, however NT3 produces a much smaller response (Kafitz *et al.*, 1999, Becker *et al.*, 1998). Perhaps this may explain why, when NT3 is blocked it does not affect memory formation at any stage as it only produces a small response and this effect is not missed, although NT3 signaling is necessary for different functions in the peripheral nervous system where it may elicit a stronger response (Coppola *et al.*, 2003). In addition, the expression profile of BDNF and NT3 and their associated Trk receptors

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in the CNS may explain the lack of effect of NT3 inhibition on memory consolidation. BDNF/Trk B and NT3/Trk C are equally expressed through out the hippocampus, the amygdala, the hypothalmus and the entorhinal cortex (Muragaki *et al.*, 1995, Klein *et al.*, 1989, Martin-Zanca *et al.*, 1990, Lamballe *et al.*, 1991). Trk B, Trk C and BDNF mRNA have been shown to be expressed throughout the entorhinal cortex and the perirhinal cortex, however NT3 mRNA does not appear to be expressed to a significant level in the perirhinal cortex (Hashimoto *et al.*, 1999). The functional significance of this has remained unclear but it is possible that this is the underlying reason behind our observation that NT3 plays no role in this type of memory.

NT4/5, along with BDNF, is a ligand at the Trk B receptor. As discussed in the introduction this causes problems when attempting to distinguish between the signaling effects of BDNF and those of NT4/5. In this study we found that inhibition of NT4/5 blocks memory consolidation. We found that anti-NT4/5 treated animals could identify the novel object when tested at 10min following training, suggesting that NT4/5 function is not essential to memory acquisition. However, when these animals were tested at 24hr we found they were unable to identify the novel object, suggesting that memory consolidation of the objects was interrupted by blocking NT4/5. These data also suggests that BDNF activation of the Trk B receptor alone is not enough to support memory consolidation. Individual blockade of neurotrophins does not affect acquisition; these data together with our other findings suggests that neurotrophins may substitute for each other during the early stages of memory formation but they are all, with the exception of NT3, required to be present for memory consolidation and storage.

Here we have shown that acquisition of recognition memory is blocked by blockade of Trk receptors, indicating a role for Trk-stimulated signaling in this process. Specific blockade of any of the neurotrophins spares acquisition. Thus we may conclude that lack of stimulation of any of Trk A, Trk B or Trk C is insufficient to block acquisition.

BDNF/Trk B the signaling has been shown to enhance synaptic transmission postsynaptically by selectively modulating the NMDA subtype of glutamate receptors in the hippocampus (Levine *et al.*, 1995). NMDA has previously been established as a

crucial modulator in LTP and memory processing (McHugh etal., 2007, Nilsson *et al.*, 2007, Wang and Salter, 1994). Neurotrophin signaling mediates rapid membrane depolarization via regulation of Ca^{2+}, Mg^{2+} and Na^{2+} currents. Membrane depolarization causes release of glutamate into the synaptic cleft activating the NMDA receptors which has been shown to lower the threshold of LTP induction (Kovalchuk *et al.*, 2002) and is necessary in synaptic plasticity (Kafitz *et al.*, 1999). It is possible that neurotrophins mediate acquisition via modulation of NMDA activation.

In contrast to our findings regarding acquisition, we have shown that simultaneous activity of all neurotrophins, with the exception of NT3, is required to allow consolidation to occur. This leads us to suggest that activation of both Trk A (by NGF) and Trk B (by both BDNF and NT4/5) and stimulation of subsequent intracellular signaling cascades, underpins consolidation of recognition memory. We will explore these signaling cascades in subsequent chapters of this thesis.

The objective of this study was to identify the roles of neurotrophins and the Trk receptors in learning and memory using the object recognition task. This study succeeded in identifying a role for BDNF, NGF and NT4/5 in consolidation of recognition memory and in establishing Trk receptor activation as an essential mediator of acquisition and consolidation of recognition memory. We also report that NT3 does not appear to be as important in memory processing as the other neurotrophins.

Chapter 4

An investigation of changes in expression of neurotrophins and their receptors following learning

4.1 Introduction

The results presented in the previous chapter suggest a fundamental role for neurotrophins in learning and memory. Inhibition of BDNF, NGF or NT4/5 blocked memory consolidation but spared acquisition, and inhibition of all the Trk receptors by Tyrphostin AG879 blocked both acquisition and consolidation of recognition memory. From these results it may be hypothesised that individually, each neurotrophin with the exception of NT3 is essential to memory consolidation but that collectively they mediate acquisition. A clear role has been established for neurotrophins in the memory formation process, however in order to reveal the cellular mechanisms mediating storage of a memory trace, memory consolidation must be assessed over time. The aim of this study is to assess whether the consolidation of recognition memory over a 24hr period is associated with changes in expression of neurotrophins and their receptors.

Memory consolidation is commonly viewed as a process lasting several hours through which memories are transformed from a labile to a more stable state (Bliss *et al.*, 1993; Riedel *et al.*, 2003; Kandel *et al* 2000; Izquierdo *et al.*, 2006). Several studies have provided evidence for a role for neurotrophins in this process. BDNF was found to be increased at 4 hours in neurons of the CA1 following contextual fear conditioning and this increase could be blocked by the NMDA receptor antagonist, MK801 (Chen *et al.*, 2007). Another study examining neurotrophins in fear conditioning recorded BDNF, NGF, NT3 and NT4/5 mRNA expression level at various time points in the amygdala and found only BDNF mRNA was increased up to 2 hours but this change had returned to control levels by 4 hours (Rattiner *et al.*, 2004). In the same study the authors also used the tyrosine kinase inhibitor K252a to block Trk B in the amygdala and successfully blocked acquisition of fear conditioning. On further investigation into the role of neurotrophins in synaptic plasticity, LTP-inducing high frequency stimulation of the dentate gyrus of awake rats was found to trigger a rapid increase in Trk B and Trk C gene expression and a delayed increase in NT3 gene expression (Bramham *et al.*, 1996).

Neurotrophin immunoreactivity is found in several structures in the brain, including the neocortex, piriform cortex, amygdaloid complex, hippocampal formation, claustrum, some thalamic and hypothalamic nuclei, the substantia nigra and some brainstem structures. Not all neurotrophins are expressed in all areas involved in learning

and memory and it has been suggested that there is anterograde transport of endogenous neurotrophins in the central nervous system (Yan *et al*., 1997).

Here we assess changes in expression of neurotrophins their Trk receptors over the 24 hour period following learning through which consolidation takes place in structures of the medial temporal lobe which are believed to be involved in consolidation of recognition memory.

4.2 Materials and Methods

Male Wistar rats aged between 2 and 4 months weighing between 250 and 350g were used in these experiments. Rats were trained in the object recognition task as described in section 2.4. In this experiment animals only performed the training phase of the experiment on day 1 and were sacrificed at selected times following exploration of objects A and B. Rats were sacrificed at 0min, 2hr, 6hr and 24hr following memory consolidation. In this study, 2 control groups were included, one of which was sacrificed directly from the home cage (termed naïve) without any exposure to the arena or objects and the second group (termed control) was sacrificed following habituation to the arena without objects. Tissue was taken and biochemical analysis for Trk receptors, P75 receptor and the neurotrophins was performed by western immunoblotting (section 2.11 and 2.12), ELISA (section 2.18 to 2.21) and RTPCR (section 2.23 and 2.24).

4.3 Results

4.3.1 Exploration of objects by Timepoint groups.

The object recognition task was used to examine consolidation of recognition memory in the rat. The task generally takes place over 2 days and assesses the ability of the rat to recognize a novel object as in chapter 1, but in this experiment animals only performed the training phase of the experiment on day 1 and were sacrificed at specific timepoints following exploration of objects A and B. Rats were sacrificed at 0min, 2hr, 6hr and 24 hr following learning. All groups displayed equal exploration of objects A and B (Figure 4.1, 0min A; 48.99 ± 1.377, B; 51.01 ± 1.377, 2hr A; 51.45 ± 1.608 B; 48.51 ± 1.602, 6hr A; 49.02 ± .778, B; 49.98 ± 1.463, 24hr A; 44.24 ± 3.769, B; 54.12 ± 3.642). The time spent exploring each object is expressed as a percentage of the total exploration time (mean ± SEM, n=6). Two control groups used for this experiment, one was a naïve group, which were sacrificed directly from the home cage and the second control group, were habituated to the arena only.

Exploration
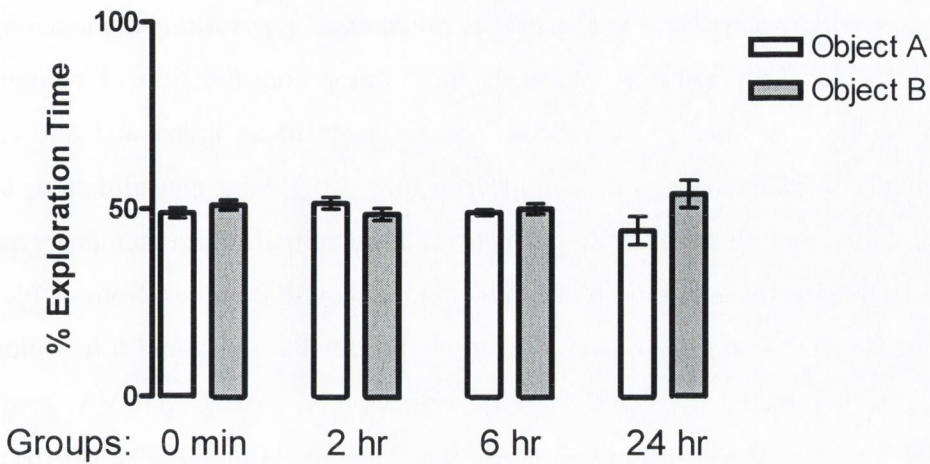
Figure 4.1 Exploration times of objects by Consolidation group

The time spent exploring each object by each of the groups was recorded and expressed as a percentage of total exploration time. Groups were only exposed to object A and B (3x5 min exposure) and then sacraficied, no test phase was carried out on these animals. Groups are differentiated by times they were sacrificed following exploration. Statistical analysis showed that there was no significance difference in the amount of time the animals spent exploring the two objects. Histograms represent mean percentage exploration time ± SEM, n=6, student unpaired t test.

Dentate Gyrus

4.3.2 BDNF message and protein expression in the dentate gyrus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of dentate gyrus from these animals were then analyzed for changes in BDNF mRNA and protein, and compared with samples from naïve and control animals. BDNF mRNA was significantly increased at 0min and 6 hr following training (Figure 4.2 A; $*p<0.05$ and $*p<0.05$ respectively; 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 0.425 ± 0.117; Control: 0.597 ± 0.084; 0min: 0.991 ± 0.101; 2hr: 0.616 ± 0.193; 6hr: 1.117 ± 0.125; 24hr: 0.771 ± 0.093). Results are expressed as means and standard error of the mean, n=6. BDNF protein was then analyzed in the dentate gyrus. Increases in BDNF protein expression were observed at all the above stated timepoints, however when statistically analyzed they proved not to be significant (Figure 4.2B, Naive; 968.9 ± 545.1, Control; 806.7 ± 365.9, 0min; 1139 ± 294.6, 2hr; 1746 ± 219.9, 6hr; 1335 ± 567.5, 24hr; 634.2 ± 149.4). Results are expressed as mean BDNF pg/µg protein $\pm$ SEM.

4.3.3 NGF message and protein expression in the dentate gyrus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of dentate gyrus from these animals were then analyzed for changes in NGF mRNA and protein and compared with samples from naïve and control animals. NGF mRNA was slightly increased at 0min and 6hr timepoints following training but this was not significant (Figure 4.3A; Naïve: 2.453 ± 0.734; Control: 1.865 ± 0.774; 0min: 3.933 ± 1.44, 2hr: 3.033 ± 0.612; 6hr: 3.999 ± 1.378; 24hr: 2.621 ± 0.842). Results are expressed as means and standard error of the mean, n=6. NGF protein was then analyzed in the dentate gyrus. Increases in NGF protein expression were observed at the 6 hr timepoint, when statistically analyzed this proved to be significant (Figure

4.3B, *p<0.05; 1-way ANOVA, *post hoc* Newman Keuls Naïve: 23.89 ± 10.37; Control: 104.5 ± 35.73; 0min: 198.9 ± 70.72; 2hr: 83.68 ± 22.93; 6hr: 275.1 ± 94.53; 24hr: 28.27 ± 7.146). Results are expressed as mean NGF pg/µg protein $\pm$ SEM.

4.3.4 NT3 message and protein expression in the dentate gyrus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of dentate gyrus from these animals were then analyzed for changes in NT3 mRNA and protein, and compared with samples from naïve and control animals. NT3 mRNA was decreased at all timepoints, and in control animals following exposure to the arena when compared to naïve animals. The 24hr timepoint was the only one, which was significantly decreased, compared to naive (Figure 4.4 A; *p<0.05; 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 3.798 ± 0.27; Control: 2.185 ± 0.273; 0min: 2.477 ± 0.481; 2hr: 2.148 ± 0.42; 6hr: 2.749 ± 0.614; 24hr: 1.806 ± 0.229). Results are expressed as means $\pm$ standard error of the mean, n=6. NT3 protein was then analyzed in the dentate gyrus. No differences in NT3 protein expression were observed between groups (Figure 4.4B). Results are expressed as mean NT3 pg/µg protein $\pm$ SEM.

4.3.5 NT4/5 message and protein expression in the dentate gyrus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of dentate gyrus from these animals were then analyzed for changes in NT4/5 mRNA and protein, and compared with naïve and control animals. NT4/5 mRNA was significantly increased at 0min and 2hr timepoints following learning (Figure 4.5A, **p<0.01 and ***p<0.0001 respectively; Naïve: 1.139 ± 0.088; Control: 0.918 ± 0.126; 0min: 1.737 ± 0.214; 2hr: 2.079 ± 0.202; 6hr: 0.937 ± 0.061; 24hr: 0.868 ± 0.154). Results are expressed as means $\pm$ standard error of the mean, n=6. NT4/5 protein was then analyzed in the dentate gyrus. No differences in NT4/5 protein

expression were observed between groups (Figure 4.5B). Results are expressed as mean NT4/5 pg/μg protein ± SEM

4.3.6 TrkA message and protein expression in the dentate gyrus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of dentate gyrus from these animals were then analyzed for changes in TrkA mRNA and protein, which was compared to naïve and control animals from the dentate gyrus. TrkA mRNA was significantly increased at 0min and 6hr following learning when compared to naïve animals (Figure 4.6 A; *p<0.05 and *P<0.05 respectively; 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 0.057 ± 0.031; Control: 0.034 ± 0.008; 0min: 0.527 ± 0.163; 2hr: 0.085 ± 0.06; 6hr: 0.672 ± 0.112; 24hr: 0.325 ± 0.143). Results are expressed as means ± standard error of the mean, n=6. TrkA protein was then analyzed in the dentate gyrus by Western Immunoblot. From the representitive TrkA blot picture (Figure 4.6B) there appears to be change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.6C). Results are expressed as percentage TrkA/Actin, mean ± SEM.

4.3.7 Trk B message and protein expression in the dentate gyrus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of dentate gyrus from these animals were then analyzed for changes in Trk B mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in Trk B mRNA following learning (Figure 4.7A). Results are expressed as means and standard error of the mean, n=6. Trk B protein was then analyzed in the dentate gyrus by Western Immunoblot. From the representitive Trk B blot picture (Figure 4.7B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.7C). Results are expressed as percentage Trk B /Actin, mean ± SEM.

4.3.8 Trk C message and protein expression in the dentate gyrus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of dentate gyrus from these animals were then analyzed for changes in Trk C mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in Trk C mRNA following learning (Figure 4.8A). Results are expressed as means ± standard error of the mean, n=6. Trk C protein was then analyzed in the dentate gyrus by Western Immunoblot. From the representitive Trk C blot picture (Figure 4.8B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.8C). Results are expressed as percentage Trk C/Actin, mean ± SEM.

4.3.9 P75 message and protein expression in the dentate gyrus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of dentate gyrus from these animals were then analyzed for changes in P75 mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in P75 mRNA following learning (Figure 4.9A). Results are expressed as means ± standard error of the mean, n=6. P75 protein was then analyzed in the dentate gyrus by Western Immunoblot. From the representitive P75 blot picture (Figure 4.9B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.9C). Results are expressed as percentage P75 /Actin, mean ± SEM.
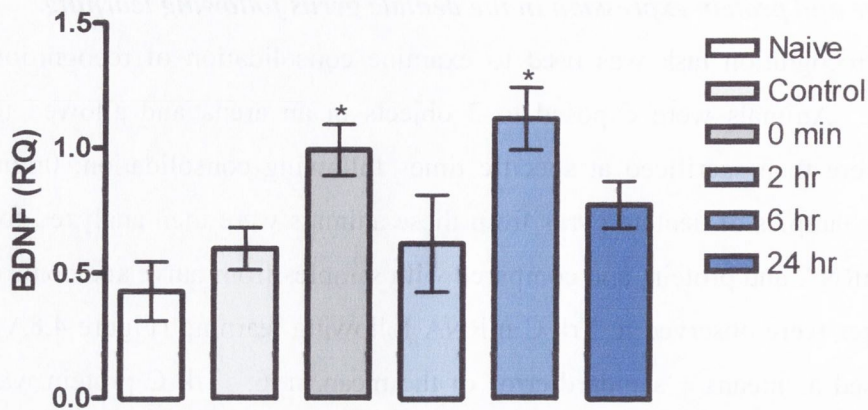
A. BDNF mRNA

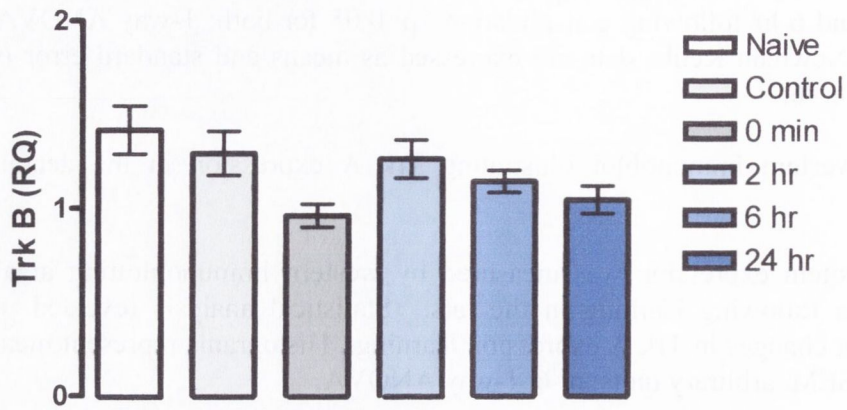## B. BDNF protein expression
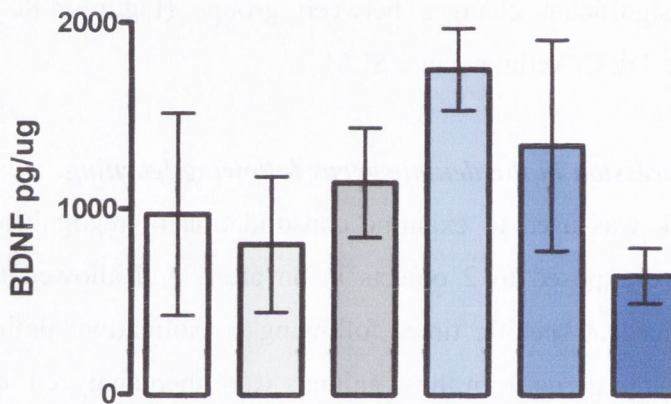
Figure 4.2 BDNF gene expression but not BDNF protein is increased in the dentate gyrus following learning in the rat.

A. BDNF mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed a significant increase in BDNF mRNA expression at 0min and 6hr following learning, *p<0.05 for both; 1-way ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.

B. BDNF expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in BDNF expression at any of the timepoints following consolidation when compared to controls. Histograms represent mean BDNF concentration ± SEM in pg/μg, 1-way ANOVA.

A. NGF mRNA
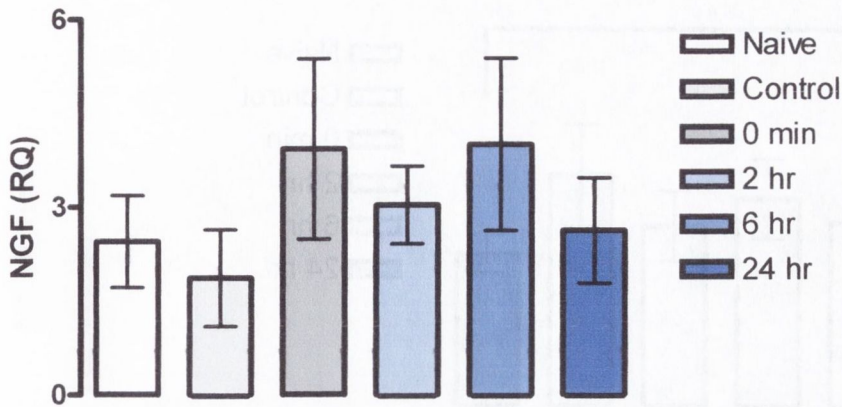
B. NGF protein expression
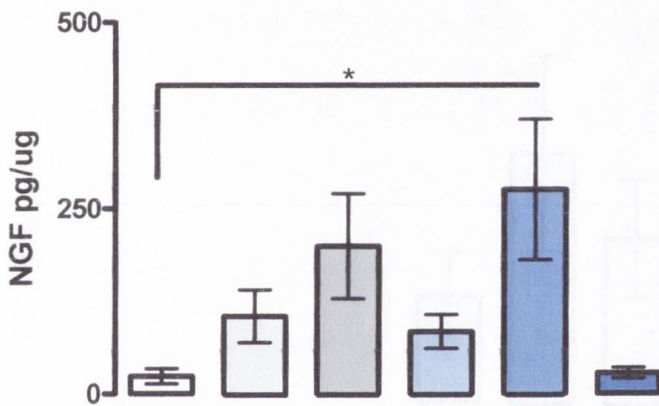
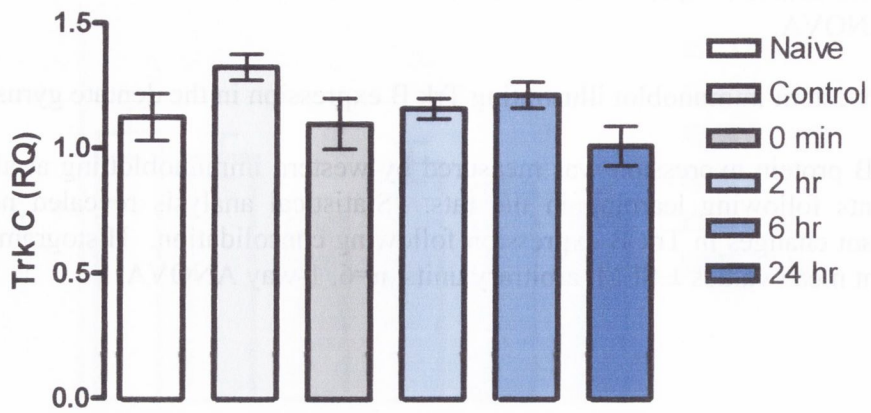**Fignure 4.3 NGF gene and protein expression in the dentate gyrus following learning in the rat.**

    A. NGF mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed no significant increases in NGF mRNA expression at timepoints following learning when compared to controls, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

    B. NGF expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed a significant increase in NGF expression at 6hr following consolidation when compared to naïve animals *p<0.05, 1-way ANOVA, *post hoc* Newman Keuls. Histograms represent mean NGF concentration ± SEM in pg/μg, n=6.
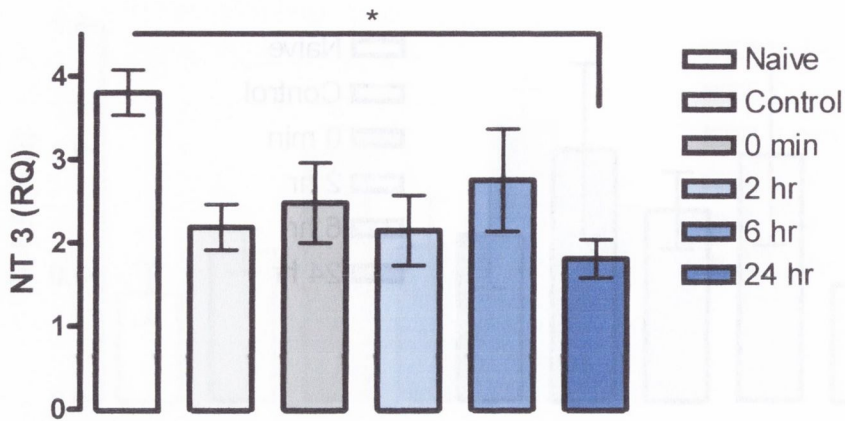
A. NT3 mRNA

B. NT3 protein expression
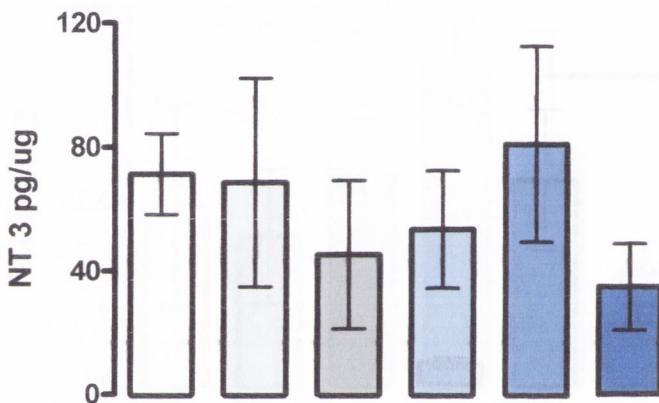
Figure 4.4 NT3 gene expression in the dentate gyrus is decreased following learning in the rat. This message is not translated into NT3 protein.

    A. NT3 mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed a significant decrease in NT3 mRNA expression at 24hr following learning when compared to naive, *$p < 0.05$; 1-way ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.

    B. NT3 expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in NT3 expression at any of the timepoints following consolidation when compared to controls. Histograms represent mean NT3 concentration ± SEM in pg/µg, 1-way ANOVA.
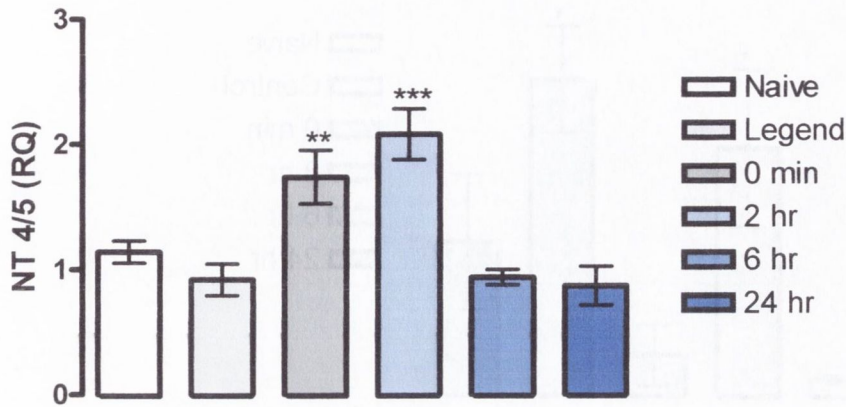
A.NT4/5 mRNA

B. NT4/5 protein expression
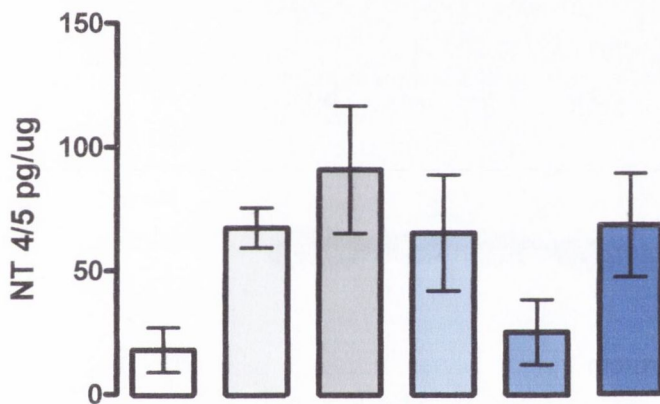
Figure 4.5 NT4/5 gene expression in the dentate gyrus is increased following learning in the rat. This message is partly translated into NT3 protein.

   A. NT4/5 mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed a significant increases in NT4/5 mRNA expression at 0min and 2hr following learning **$p<0.01$ & ***$p<0.0001$ respectively; 1-way ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.
   B. NT4/5 expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in NT4/5 expression following consolidation. Histograms represent mean BDNF concentration ± SEM in pg/µg, n=6, 1-way ANOVA.

A. Trk A mRNA
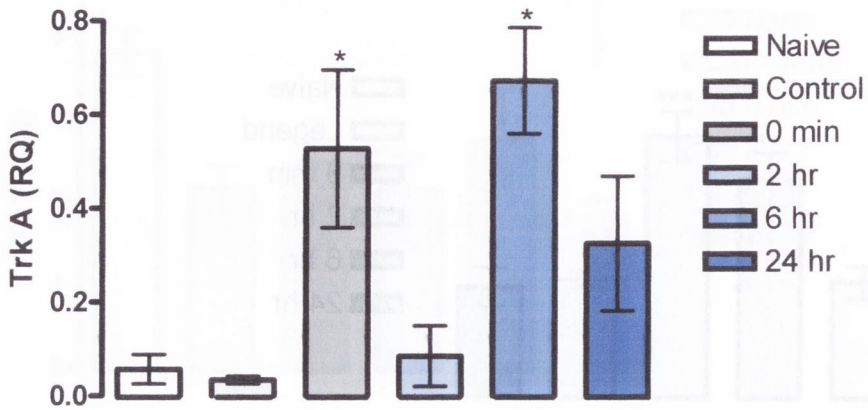
B. Western immunoblot

Trk A

Actin

C. Histograms representing Trk A protein
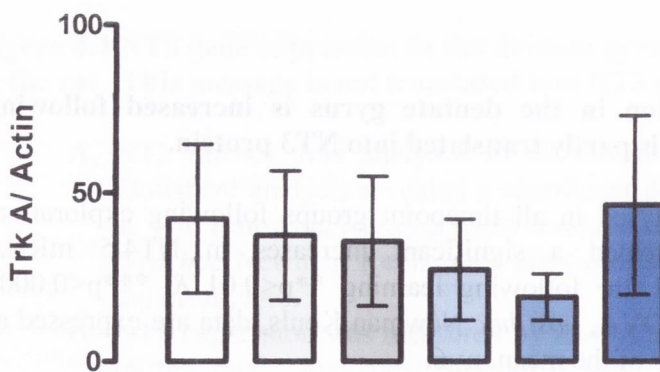
Figure 4.6 Trk A mRNA expression but not Trk A protein is increased in the dentate gyrus following learning in the rat

A. Trk A mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed a significant increase in Trk A mRNA expression at 0min and 6 hr following consolidation *p<0.05 for both; 1-way ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.

B. Sample western immunoblot illustrating Trk A expression in the dentate gyrus.

C. Trk A protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk A expression learning. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way ANOVA.

A. Trk B mRNA

B. Western immunoblot

Trk B

Actin

C. Histograms representing Trk B protein expression
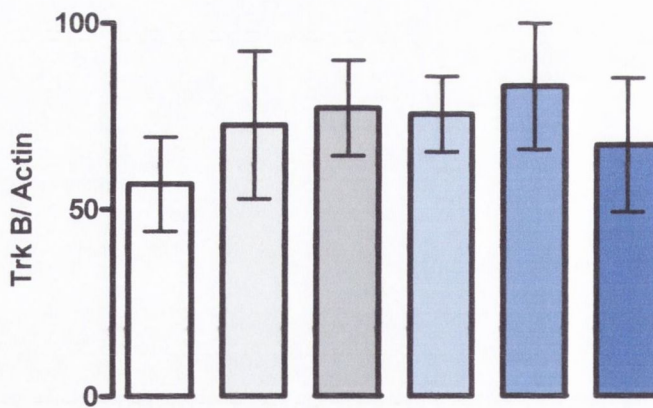
Figure 4.7 There is no change in Trk B mRNA or Trk B protein expression in the dentate gyrus following learning in the rat

A. Trk B mRNA was analysed in all timepoint groups following exploration

Statistical analysis revealed no significant changes in Trk B mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. Sample western immunoblot illustrating Trk B expression in the dentate gyrus

C. C. Trk B protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk B expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way ANOVA.

A. Trk C mRNA

B. Western immunoblot

Trk C

Actin

C. Histograms representing Trk C protein expression
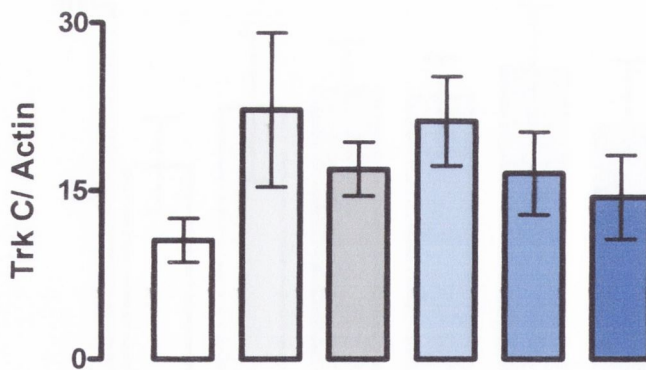
Figure 4.8 There is no change in Trk C mRNA or Trk C protein expression in the dentate gyrus following learning in the rat

A. Trk C mRNA was analysed in all timepoint groups following exploration

Statistical analysis revealed no significant changes in Trk C mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. Sample western immunoblot illustrating Trk C expression in the dentate gyrus

C. Trk C protein expression was measured by western immunoblotting at all
Statistical analysis
revealed no significant changes in Trk C expression following consolidation.
Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way-
ANOVA

.
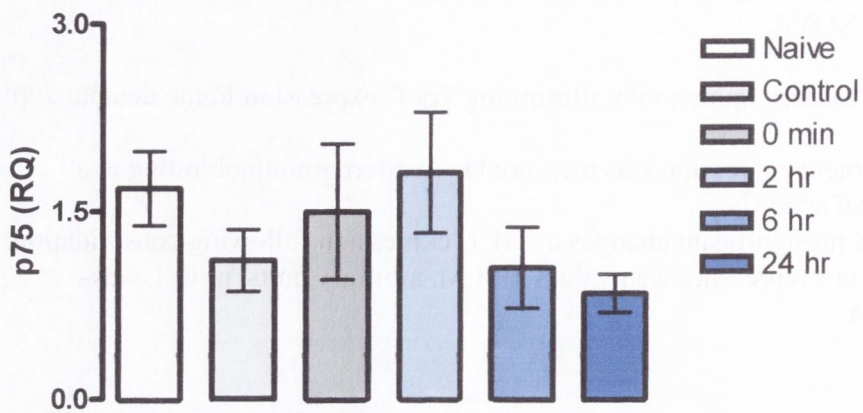
A. P75 mRNA

B. Western immunoblot

P75

Actin

C. P75 Histograms representing P75 protein expression
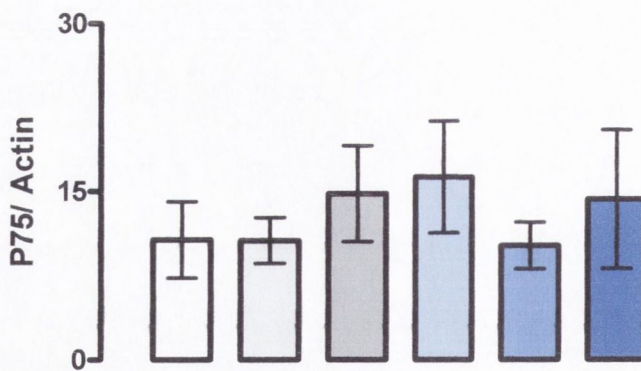
Figure 4.9 There is no change in p75 mRNA or p75 protein expression in the dentate gyrus following learning in the rat

A. P75 mRNA was analysed in all timepoint groups following exploration

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Statistical analysis revealed no significant changes in P75 mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. Sample western immunoblot illustrating P75 expression in the dentate gyrus

C. P75 protein expression was measured by western immunoblotting at all timepoints following learning in the rat. Statistical analysis revealed no significant changes in P75 expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way-ANOVA

Hippocampus

4.3.10 BDNF message and protein expression in the hippocampus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of hippocmpus from these animals were then analyzed for changes in BDNF mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in BDNF mRNA following learning (Figure 4.10A). Results are expressed as means ± standard error of the mean, n=6. BDNF protein was then analyzed in the hippocampus. Significant Increases in BDNF protein expression were observed in the control group and 2hrs timepoint when compared to naïve animals (Figure 4.10B, *p<0.05 and **p<0.01 respectively; 1-way ANOVA, *post hoc* Newman Keuls; Naïve: 123.6 ± 31.87; Control: 254.7 ± 31.64; 0min: 135.4 ± 29.64; 2hr: 268.8 ± 41.48; 6hr: 123.8 ± 15.95; 24hr: 119.2 ± 15.52). Results are expressed as mean BDNF pg/µg protein ± SEM.

4.3.11 NGF message and protein expression in the hippocampus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of hippocampus from these animals were then analyzed for changes in NGF mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in NGF mRNA following learning (Figure 4.11A). Results are expressed as means ± standard error of the mean, n=6. NGF protein was then analyzed in the hippocampus. No changes in NGF protein expression were observed between groups (Figure 4.11B). Results are expressed as mean NGF pg/µg protein ± SEM.

4.3.12 NT3 message and protein expression in the hippocampus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of hippocampus from these animals were then analyzed for changes in NT3 mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in NT3 mRNA following learning (Figure 4.12A). Results are expressed as means ± standard error of the mean, n=6. NT3 protein was then analyzed in the hippocampus. No changes in NT3 protein expression were observed between groups (Figure 4.12B). Results are expressed as mean NT3 pg/µg protein ± SEM.

4.3.13 NT4/5 message and protein expression in the hippocampus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of hippocampus from these animals were then analyzed for changes in NT4/5 mRNA and protein, and compared with samples from naïve and control animals. NT4/5 mRNA was significantly increased at 2hr following learning (Figure 4.13A, *p<0.05; Naïve: 1.174 ± 0.219; Control: 1.217 ± 0.227; 0min: 2.186 ± 0.688; 2hr: 2.779 ± 0.506; 6hr: 1.339 ± 0.249; 24hr: 1.386 ± 0.265). Results are expressed as means ± standard error of the mean, n=6. NT4/5 protein levels were undetectable for this brain region when measured by ELISA.

4.3.14 TrkA message and protein expression in the hippocampus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of hippocampus from these animals were then analyzed for changes in Trk A mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in Trk A mRNA following learning (Figure 4.14A).

Results are expressed as means ± standard error of the mean, n=6. Trk A protein was then analyzed in the hippocampus by Western Immunoblot. From the representitive Trk A blot picture (Figure 4.14B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.14C). Results are expressed as percentage Trk A/Actin, mean ± SEM.

4.3.15 Trk B message and protein expression in the hippocampus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of hippocampus from these animals were then analyzed for changes in Trk B mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in Trk B mRNA following learning (Figure 4.15A). Results are expressed as means ± standard error of the mean, n=6. Trk B protein was then analyzed in the hippocampus by Western Immunoblot. From the representitive Trk B blot picture (Figure 4.15B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.15C). Results are expressed as percentage Trk B/Actin, mean ± SEM.

4.3.16 Trk C message and protein expression in the hippocampus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of hippocampus from these animals were then analyzed for changes in Trk C mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in Trk C mRNA following learning (Figure 4.16A). Results are expressed as means ± standard error of the mean, n=6. Trk C protein was then analyzed in the hippocampus by Western Immunoblot. From the representitive Trk C blot picture (Figure 4.16B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.16C). Results are expressed as percentage Trk C/Actin, mean ± SEM.

4.3.17 P75 message and protein expression in the hippocampus following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of hippocampus from these animals were then analyzed for changes in P75 mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in P75 mRNA following learning (Figure 4.17A). Results are expressed as means ± standard error of the mean, n=6. P75 protein was then analyzed in the hippocampus by Western Immunoblot. From the representitive P75 blot picture (Figure 4.17B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.17C). Results are expressed as percentage P75/Actin, mean ± SEM.
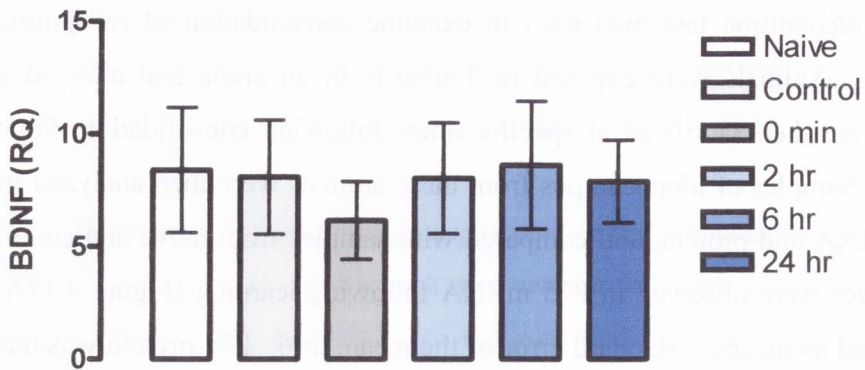
A. BDNF mRNA

B. BDNF protein expression
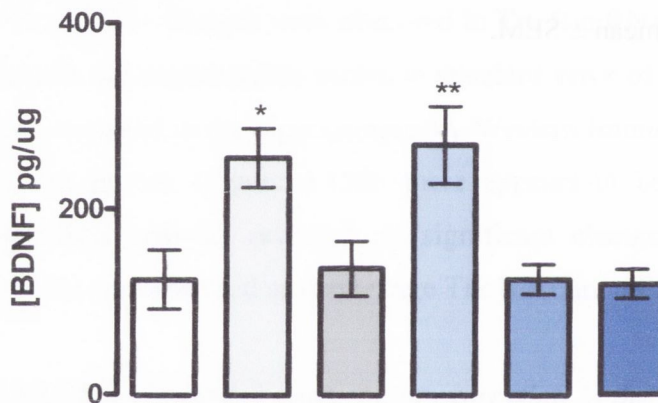
Figure 4.10 No changes were observed in BDNF gene expression in the hippocampus following learning in the rat, however, BDNF protein increased following learning.
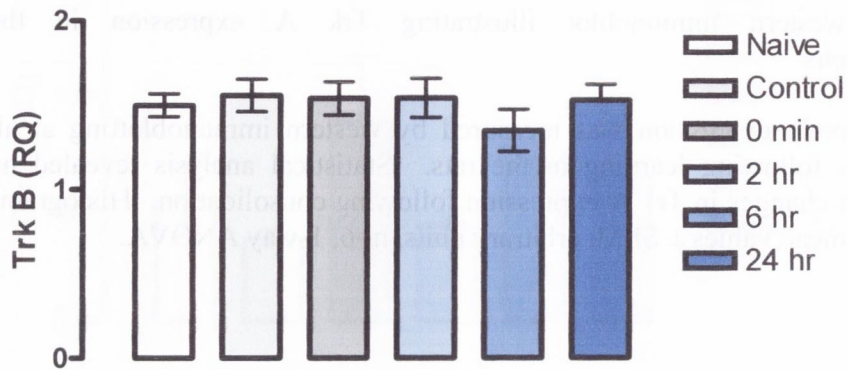
A. BDNF mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed no significant changes in BDNF mRNA expression following learning, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. BDNF expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed significant increases in BDNF expression in the control group and at 2hr following consolidation compared to naïve animals, *p<0.05 and **p<0.01 respectively; 1-way ANOVA; *post hoc* Newman Keuls. Histograms represent mean BDNF concentration ± SEM in pg/μg, n=6.
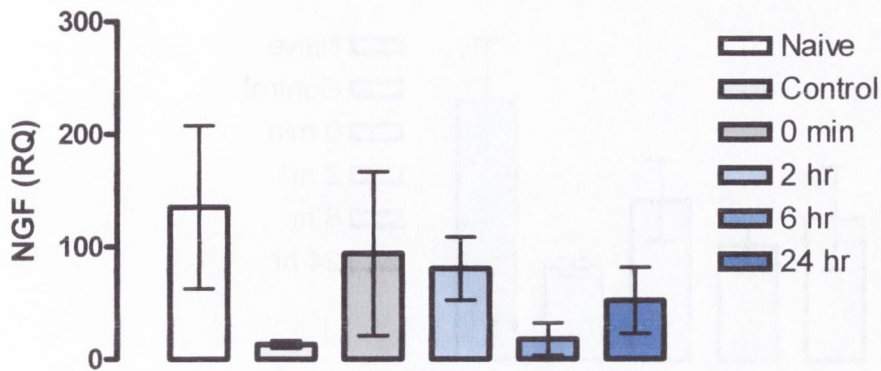
A. NGF mRNA

B. NGF protein expression
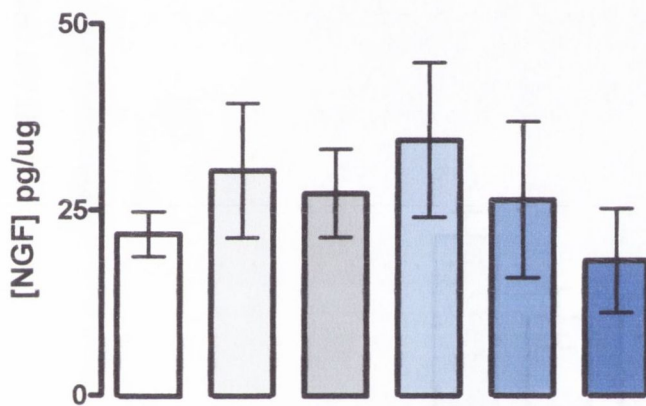
Figure 4.11 Following learning in the rat no changes were observed in NGF gene or protein expression in the hippocampus.

A. NGF mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed no significant increases in NGF mRNA expression at timepoints following learning when compared to controls, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. NGF expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in NGF expression at timepoints recorded. Histograms represent mean NGF concentration ± SEM in pg/μg, n=6, 1-way ANOVA.
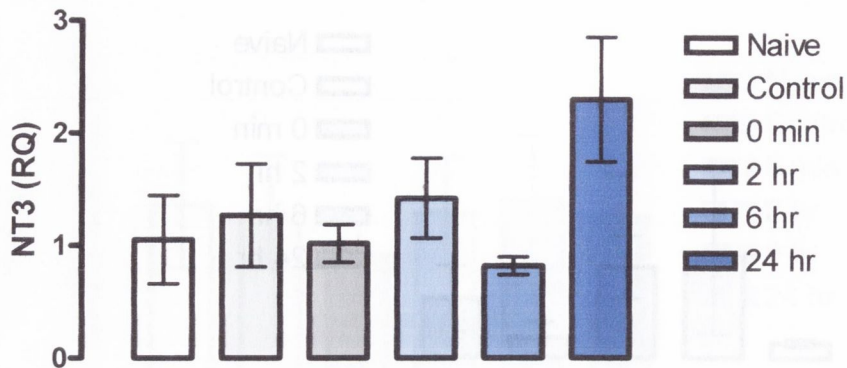
A. NT3 mRNA

B. NT3 protein expression
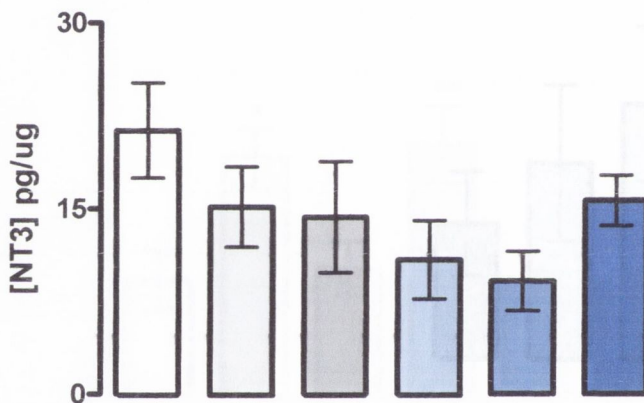
Figure 4.12 No changes were observed in NT3 gene expression or NT3 protein expression in the hippocampus following learning in the rat.

A. NT3 mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed no significant changes in NT3 mRNA expression at 24hr following learning when compared to naive, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. NT3 expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in NT3 expression at any of the timepoints following consolidation when compared to controls. Histograms represent mean NT3 concentration ± SEM in pg/μg, 1-way ANOVA.
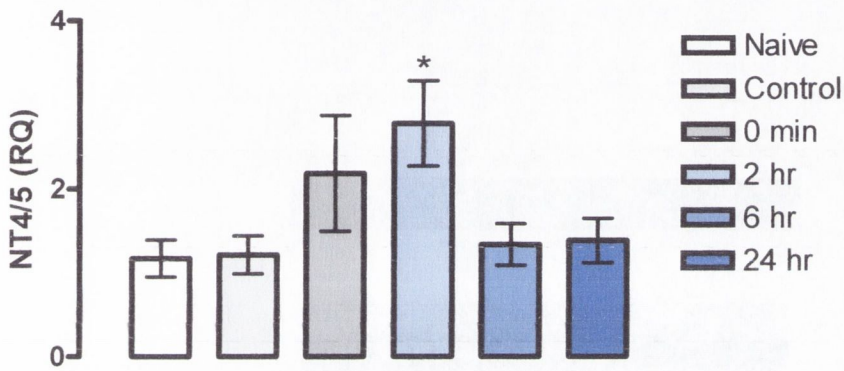
A. NT4/5 mRNA

Figure 4.13 NT4/5 gene expression in the hippocampus is increased following learning in the rat. NT4/5 protein levels were undetectable in the hippocampus by ELISA.

A. NT4/5 mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed a significant increases in NT4/5 mRNA expression at 2hr following learning **p<0.05; 1-way ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.
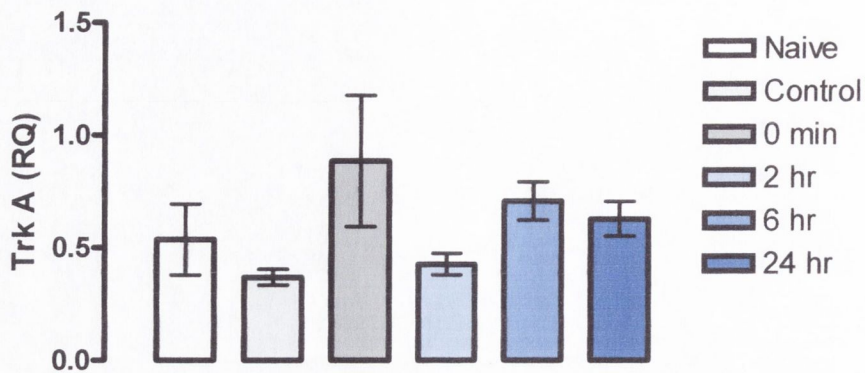
A Trk A mRNA

B. Western immunoblot

Trk A

Actin
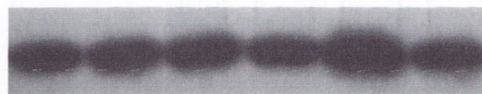
C. Histograms representing Trk A protein
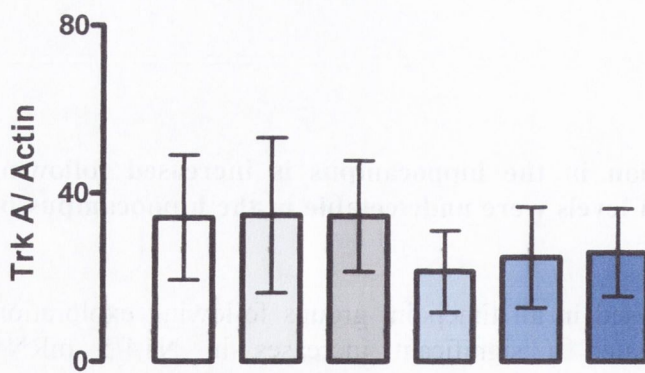
Figure 4.14 No changes were observed in Trk A gene expression or Trk A protein in the hippocampus following learning in the rat

A. Trk A mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed no significant increase in Trk A mRNA at any of

the timepoints recorded following consolidation, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.
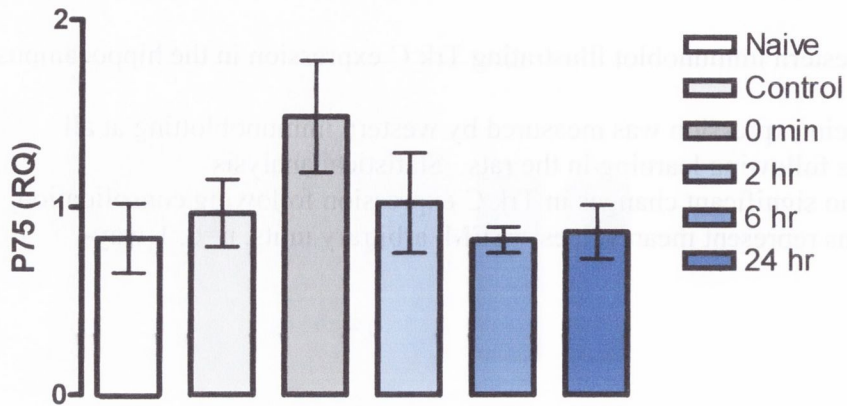
B. Sample western immunoblot illustrating Trk A expression in the hippocampus.

C. Trk A protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk A expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way ANOVA.

## A. Trk B mRNA

B. Western immunoblot
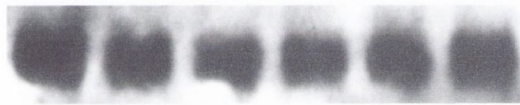
Trk B

Actin
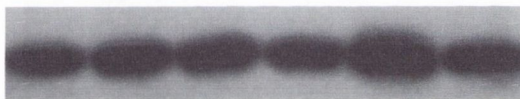
C. Histograms representing Trk B protein
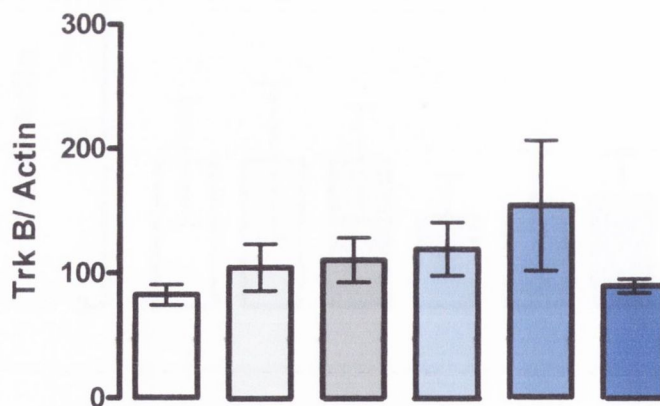
Figure 4.15 There is no change in Trk B mRNA or Trk B protein expression in the hippocampus following learning in the rat

A. Trk B mRNA was analysed in all timepoint groups following exploration Statistical analysis revealed no significant changes in Trk B mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. Sample western immunoblot illustrating Trk B expression in the hippocampus

C. Trk B protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk B expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way ANOVA.
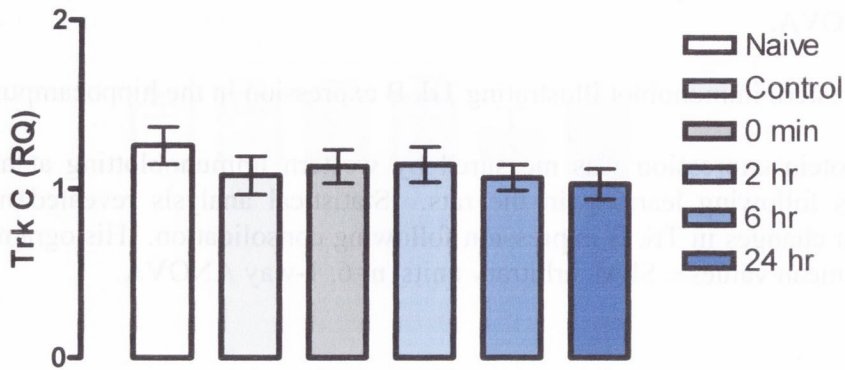
A. Trk C mRNA

B. Western immunoblot

Trk C

Actin
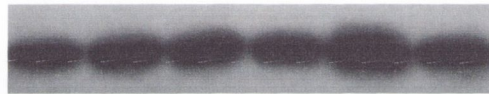
C. Histograms representing Trk C protein
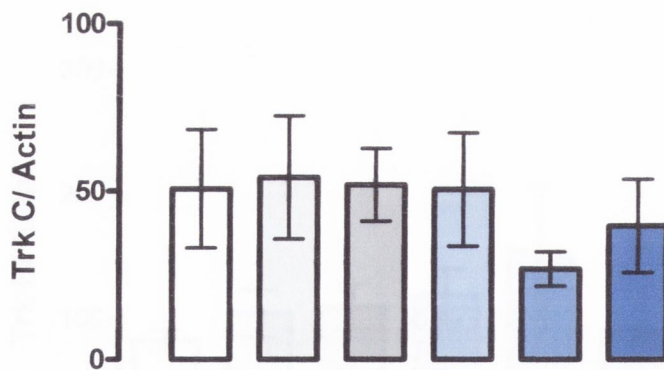
Figure 4.16 There is no change in Trk C mRNA or Trk C protein expression in the hippocampus following learning in the rat

A. Trk C mRNA was analysed in all timepoint groups following exploration

Statistical analysis revealed no significant changes in Trk C mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. Sample western immunoblot illustrating Trk C expression in the hippocampus

C. Trk C protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk C expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way-ANOVA.

A. P75 mRNA

B. Western immunoblot

P75

Actin

C. Histogram representing P75 protein
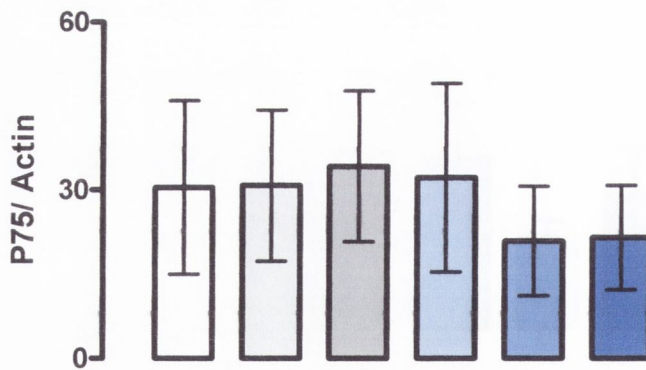
Figure 4.17 There is no change in p75 mRNA or p75 protein expression in the hippocampus following learning in the rat

B. P75 mRNA was analysed in all timepoint groups following exploration

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Statistical analysis revealed no significant changes in P75 mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. Sample western immunoblot illustrating P75 expression in the hippocampus

C. P75 protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in P75 expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way-ANOVA.

Perirhinal Cortex

4.3.18 BDNF message and protein expression in the perirhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of perirhinal cortex from these animals were then analyzed for changes in BDNF mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in BDNF mRNA following learning (Figure 4.18A). Results are expressed as means ± standard error of the mean, n=6. BDNF protein was then analyzed in the perirhinal cortex. No differences in BDNF protein expression were observed between groups (Figure 4.18B). Results are expressed as mean BDNF pg/μg protein ± SEM.

4.3.19 NGF message and protein expression in the perirhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of perirhinal cortex from these animals were then analyzed for changes in NGF mRNA and protein, and compared with samples from naïve and control animals. NGF mRNA was significantly increased at 2hr following learning when compared to the other timepoints but not controls (Figure 4.19A; *p<0.05; 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 0.82 ± 0.102; Control: 0.807 ± 0.118; 0min: 0.395 ± 0.088; 2hr: 1.295 ± 0.317; 6hr: 0.587 ± 0.165; 24hr: 0.499 ± 0.182). Results are expressed as means ± standard error of the mean, n=6. NGF protein was then analyzed in the perirhinal cortex.. No changes in NGF protein expression were observed between groups (Figure 4.19B). Results are expressed as mean NGF pg/μg protein ± SEM.

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4.3.20 NT3 message and protein expression in the perirhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of perirhinal cortex from these animals were then analyzed for changes in NT3 mRNA and protein, and compared with samples from naïve and control animals. NT3 mRNA was decreased at all timepoints, including control following learning when compared to naïve animals. The 24hr timepoint was the only one, which was significantly decreased, compared to naive (Figure 4.20 A; $*p<0.05$; 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 4.047 ± 0.321; Control: 2.386 ± 0.276; 0min: 2.716 ± 0.484; 2hr: 2.297 ± 0.472; 6hr: 2.968 ± 0.675; 24hr: 2.023 ± 0.258). Results are expressed as means $\pm$ standard error of the mean, n=6. NT3 protein was then analyzed in the perirhinal cortex. NT3 protein expression was significantly decreased at all timepoints taken including the control group when compared to naïve animals (Figure 4.20B; $*p<0.05$; 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 57.78 ± 10.05; Control: 14.9 ± 6.825; 0min: 14.05 ± 4.43; 2hr: 11.88 ± 1.595; 6hr: 14.41 ± 3.718; 24hr: 15.97 ± 5.97). Results are expressed as mean NT3 pg/µg protein $\pm$ SEM.

4.3.21 NT4/5 message and protein expression in the perirhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of perirhinal cortex from these animals were then analyzed for changes in NT4/5 mRNA and protein, and compared with samples from naïve and control animals. NT4/5 mRNA was significantly increased at 0 min and 2 hrs following learning (Figure 4.21A, $**p<0.01$ and $***p<0.0001$ respectively; 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 1.132 ± 0.092; Control: 0.965 ± 0.144; 0min: 1.765 ± 0.22: 2hr: 2.143 ± 0.207; 6hr: 0.934 ± 0.078; 24hr: 0.885 ± 0.167). Results are expressed as means $\pm$ standard error of the mean, n=6. NT4/5 protein levels were undetectable for this brain region when measured by ELISA.

4.3.22 TrkA message and protein expression in the perirhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of perirhinal cortex from these animals were then analyzed for changes in Trk A mRNA and protein, and compared with samples from naïve and control animals. Trk A mRNA was decreased at all timepoints including the control group following learning when compared to naïve animals, however only the 2hr group was significantly reduced (Figure 4.22A, *p<0.05, 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 1.996 ± 0.529; Control: 0.796 ± 0.107; 0min: 0.938 ± 0.337; 2hr: 0.632 ± 0.118; 6hr: 1.021 ± 0.186; 24hr: 1.044 ± 0.341). Results are expressed as means ± standard error of the mean, n=6. Trk A protein was then analyzed in the perirhinal cortex by Western Immunoblot. From the representitive Trk A blot picture (Figure 4.22B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.22C). Results are expressed as percentage TrkA/Actin, mean ± SEM.

4.3.23 Trk B message and protein expression in the perirhinal cortex following learning

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of perirhinal cortex from these animals were then analyzed for changes in Trk B mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in Trk B mRNA following learning (Figure 4.23A). Results are expressed as means ± standard error of the mean, n=6. Trk B protein was then analyzed in the perirhinal cortex by Western Immunoblot. From the representitive Trk B blot picture (Figure 4.23B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.23C). Results are expressed as percentage Trk B/Actin, mean ± SEM.

4.3.24 Trk C message and protein expression in the perirhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of perirhinal cortex from these animals were then analyzed for changes in Trk C mRNA and protein, and compared with samples from naïve and control animals. Trk C mRNA was slightly increased following learning with the 0min group significantly increased compared to controls (Figure 4.24A, $*p<0.05$, 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 0.837 ± 0.044; Control: 1.01 ± 0.059; 0min: 1.049 ± 0.07; 2hr: 1.008 ± 0.02; 6hr: 0.915 ± 0.02; 24hr: 0.932 ± 0.01). Results are expressed as means ± standard error of the mean, n=6. Trk C protein was then analyzed in the perirhinal cortex by Western Immunoblot. From the representitive Trk C blot picture (Figure 4.24B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.24C). Results are expressed as percentage Trk C/Actin, mean ± SEM.

4.3.25 P75 message and protein expression in the perirhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of perirhinal cortex from these animals were then analyzed for changes in P75 mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in P75 mRNA following learning (Figure 4.25A). Results are expressed as means ± standard error of the mean, n=6. P75 protein was then analyzed in the perirhinal cortex by Western Immunoblot. From the representitive blot picture (Figure 4.25B) there appears to be a decrease in P75 protein expression in all groups compared to naive. Statistical analysis revealed this change was not significant (Figure 4.25C). Results are expressed as percentage P75/Actin, mean ± SEM
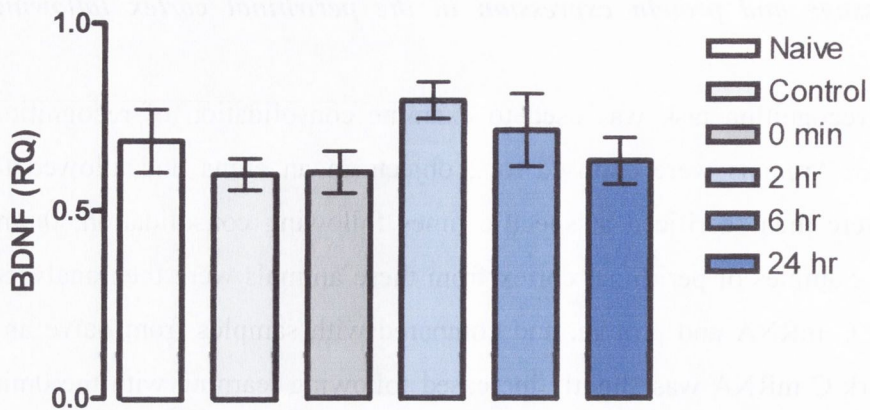
A. BDNF mRNA

B. BDNF protein expression
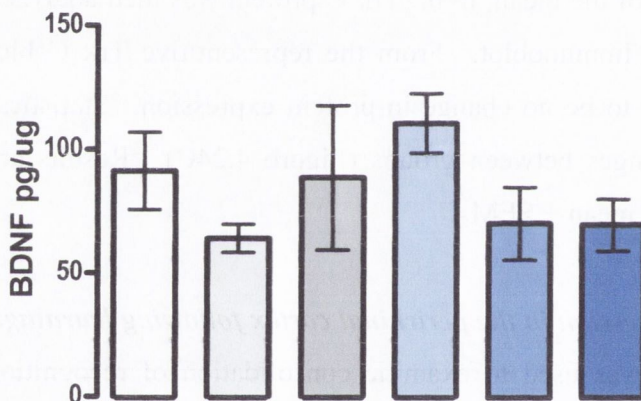
Figure 4.18 No changes were observed in BDNF gene or BDNF protein expression in the perirhinal cortex following learning in the rat.
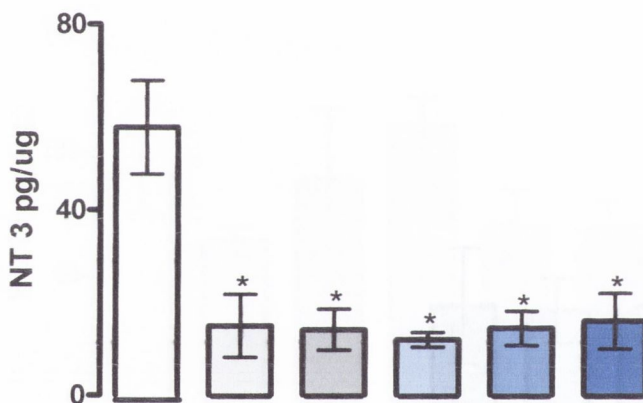
A. BDNF mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed no significant changes in BDNF mRNA expression following learning, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. BDNF expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in BDNF expression at any of the timepoints recorded. Histograms represent mean BDNF concentration ± SEM in pg/μg, n=6, 1-way ANOVA.
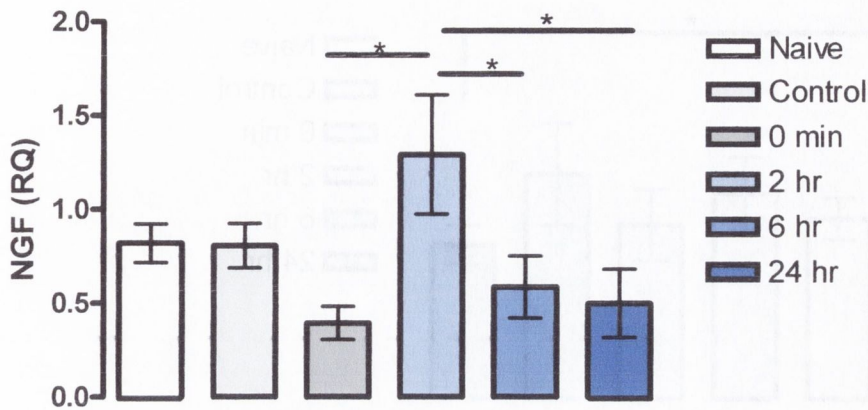
A. NGF mRNA

B. NGF protein expression
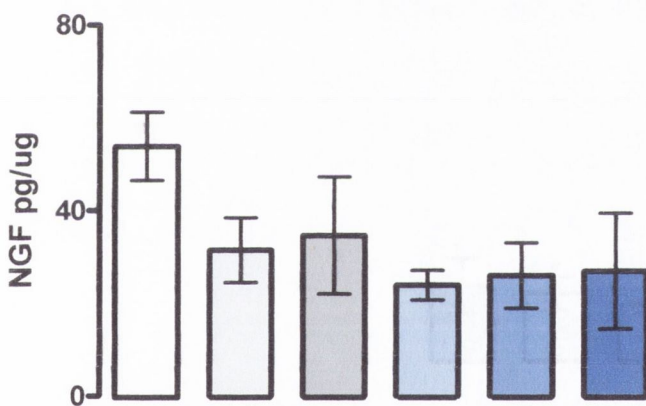
Figure 4.19 Following learning in the rat increases in NGF gene but not NGF protein expression were observed in the perirhinal cortex.

A. NGF mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed a significant increases in NGF mRNA expression at 2hr following learning when compared to 0min, 6hr and 24hr timepoints, *$p < 0.05$; 1-way ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.

B. NGF expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in NGF expression at timepoints recorded. Histograms represent mean NGF concentration ± SEM in pg/μg, n=6, 1-way ANOVA.
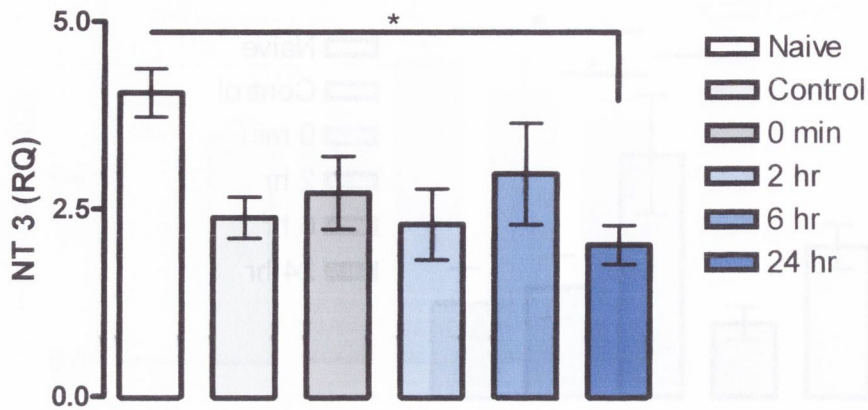
A. NT3 mRNA

B. NT3 protein expression

Figure 4.20 Learning in the rat results in decreased NT3 gene expression and NT3 protein expression in the perirhinal cortex

A. NT3 mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed a significant decrease in NT3 mRNA expression at 24hr following learning when compared to naive, *$p<0.05$; 1-way ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.

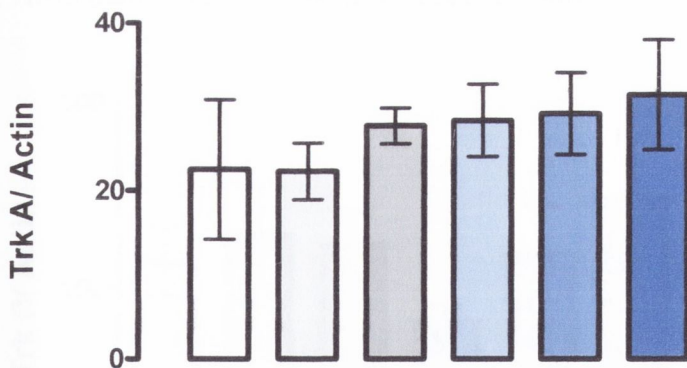B. NT3 expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed a significant decrease in NT3 expression at all of the timepoints and control animals following consolidation when compared to naïve *$p<0.05$; 1-way ANOVA, *post hoc* Newman Keuls.

Histograms represent mean NT3 concentration ± SEM in pg/μg, 1-way ANOVA.

A. NT4/5 mRNA

Figure 4.21 NT4/5 gene expression in the perirhinal cortex is increased following learning in the rat. NT4/5 protein levels were undetectable in the perirhinal cortex by ELISA.

A. NT4/5 mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed a significant increases in NT4/5 mRNA expression at 2hr and at 6hr following learning **p<0.01 & ***p<0.0001 respectively; 1-way ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.

A. Trk A mRNA

B. Western immunoblot

Trk A

Actin
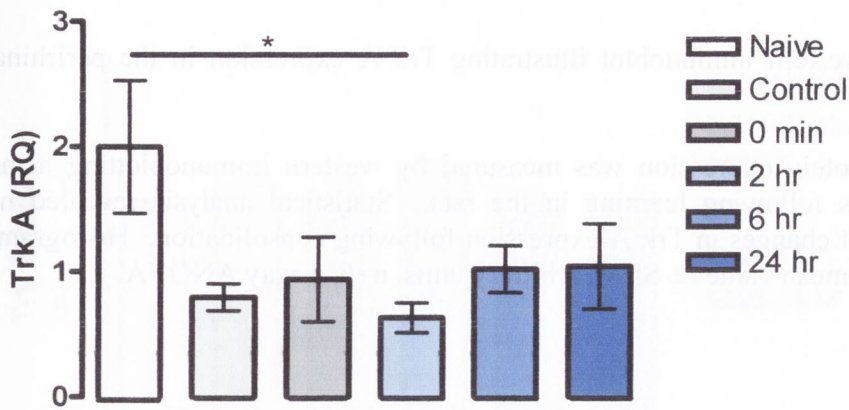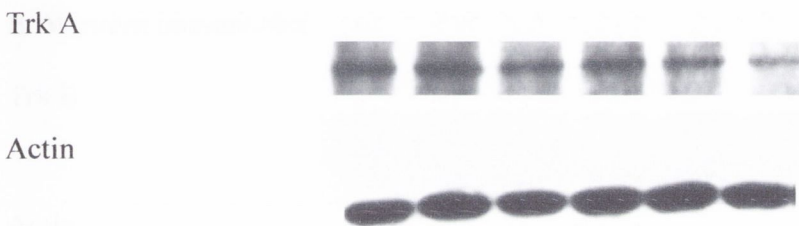
C. Histograms represent Trk A protein expression
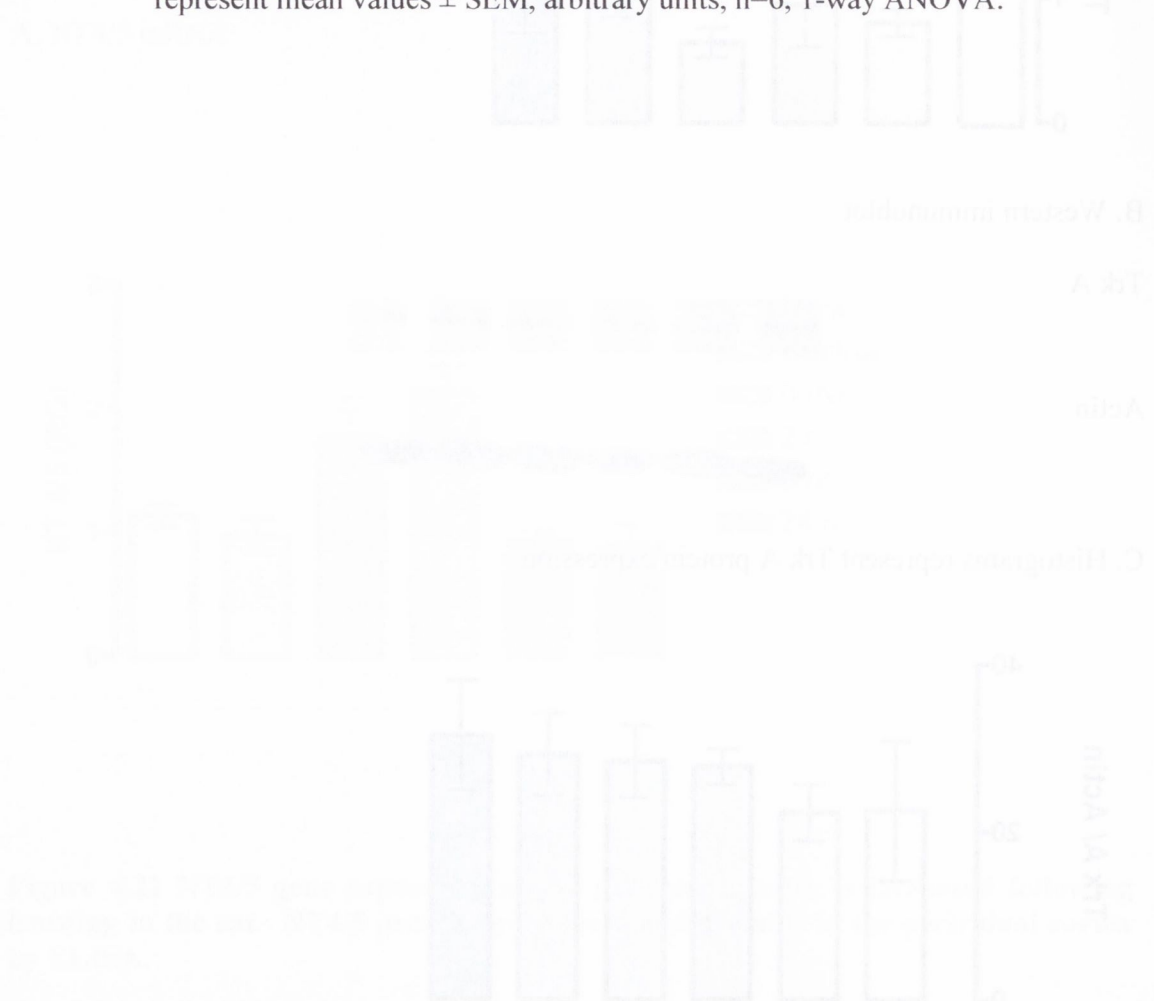

Figure 4.22 Trk A gene expression but not Trk A protein was decreased in the perirhinal cortex following learning in the rat

A. Trk A mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed a significant decrease in Trk A mRNA at 2hr timepoint following consolidation when compared to naïve *p<0.05; 1-way

ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.

B. Sample western immunoblot illustrating Trk A expression in the perirhinal cortex.

C. Trk A protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk A expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way ANOVA.
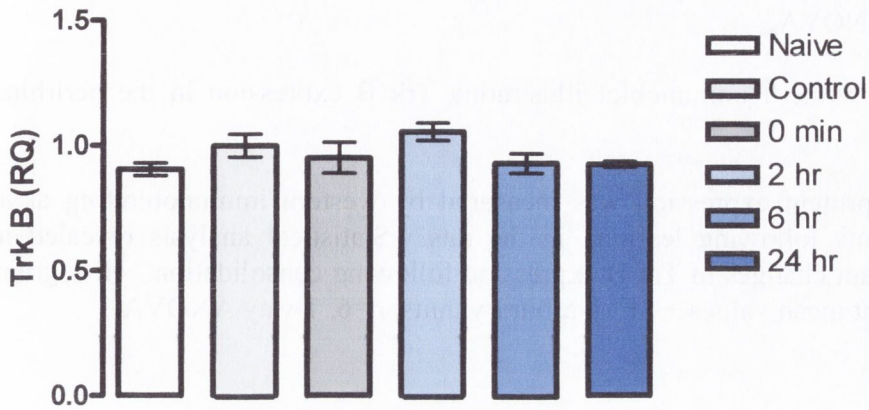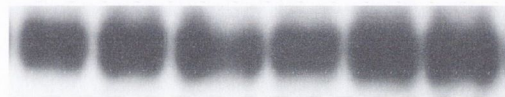
A. Trk B mRNA

B. Western immunoblot

Trk B

Actin

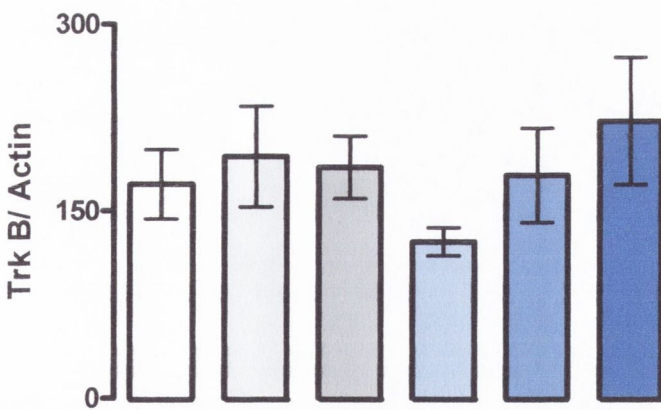## C. Histograms represent Trk B protein expression

Figure 4.23 There is no change in Trk B mRNA or Trk B protein expression in the perirhinal cortex following learning in the rat

A. Trk B mRNA was analysed in all timepoint groups following exploration Statistical analysis revealed no significant changes in Trk B mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. Sample western immunoblot illustrating Trk B expression in the perirhinal cortex

C. Trk B protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk B expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way ANOVA.
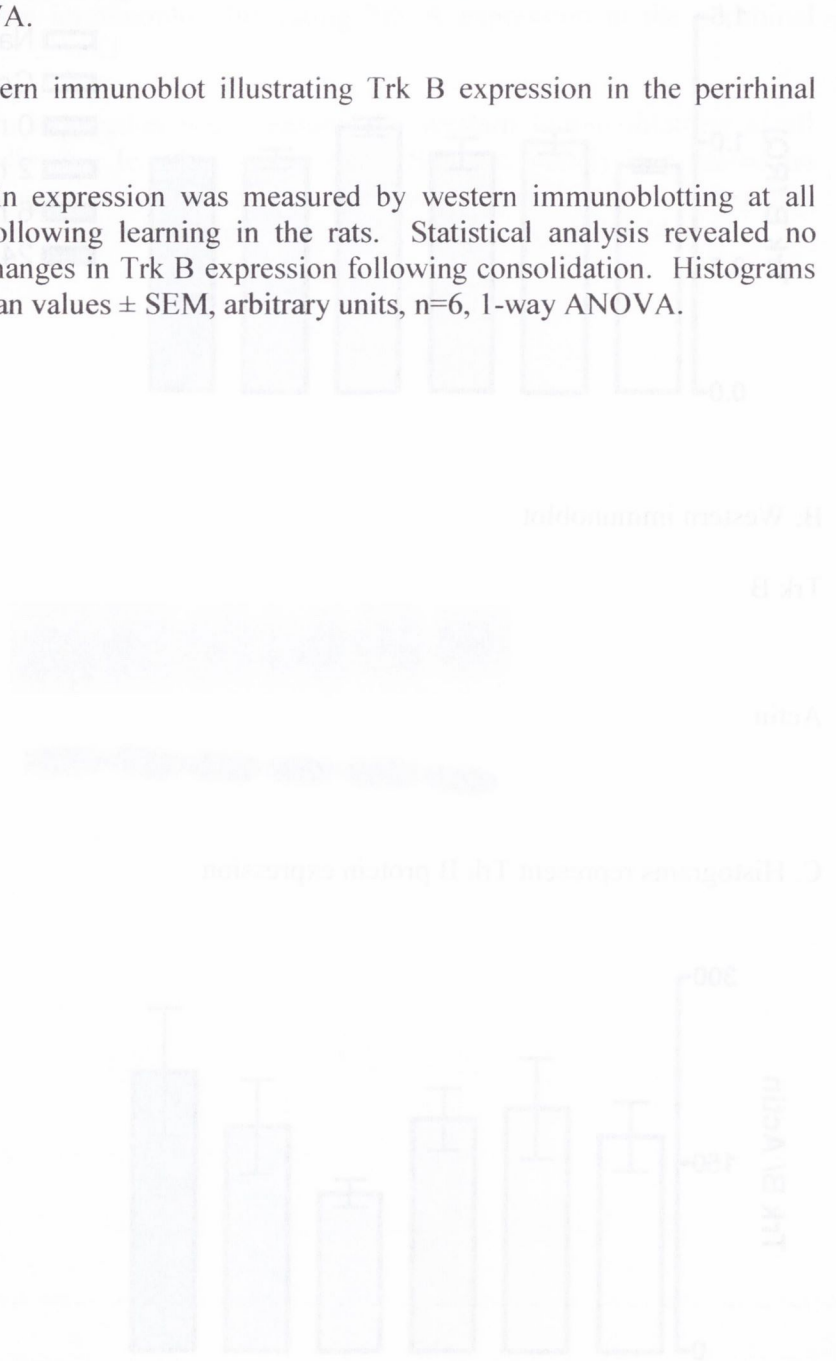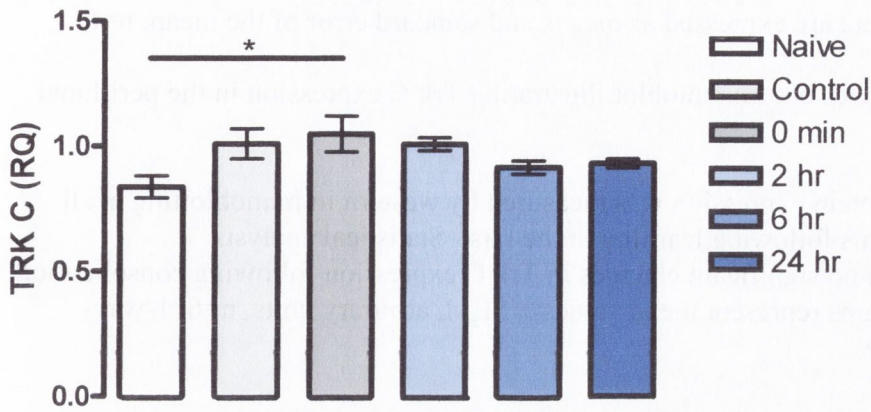
A. Trk C mRNA

B. Western immunoblot

Trk C

Actin

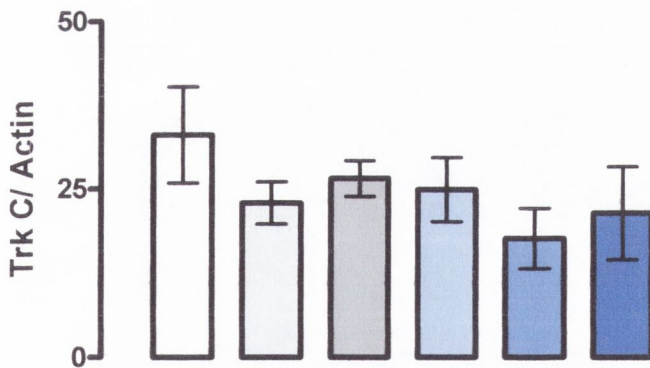C. Histograms represents Trk C protein expression

Figure 4.24 Trk C mRNA but not Trk C protein expression is increased in the perirhinal cortex following learning in the rat

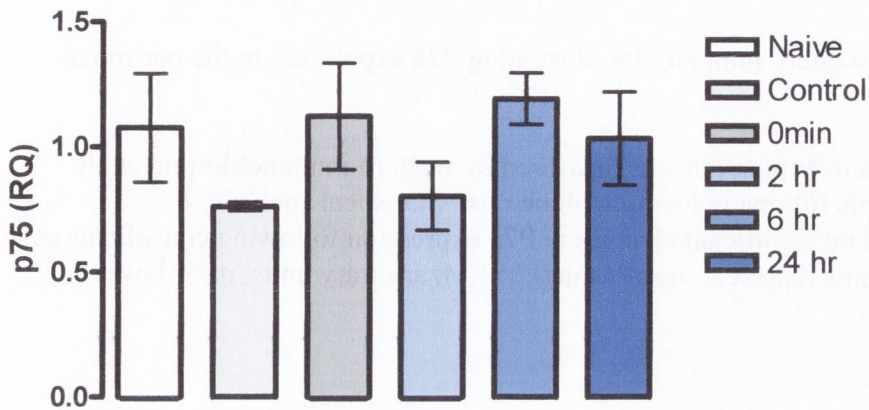A. Trk C mRNA was analysed in all timepoint groups following exploration

Statistical analysis revealed a significant increase in Trk C mRNA expression at 0min when compared to naïve *p<0.05; 1-way ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.

B. Sample western immunoblot illustrating Trk C expression in the perirhinal cortex

C. Trk C protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk C expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way-ANOVA.

A. P75 mRNA

B. Western immunoblot

P75

Actin

C. Histograms represent P75 protein expression

Figure 4.25 There is no change in p75 mRNA or p75 protein expression in the perirhinal cortex following learning in the rat

A. P75 mRNA was analysed in all timepoint groups following exploration

Statistical analysis revealed no significant changes in P75 mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. Sample western immunoblot illustrating P75 expression in the perirhinal cortex

C. P75 protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in P75 expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way-ANOVA.
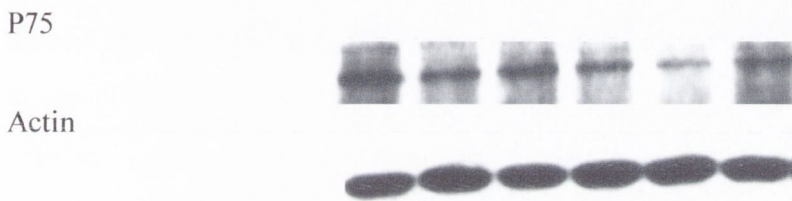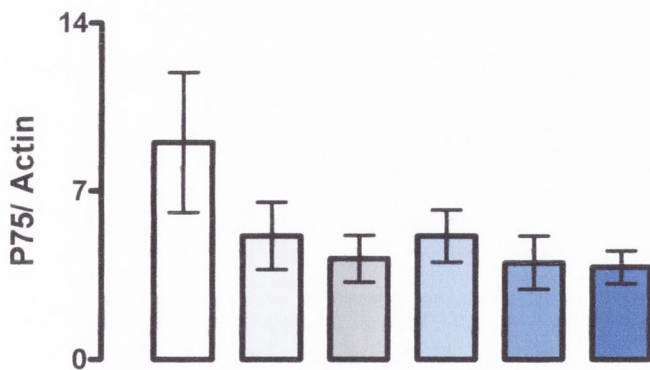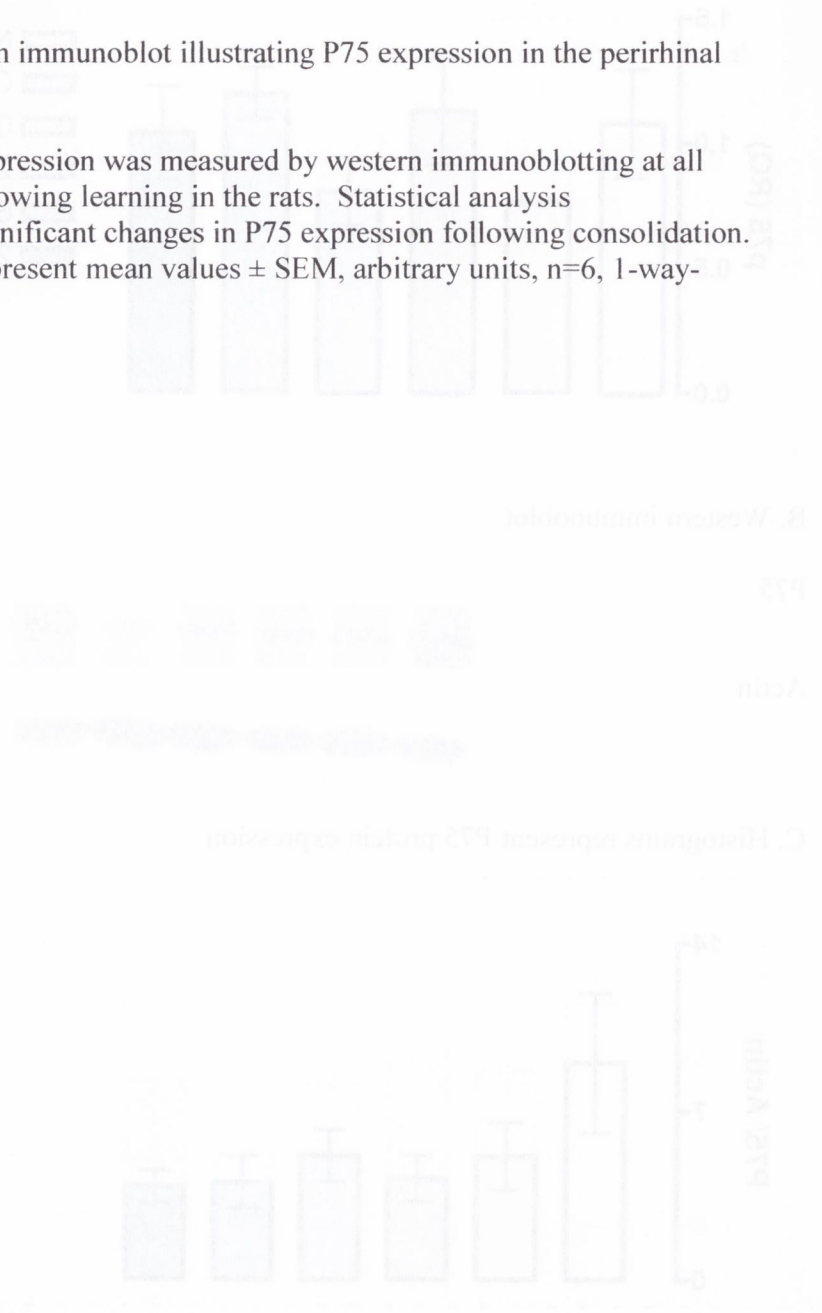
Entorhinal Cortex

4.3.26 BDNF message and protein expression in the entorhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of entorhinal cortex from these animals were then analyzed for changes in BDNF mRNA and protein, and compared with samples from naïve and control animals. BDNF mRNA was significantly increased at the 6hr timepoint following learning (Figure 4.26 A; $*p<0.05$; 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 0.337 ± 0.08; Control: 0.232 ± 0.04; 0min: 0.614 ± 0.297; 2hr: 0.471 ± 0.08; 6hr: 0.639 ± 0.113; 24hr: 0.273 ± 0.032). Results are expressed as means $\pm$ standard error of the mean, n=6. BDNF protein was then analyzed in the entorhinal cortex. No differences in BDNF protein expression were observed between groups (Figure 4.26B). Results are expressed as mean BDNF pg/µg protein $\pm$ SEM.

4.3.27 NGF message and protein expression in the entorhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of entorhinal cortex from these animals were then analyzed for changes in NGF mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in NGF mRNA following learning (Figure 4.27A). Results are expressed as means $\pm$ standard error of the mean, n=6. NGF protein was then analyzed in the entorhinal cortex. No changes in NGF protein expression were observed following learning (Figure 4.27B). Results are expressed as mean NGF pg/µg protein $\pm$ SEM.

4.3.28 NT3 message and protein expression in the entorhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of entorhinal cortex from these animals were then analyzed for changes in NT3 mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in NT3 mRNA following learning when compared to naïve animals (Figure 4.28A). Results are expressed as means ± standard error of the mean, n=6. NT3 protein was then analyzed in the entorhinal cortex. NT3 protein expression was significantly increased at the 24hr timepoint when compared to the 6hr and 0min timepoints (Figure 4.28B; *p<0.05; 1-way ANOVA, *post hoc* Newman Keuls, Naïve: 16.47 ± 3.138; Control: 17.61 ± 3.65; 0min: 13.94 ± 2.69; 2hr: 22.37 ± 3.47; 6hr: 11.08 ± 3.38; 24hr: 35.04 ± 6.831). Results are expressed as mean NT3 pg/µg protein ± SEM.

4.3.29 NT4/5 message and protein expression in the entorhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of entorhinal cortex from these animals were then analyzed for changes in NT4/5 mRNA and protein, and compared with samples from naïve and control animals. No significant changes in NT4/5 mRNA were observed following learning (Figure 4.29A). Results are expressed as means ± standard error of the mean, n=6. NT4/5 protein levels were undetectable for this brain region when measured by ELISA.

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4.3.30 TrkA message and protein expression in the entorhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of entorhinal cortex from these animals were then analyzed for changes in Trk A mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in Trk A mRNA following learning (Figure 4.30A). Results are expressed as means ± standard error of the mean, n=6. Trk A protein was then analyzed in the entorhinal cortex by Western Immunoblot. From the representitive Trk A blot picture (Figure 4.30B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.30C). Results are expressed as percentage Trk A/Actin, mean ± SEM.

4.3.31 TrkB message and protein expression in the entorhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of entorhinal cortex from these animals were then analyzed for changes in Trk B mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in Trk B mRNA following learning (Figure 4.31A). Results are expressed as means ± standard error of the mean, n=6. Trk B protein was then analyzed in the entorhinal cortex by Western Immunoblot. From the representitive Trk B blot picture (Figure 4.31B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.31C). Results are expressed as percentage Trk B/Actin, mean ± SEM.

4.3.32 Trk C message and protein expression in the entorhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificied at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of entorhinal cortex from these animals were then analyzed for changes in Trk C mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in Trk C mRNA following learning (Figure 4.32A). Results are expressed as means ± standard error of the mean, n=6. Trk C protein was then analyzed in the entorhinal cortex by Western Immunoblot. From the representsitive Trk C blot picture (Figure 4.32B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.32C). Results are expressed as percentage Trk C/Actin, mean ± SEM.

4.3.33 P75 message and protein expression in the entorhinal cortex following learning.

The object recognition task was used to examine consolidation of recognition memory in the rat. Animals were exposed to 2 objects in an arena and allowed to explore, animals were then sacrificed at specific times following consolidation; 0min, 2hr, 6hr and 24hr. Samples of entorhinal cortex from these animals were then analyzed for changes in P75 mRNA and protein, and compared with samples from naïve and control animals. No changes were observed in P75 mRNA following learning (Figure 4.33A). Results are expressed as means ± standard error of the mean, n=6. P75 protein was then analyzed in the entorhinal cortex by Western Immunoblot. From the representitive P75 blot picture (Figure 4.33B) there appears to be no change in protein expression. Statistical analysis revealed no significant changes between groups (Figure 4.33C). Results are expressed as percentage P75/Actin, mean ± SEM.
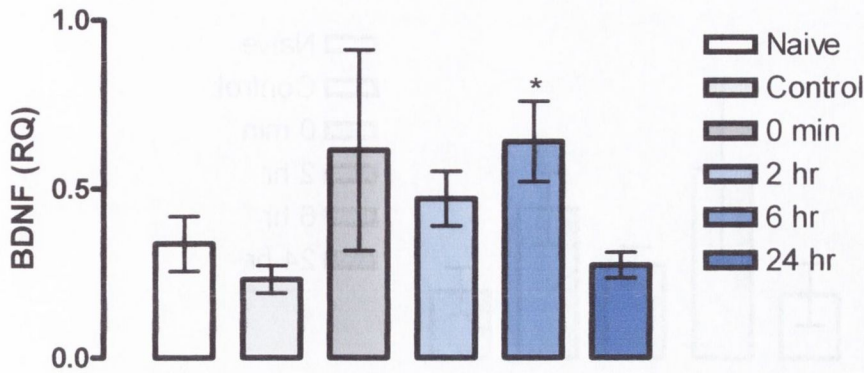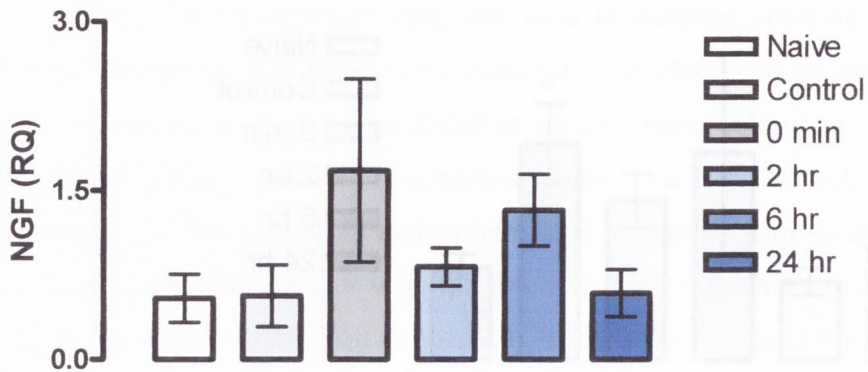
A. BDNF mRNA

B. BDNF protein expression
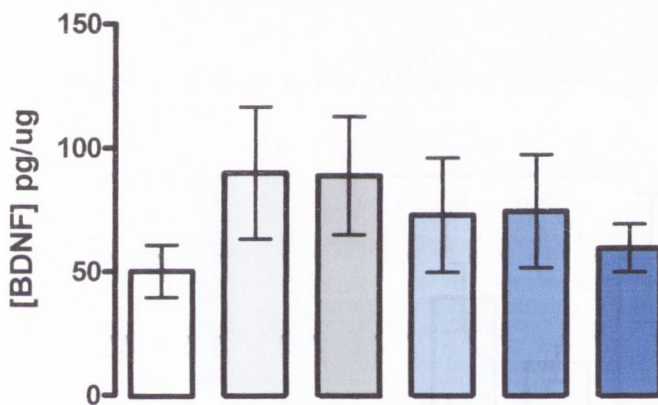
Figure 4.26 BDNF gene expression but not BDNF protein expression was increased in the entorhinal cortex following learning in the rat.

A. BDNF mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed a significant increase in BDNF mRNA expression at 2hr following learning *$p<0.05$; 1-way ANOVA, *post hoc* Newman Keuls, data are expressed as means and standard error of the mean, n=6.

B. BDNF expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in BDNF expression at any of the timepoints recorded. Histograms represent mean BDNF concentration ± SEM in pg/µg, n=6, 1-way ANOVA.

A. NGF mRNA

B. NGF protein expression

Figure 4.27 No changes were observed in NGF gene or NGF protein expression in the entorhinal cortex following learning in the rat.

A. NGF mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed no significant changes in NGF mRNA expression following learning, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.
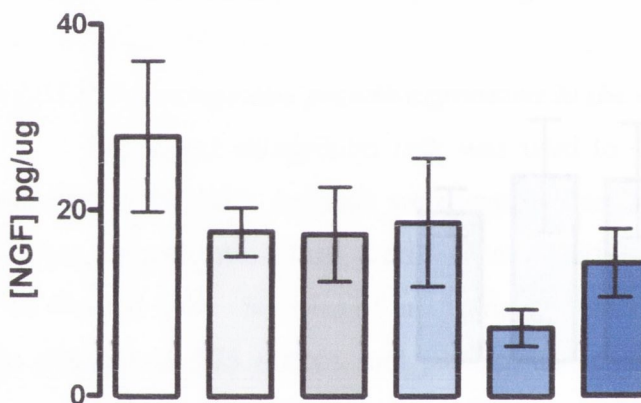
B. NGF expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in NGF expression at timepoints recorded. Histograms represent mean BDNF concentration ± SEM in pg/µg, n=6, 1-way ANOVA.
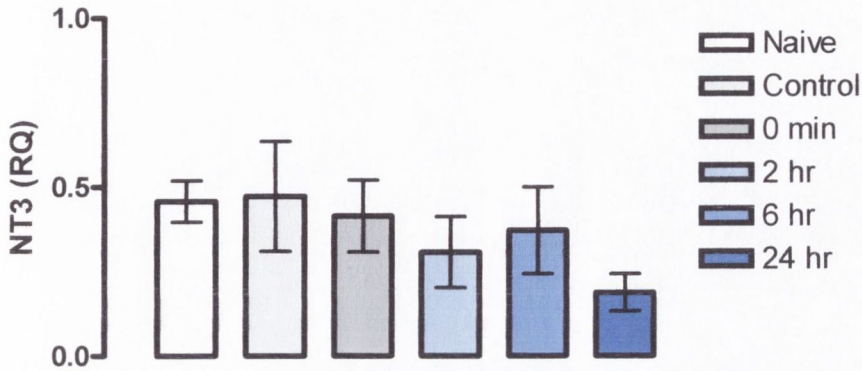
A. NT3 mRNA

B. NT3 protein expression

Figure 4.28 Learning in the rat results in increased NT3 protein expression but not NT3 gene expression in the entorhinal cortex
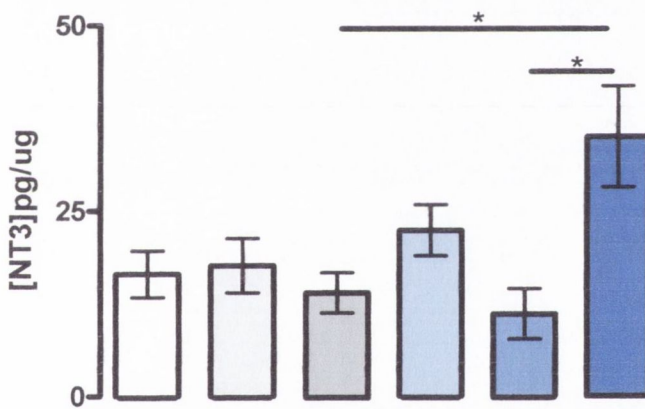
A. NT3 mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed no significant changes in NT3 mRNA expression following learning, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. NT3 expression was measured by ELISA at all timepoints following learning in the rats. Statistical analysis revealed a significant increase in NT3 expression at 24hr following consolidation when compared to 2hr and 0min

*p<0.05; 1-way ANOVA, *post hoc* Newmann Keuls. Histograms represent mean BDNF concentration ± SEM in pg/µg, n=6.

A. NT4/5 mRNA

Figure 4.29 NT4/5 gene expression in the entorhinal cortex following learning in the rat. NT4/5 protein levels were undetectable in the entorhinal cortex by ELISA.

A. NT4/5 mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed no significant increases in NT4/5 mRNA expression following learning, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

A. Trk A mRNA
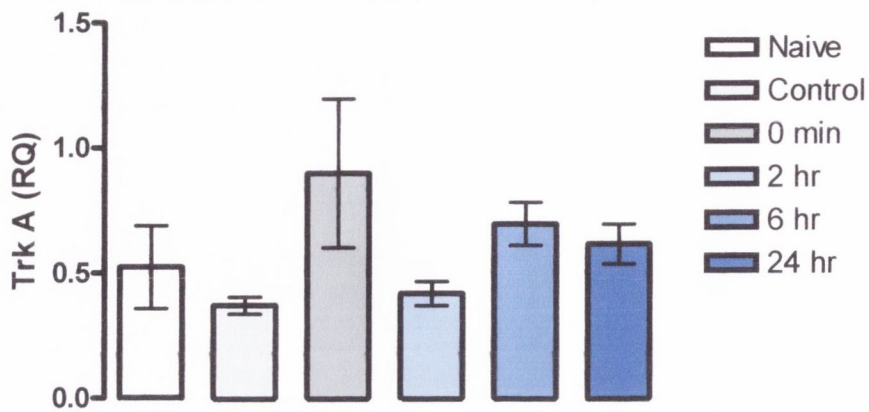
B. Western immunoblot

Trk A

Actin

C. Histogram represents Trk A protein expression

Figure 4.30 Trk A gene and Trk A protein expression in the entorhinal cortex following learning in the rat

A. Trk A mRNA was analysed in all timepoint groups following exploration. Statistical analysis revealed no significant changes in Trk A mRNA following

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consolidation when compared to naïve, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. Sample western immunoblot illustrating Trk A expression in the entorhinal cortex.

C. Trk A protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk A expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way ANOVA.

A. Trk B mRNA

B. Western immunoblot

Trk B

Actin
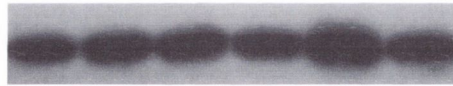
C. Histogram represents Trk B protein expression
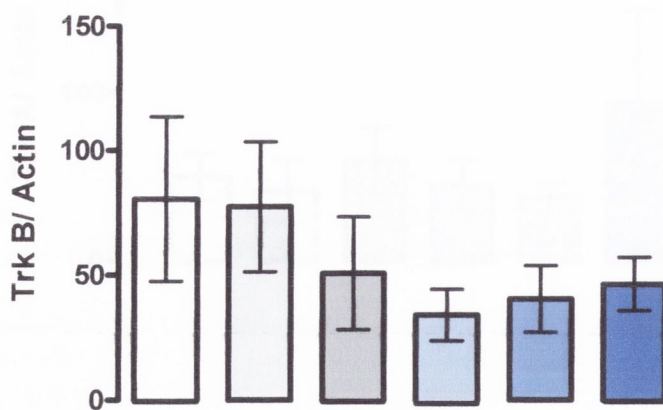
Figure 4.31 There is no change in Trk B mRNA or Trk B protein expression in the entorhinal cortex following learning in the rat

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A. Trk B mRNA was analysed in all timepoint groups following exploration Statistical analysis revealed no significant changes in Trk B mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

B. Sample western immunoblot illustrating Trk B expression in the entorhinal cortex

C. Trk B protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk B expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way ANOVA.

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A Trk C mRNA

B. Western immunoblot

Trk C

Actin

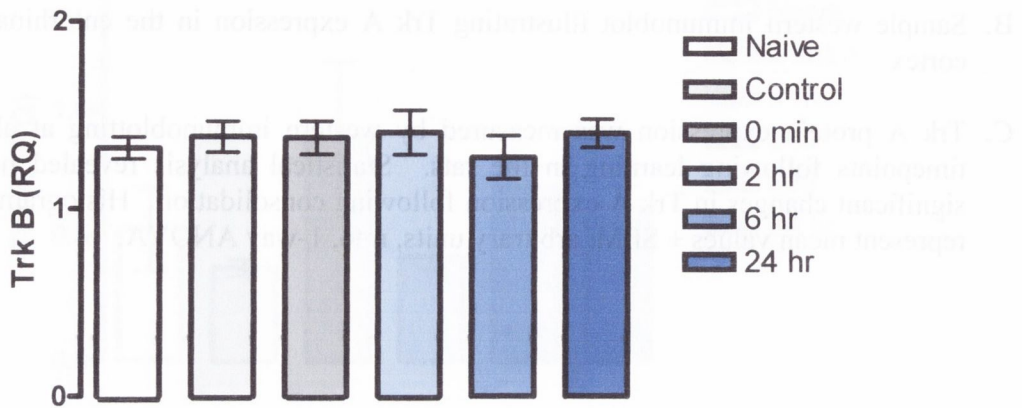C. Histogram representing Trk C protein expression

Figure 4.32 Trk C mRNA and Trk C protein expression in the entorhinal cortex following learning in the rat

    A. Trk C mRNA was analysed in all timepoint groups following exploration

Statistical analysis revealed no significant changes in Trk C mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.
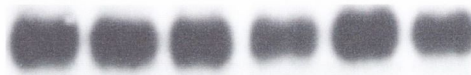
B. Sample western immunoblot illustrating Trk C expression in the entorhinal cortex

C. Trk C protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in Trk C expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way-ANOVA.

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A. P75 mRNA

B. Western immunoblot

P75

Actin

C. Histogram representing P75 protein expression

Figure 4.33 There is no change in p75 mRNA or p75 protein expression in the entorhinal cortex following learning in the rat

C. P75 mRNA was analysed in all timepoint groups following exploration

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Statistical analysis revealed no significant changes in P75 mRNA expression, data are expressed as means and standard error of the mean, n=6, 1-way ANOVA.

D. Sample western immunoblot illustrating P75 expression in the entorhinal cortex

C. P75 protein expression was measured by western immunoblotting at all timepoints following learning in the rats. Statistical analysis revealed no significant changes in P75 expression following consolidation. Histograms represent mean values ± SEM, arbitrary units, n=6, 1-way-ANOVA.

4.4 Discussion

From the cellular prespective, memory is not aquired in the form in which it is finally stored, instead memory formation is a temporally graded process during which new information becomes consolidated over time. Consolidation is a process essential to formation of long-lasting memories. Memory consolidation is believed to be a multi-step process comprised of complex molecular cascades leading to more robust and effective inter-neuronal communication. Learning induced synthesis of new proteins is ascribed to play a pivotal role in the consolidation process (Bekinschtein *et al.*, 2007) but detailed information covering specific proteins and timing of their translation is still lacking. Having previously identified that activation of Trk receptors by neurotrophins is essential to acquisition and consolidation of recognition memory, in this study we have aimed to reveal the changes in neurotrophin and neurotrophin receptor expression over a 24hr period of memory consolidation in four different brain regions, the dentate gyrus, hippocampus, perirhinal cortex and the entorhinal cortex, which have all previously been linked to memory formation (Jun *et al*, 1998; Smith and Mizumri, 2006, Winters *et al.*, 2007, Khawaja *et al.*, 2007).

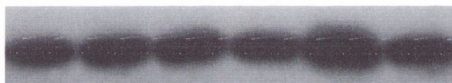Both early and late stages of memory consolidation and LTP have been shown to be dependent upon protein synthesis (Lu *et al.*, Commarota *et al.*, 2007, Herandez *et al.*, 2002, Scharf *et al.*, 2002). During memory formation, protein synthesis is thought to be required to transform newly learned information into stable synaptic modifications. Neurotrophins have been extensively implicated in synaptic plasticity and memory; this is supported by the findings from our earlier study. It has been demonstrated that neurotrophin activation is essential for protein synthesis which is essential to LTP and memory processing (Patterson *et al.*, 1996, Tyler *et al.*, 2002, Morris *et al.*, 2003). Of particular interest to this study consolidation and reconsolidation of recognition memory has been shown to be protein synthesis dependent (Kelly *et al.*, 2003). Protein synthesis is believed to modulate synapse formation and dendritic spine growth, which are processes thought to underlie memory storage (Bamji *et al.*, 2006, Tyler and Pozzo-Miller, 2003).

Here we chose to analyze expression of neurotrophins and their receptors in the denate gyrus, the hippocampus, the perirhinal cortex and the entorhinal cortex. As

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previously mentioned in the introduction, these are all regions of the medial temporal lobe that are regarded to be essential to memory processing. Lesions to any one of these brain regions have been shown to disrupt consolidation (Broadbent *et al*, 2004, Bussey *et al*, 1999, Brown *et al*., 1999, Aggleton and Brown, 1999). The dentate gyrus and the perirhinal cortex appear to be the most extensively investigated regarding recognition memory (Kelly *et al*., 2003, Cowell *et al*., 2006, Furtk *et al*., 2007).

The dentate gyrus is the brain region where LTP was first recorded via the perforant path. This was the first brain region where we assessed neurotrophin and Trk receptor expression and protein. Here we found an immediate increase in BDNF mRNA at 0min along with an increase at 6hr, although not significant this was accompanied by increases in BDNF protein at 0min 2hr and 6hr, with levels returning to normal by 24hr. It was interesting to see an immediate increase in BDNF mRNA following learning with matching protein increases. It is possible that the rapid increase in protein is from BDNF released from intracellular stores as previously shown (Xu *et al*., 2000, Alonso *et al*., 2002). In this study, we have assessed protein expression in homogenate and have not attempted to assess more specifically the localization of expression of our proteins of interest. Other studies have shown increases in BDNF mRNA up to 12hr after acquisition in the hippocampus whole, suggesting this later increase is associated with long term memory storage (Bekinschtein *et al*., 2007).

Similarly with NGF increases in mRNA expression were observed immediately (0min) and at 6hr following training, with increases in NGF protein observed at 6hr following learning. This was accompanied by an increase in Trk A mRNA expression at both 0min and 6hr. These increases in NGF/Trk A expression further support the evidence which exists, suggesting a role for NGF/Trk A signaling in memory processing. NGF expression has previously been shown to be upregulated at 2hr following LTP (Bramham *et al*., 1996) and it has also been reported that inhibition of NGF block induction of LTP (Maguire *et al*., 1999).

Surprisingly NT3 mRNA was found to be decreased in all groups, including the controls (habituated to arena), compared to naïve animals (killed directly from home cage), which suggests that exposure to new environments, even in the absence of objects, results in downregulation of NT3. On the basis of this finding we can tentatively

hypothesise a role for regulation of NT3 expression in dentate gyrus in novelty detection, however the functional significance of this decreased expression of NT3 remains to be elucidated. Alternatively previous studies have shown decreases in NT3 expression, both mRNA and protein, in response to seizure activity in the dentate gyrus (Rocamora et al., 1994, Bramham et al., 1996). Seizures occur as a result of over-excitation of the synapse, perhaps the increased synaptic excitability which is essential to LTP and memory consolidation can have similar effects on NT3 expression.

Previously we have identified a role for NT4/5 in consolidation of recognition memory. Other studies have also implicated NT4/5 in induction of LTP, with NT4/5[-/-] mice impaired in LTP (Xie et al., 2000). Here we found NT4/5 was significantly increased immediately following training and this increase remained for up to 2 hrs there after further supporting the evidence that NT4/5 modulates memory consolidation.

The hippocampus was the second region analyzed for changes in neurotrophin expression following consolidation. Here the hippocampus consists mainly of the CA1, CA2 and CA3 regions as the dentate gyrus has been subdissected from the hippocampus formation. There is ample evidence in the literature declaring the hippocampus as a central brain region in memory processing (Jun et al, 1998; Smith and Mizumri, 2006; Dash et al, 2002). We observed very little changes in mRNA expression of any of the neurotrophins in the hippocampus suggesting that the dentate gyrus, a sub-region of the hippocampus may be more involved in processing this type of memory. An increase in NT4/5 mRNA was observed at 2 hr following training in the object recognition task. Although, to our knowledge NT4/5 mRNA has never before been analyzed following consolidation, other studies have reported NT4/5 mRNA in the hippocampus (Timmusk et al., 1993), unfortunately we have no data on protein for this region as NT4/5 protein expression as measured by ELISA was below detectable levels.

The only neurotrophin protein change detected in this region was in BDNF. In many other reports BDNF has been identified as an important modulator of synaptic plasticity in the hippocampus (Knipper et al, 1994; Levine et al, 1995; Takei et al, 1997, Roux et al., 2006, Macbeth et al., 2007). Here we report increase in the control group which were habituated to the arena and also at 2hr following exploration/learning of the

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objects. This increase further supports the data in the literature. Habituation to the arena may have acted as a mild, acute form of enriched environment, which has previous been shown to increase BDNF levels in the hippocampus (Rossi *et al*., 2006).

Neurotrophin and receptor expression were assessed in the perirhinal cortex, an area of the brain largely associated with recognition memory, with lesions of this region resulting in an impairment of recognition memory (Mumby *et al*., 2007).Surprisingly no changes were observed in BDNF mRNA expression in the perirhinal cortex with only slight increases in BDNF protein expression observed immediately and 2hr following learning. Previous studies have reported widespread BDNF expression in the perirhinal cortex (Hashimoto *et al*., 2000) with increased BDNF release following induction of LTP in slices of the perirhinal cortex (Aicardi *et al*., 2004).

NGF mRNA expression was increased at 2hr following learning, this did not appear to translate into protein increases at this or later timepoints, however other studies investigating memory consolidation reported increases in NGF expression between 24hr and 1 week following learning (Woolfe *et al*., 1999) suggesting that NGF is more important in long term memory consolidation. Interestingly Trk A mRNA levels appear to be decreased in all groups compared to control groups. This novel finding may again be linked with novelty detection, as discussed above in the case of NT3 expression in dentate gyrus, however this is purely speculative and no evidence exists in the literature as yet to support this. Further experiments are warranted to clarify any functional significance of this observed change.

As previously found in the dentate gyrus, NT3 mRNA and protein were decreased in all groups including the control compared to naïve animals, again we can only speculate that perhaps this may be a cellular event associated with novelty-detection. Interestingtly we see opposite effects with the Trk C receptor mRNA expression with increases observed immediately following learning, although previous studies have reported large differences in the expression patterns of NT3 and TrkC in the perirhinal cortex (Bramham *et al*., 1996, Okuno *et al*., 1994).

There are very few studies investigating the effects of NT4/5 with little or none having identified expression of this neurotrophin in the perirhinal cortex. In this study

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we detected NT4/5 mRNA in this region and report that expression was significantly increased immediately following training and this increase remained for up to 2 hr following learning but had disappeared by 6hr, indicating that NT4/5 is involved in the early stages of memory consolidation. Unfortunately NT4/5 protein levels were undetectable by ELISA for this region so even if small changes occurred we could not detect them.

The Entorhinal cortex, has been seen as an impotent modulator in memory processing as it mediates the signaling input and output of the hippocampus. Here we analyzed neurotrophins and Trk receptor expression to investigate if the entorhinal cortex has a direct link to recognition memory processing.

Firstly we identified increases in BDNF mRNA at 6hr following learning with slight increases in BDNF in all groups compared to naïve animals following either habituation or learning. In support of these findings, previous studies have observed increases in BDNF mRNA at 4.5hr following memory consolidation (Broad et al., 2002).

NT3 protein expression was increased at 24 hr following learning, suggesting that perhaps NT3 is more involved in the late phase of memory consolidation where synaptic modulation is believed to take place. It has previously been reported that NT3 can modulate structural and functions changes at a synapse and it may also increase the number of synaptic sites of a neuron (Je et al., 2005). Perhaps the changes in NT3 expression and protein would be detected at a later time. Other studies have suggested that memory consolidation is a process that takes place over days and even weeks (Woolfe et al., 2001) and that NT3 may play a functional role in this later stage of memory consolidation.

Overall it appears that the greatest changes in neurotrophin expression were observed in the dentate gyrus and the perirhinal cortex, both are brain regions which have been largely associated with recognition memory in previous studies (Kelly et al., 2003, Cowell et al., 2006, Furtk et al., 2007). We also observed that the most notable changes in the neurotrophins were immediate or in the early hours following memory consolidation with BDNF displaying the most prevalent modifications.

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In this study we found a number of increases in neurotrophin mRNA expression which rarely transcribed into parallel changes in protein expression. We now believe that the time gaps of 2 hours, 4 hours and then 18 hours, which were chosen in the context of the published literature, constituted time windows that were too large to detect protein expression following observed changes in mRNA expression. Having found that NT4/5 protein expression levels were undetectable in all brain regions, with the exception of the dentate gyrus, we found that Katoh-Semba and colleagues (2003) on examining NT4/5 protein in many brain structures found that its levels are extremely low in many brain structures. This information together with our results from the previous study showing that anti-NT4/5 blocks memory acquisition, suggests that NT4/5 must have extremely potent signaling effects on the Trk B receptor to elicit such a response give its low expression.

It has to be noted that very little changes in Trk receptor expression were observed in any of the brain regions analyzed. Here we only measured the expression of the full receptor forms and not the phosphorylation of the Trk receptors. It would be of great interest to identify Trk receptor phosphorylation during memory consolidation to identify which receptors are activated when and in which brain region. Unfortunately because of Trk receptor homology there are no available antibodies on the market which are reliable in differentiating between the individual receptors. Analysis of the p75 receptor which is capable of binding any one of the neurotrophins revealed that p75 expression does not appear to change as a result of memory processing. The elusive effects p75 receptor signaling remain unclear, however previous studies have implicated p75 signaling with apoptosis and cell damage, and upregulation of p75 expression is associated with impairment of LTP and learning (Hennigan et al., 2007). From the literature p75 signaling does not appear to be involved in the mediation of recognition memory. In our lab we have previously observed that blocking of the p75 receptor enhances both LTP and spatial learning (Hennigan et al., unpublished data). Together the data presented/discussed here suggests that p75 upregulation may have a negative impact on synaptic plasticity and cognition, although further investigation is required to address this issue.

The objective of this study was to access neurotrophin and Trk receptor expression over a 24hr period following learning in the rat, through which this time consolidation takes place. From our findings it appears that neurotrophins are essential to memory processing, and it is possible the immediate effects of neurotrophin release and signaling that are most important in mediating protein synthesis which is essential to synaptic plasticity (Kang and Schuman, 1996). This will be addressed in our subsequent studies.

Chapter 5

Neurotrophins and recognition memory: investigation of
the underlying intracellular signaling mechanisms

5.1 Introduction

In Chapter 3 and 4 we have provided evidence that neurotrophins and their activation of the Trk receptors may play a vital role in consolidation of recognition memory. In chapter 3 we showed that inhibition of BDNF, NGF or NT4/5 blocked memory consolidation and that inhibition of all the Trk receptors by Tyrphostin AG879 blocked both acquisition and consolidation of recognition memory. From chapter 4 there is substantial evidence supporting the role of neurotrophins in learning and memory with significant increases in neurotrophin mRNA in the dentate gyrus, hippocampus, perirhinal cortex and the entorhinal cortex within 24hr of learning taking place. As previously mentioned, synthesis of these proteins plays a pivotal role in memory formation but the key intracellular signaling molecules which enable this process have not been well documented for this type of memory. Assessment of the role of such neurotrophin-stimulated signaling cascades is the aim of this study.

Neurotrophins, upon binding to the Trk receptors, activate a cascade of signaling events which are also believed to be important in expression of various forms of synaptic plasticity. Extracellular signal-regulated kinases (ERKs) are members of the mitogen-activated protein kinase (MAPK) superfamily and form a major signal transduction pathway mediating extracellular stimuli, among which is the binding of neurotrophins to their preferred Trk receptor, to transcription in the nucleus (Schaeffer and Weber, 1999). ERKs were originally discovered as regulators of cell division and differentiation but, like neurotrophins, were soon reported to play a major role in synaptic plasticity, learning and memory (Atkins *et al.*, 1998; Sweatt, 2001, 2004; Mazzucchelli *et al.*, 2002).

ERK activation, which is stimulated by ligand binding of the Trk receptors has been shown to be involved in other types of hippocampal-dependent memory. Blum and colleagues in 1999 displayed evidence showing a role for ERK in long term spatial memory. Another study showed that rats injected with the ERK inhibitor, UO126, were unable to distinguish a familiar from a novel object. The authors found increased ERK phosphorylation in various structures of the medial temporal lobe implicating the ERK/MAPK pathway in the signaling cascades activated during memory consolidation (Kelly *et al.*, 2003). In the same study the authors also established a possible role for the ERK/MAPK pathway in reconsolidation of long term recognition memory, finding that

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the inhibitor UO126 effectively blocks reconsolidation of recognition memory, and this was shown to be dependent on reactivation of the memory trace by brief reexposure to the objects. In addition to activation of certain kinases measurement of immediate early gene (IEG) activation such as c-fos across brain regions provides a means of visualizing the pattern of neural activation resulting from specific behaviours in animal models. Previous studies have shown increased c-fos synthesis following place and response learning in the hippocampus and the dorsal striatum (Gill *et al.*, 2007). In this study the aim was to assess whether activation of ERK and c-fos downstream of Trk receptor activation is important in mediating the cellular changes associated with both consolidation and reconsolidation of recognition memory.

5.2 Materials and Methods

Male Wistar rats aged between 2 and 4 months weighing between 250 and 350g were used in these experiments. Rats were trained in the object recognition task as described in section 2.4. In this experiment 4 groups were included, the first group, named our consolidation group, was sacrificed following training on day one of the task the second group named consolidation and learning, was sacrificed following testing on day 2 of the task, the third group named reconsolidation was sacrificed on day 2 of the task following re-exposure to objects A and B, the final group, the control group was habituated to the arena only in the absence of objects. Tissue was taken and biochemical analysis for ERK, c-fos and BDNF was performed by western immunoblotting (section 2.13 and 2.14) and ELISA (section 2.18).

5.3 Results

Exploration Results

5.3.1 Exploration time of objects by consolidation group

Animals in the consolidation group were exposed to objects A and B on day 1 of the experiment only. Animals showed equal exploration times of objects A and B (Figure 5.1, student *t*-test; Consolidation group, A: 48.44 ± 9.052%; B: 51.56 ± 9.052%) Results are expressed as mean ± SEM, n=6.
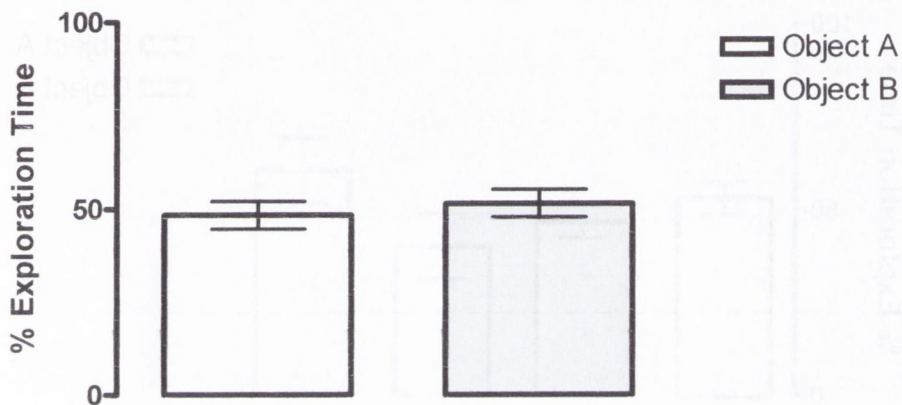
5.3.2 Exploration time of objects by reconsolidation group

Rats in the reconsolidation group were exposed to objects A and B on day 1 and objects A and B again on day 2. Statistical analysis showed there was no significant difference between exploration time of object A or B on day1, or day 2 (Figure 5.2, student *t*-test; Reconsolidation group day 1, A: 53.26 ± 10.71%; B: 46.74 ± 10.71%; day 2, A: 40.00 ± 21.43%; B: 60.00 ± 21.43%). Results are expressed as mean ± SEM, n=6.

5.3.3 Exploration times of objects by consolidation and learning group

Rats in the learning and consolidation group were exposed to objects A and B on day 1 and to objects A and C (a novel object) on day 2. Statistical analysis revealed there was equal exploration of the objects on day 1 but on day 2 rats had a significant preference for the novel object C (Figure 5.3, ***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls, consolidation A: 48.44 ± 9.052%; B: 51.66 ± 9.052%; consolidation & learning A: 19.78 ± 10.86%; C: 80.22 ± 10.86%). Results are expressed as mean ± SEM, n=6.

Figure 5.1 Exploration times of objects by Consolidation group

The time spent exploring each object by the Consolidation group was recorded and expressed as a percentage of total exploration time. Exploration consisted of 3x5 min periods. Statistical analysis showed that there was no significance difference in the amount of time the animals spent exploring the two objects. Histograms represent mean percentage exploration time ± SEM, n=6, student unpaired t-test.
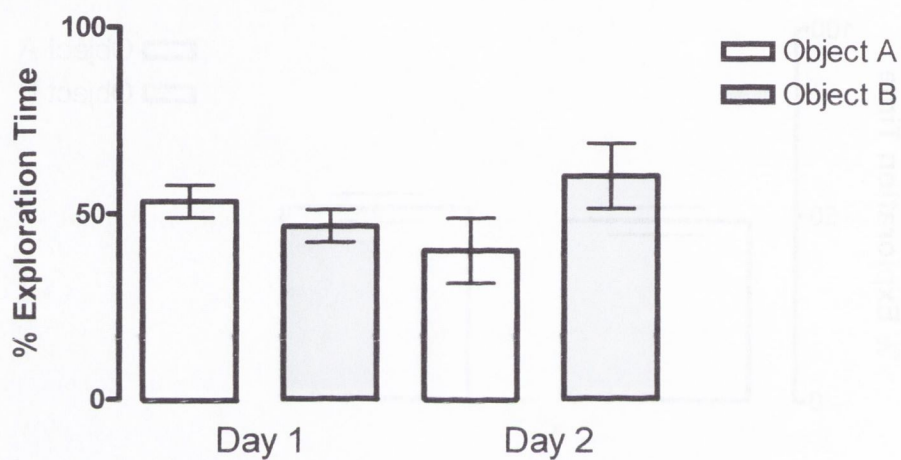
Figure 5.2 Exploration time of objects by Reconsolidation group

The time spent exploring each object by the reconsolidation group on day 1 and day 2 was recorded and is expressed as a percentage of total exploration time. Statistical analysis showed that there was no significant difference in the amount of time the rats spent exploring the two objects on either day. Histograms represent mean percentage exploration time ± SEM, n=6, unpaired student t-test.
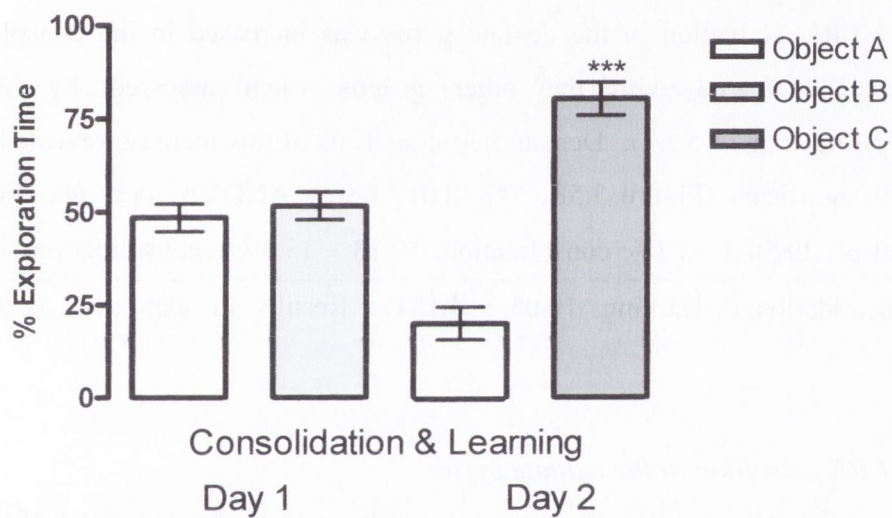
Figure 5.3 Exploration time of objects by Consolidation and Learning group

The time spent exploring each object by the consolidation and learning group was recorded and is expressed as a percentage of total exploration time. Statistical analysis showed that there was significantly greater exploration time of the novel object, C on day 2 of the experiment ($80.22 \pm 4.43\%$). Histograms represent mean percentage exploration time $\pm$ SEM, n=6, *** $P<0.0001$.

Dentate Gyrus

5.3.4 BDNFrelease in the dentate gyrus

KCl-stimulated release of BDNF was significantly increased in the consolidation group when compared with the other groups (Figure 5.4, *p<0.05; 1-way ANOVA, *post hoc* Newman Keuls, control: 585 ± 325.9 pg/ml; consolidation: 3147 ± 784; reconsolidation: 1937 ± 452.2; consolidation & learning: 2360 ± 761.7). Results are expressed as mean ± SEM, n=6.

5.3.5 p44 ERK activation in the dentate gyrus

P44 ERK activation in the dentate gyrus was increased in the consolidation group when compared with the other groups when assessed by Western Immunoblotting (Figure 5.5A). Densitometric analysis of this increase revealed it was statistically significant (Figure 3.5B, **p<0.01; 1-way ANOVA, *post hoc* Newman Keuls, control: 20.56 ± 12.3%; consolidation: 59.13 ± 15.39; reconsolidation: 18.36 ± 13.41; consolidation & learning: 10.05 ± 4.134). Results are expressed as mean ± SEM, n=6.

5.3.6 p42 ERK activation in the dentate gyrus

No statistically significant changes were observed in the activation of the p42 ERK isoform between any of the groups when analysed by Western Immunoblotting (Figure 5.6A). Densometric analysis of the immunoblot revealed there were no statistically significant differences between any of the groups. Results are expressed as mean ± SEM, n=6, 1-way ANOVA (Figure 5.6B)

5.3.7 C-fos activation in the dentate gyrus

C-fos activation in the dentate gyrus was increased in the consolidation group when compared with the other groups when assessed by Western Immunoblotting (Figure 5.7A). Densitometric analysis of this increase revealed it was statistically significant (Figure 5.7B, **p<0.01; 1-way ANOVA, *post hoc* Newman Keuls, control:

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4.349 ± 1.789%; consolidation: 32.14 ± 11.40; reconsolidation: 11.02 ± 6.599; consolidation & learning: 14.43 ± 7.410). Results are expressed as mean ± SEM, n=6.

Figure 5.4 BDNF release in the dentate gyrus

BDNF release in the dentate gyrus was measured across all groups (*n=6* for all groups), that were sacrificied on completion of experiments on day 1 and day 2. Statistical analysis revealed a significant increase in BDNF concentrations in the Consolidation group (* $P<0.05$), no significant increases were observed in the other groups. Histograms represent mean BDNF concentration ± SEM in pg/ml, 1-way ANOVA.

pERK

TERK
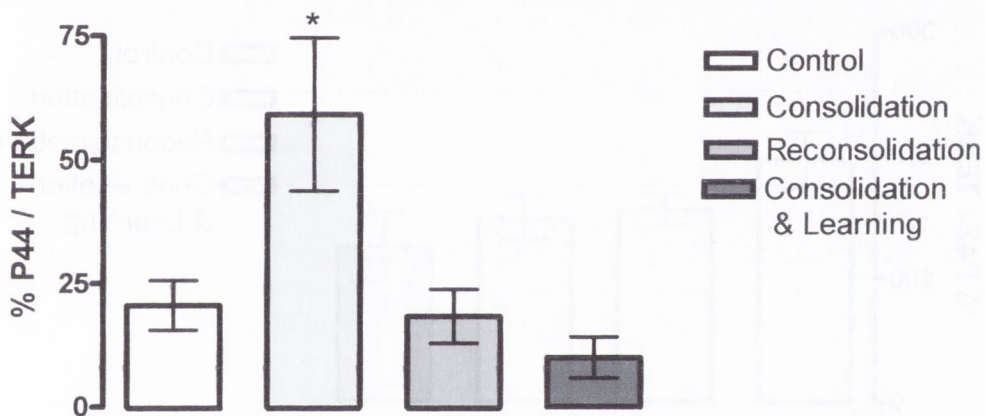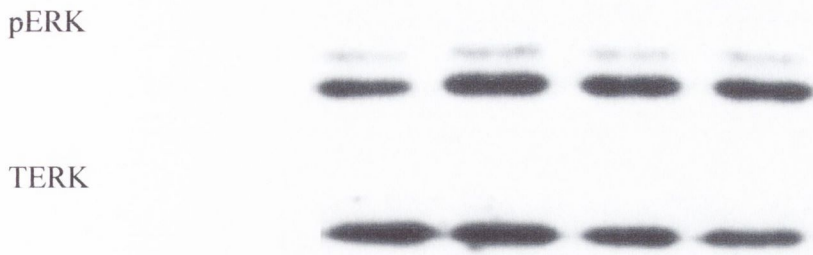
Figure5.5 p44 ERK in the dentate gyrus

A. Sample Western Immunoblot illustrating p44 ERK activation in the dentate gyrus

B. pERK was analysed in the dentate gyrus of all groups, n=6. The expression of the p44 isoform of pERK is given as a percentage of total ERK. Statistical analysis revealed a significant increase in the Consolidation group (* $P<0.01$), with no significant increase in the other groups. Histograms represent mean values ± SEM, 1-way ANOVA.

PERK

TERK
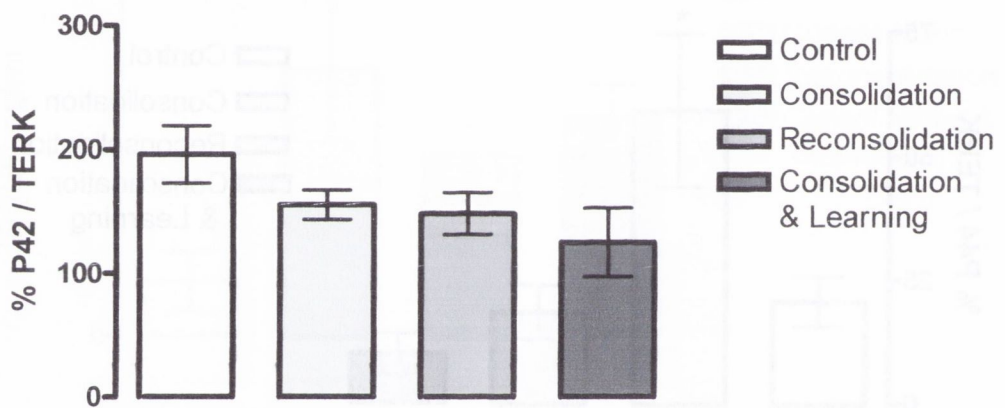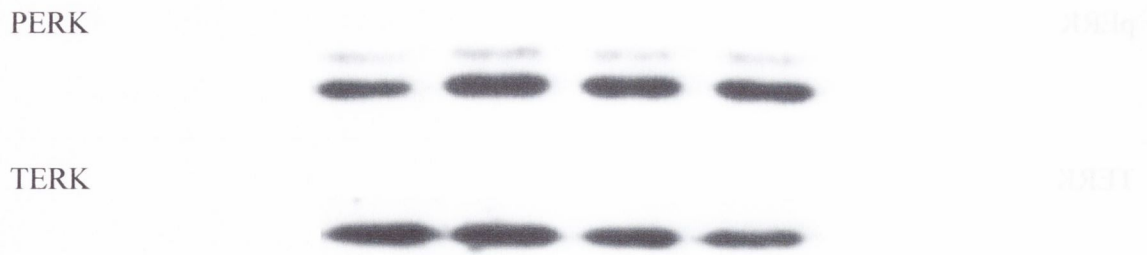
Figure5.6 p42 ERK in the dentate gyrus

A. Sample Western Immunoblot illustrating p42 ERK activation in the dentate gyrus

B. pERK was analysed in the dentate gyrus of all groups. The expression of the p42 isoform of pERK is given as a percentage of total ERK, *n=6* in all groups. Statistical analysis revealed no significant differences between the four groups. Histograms represent mean values ± SEM.
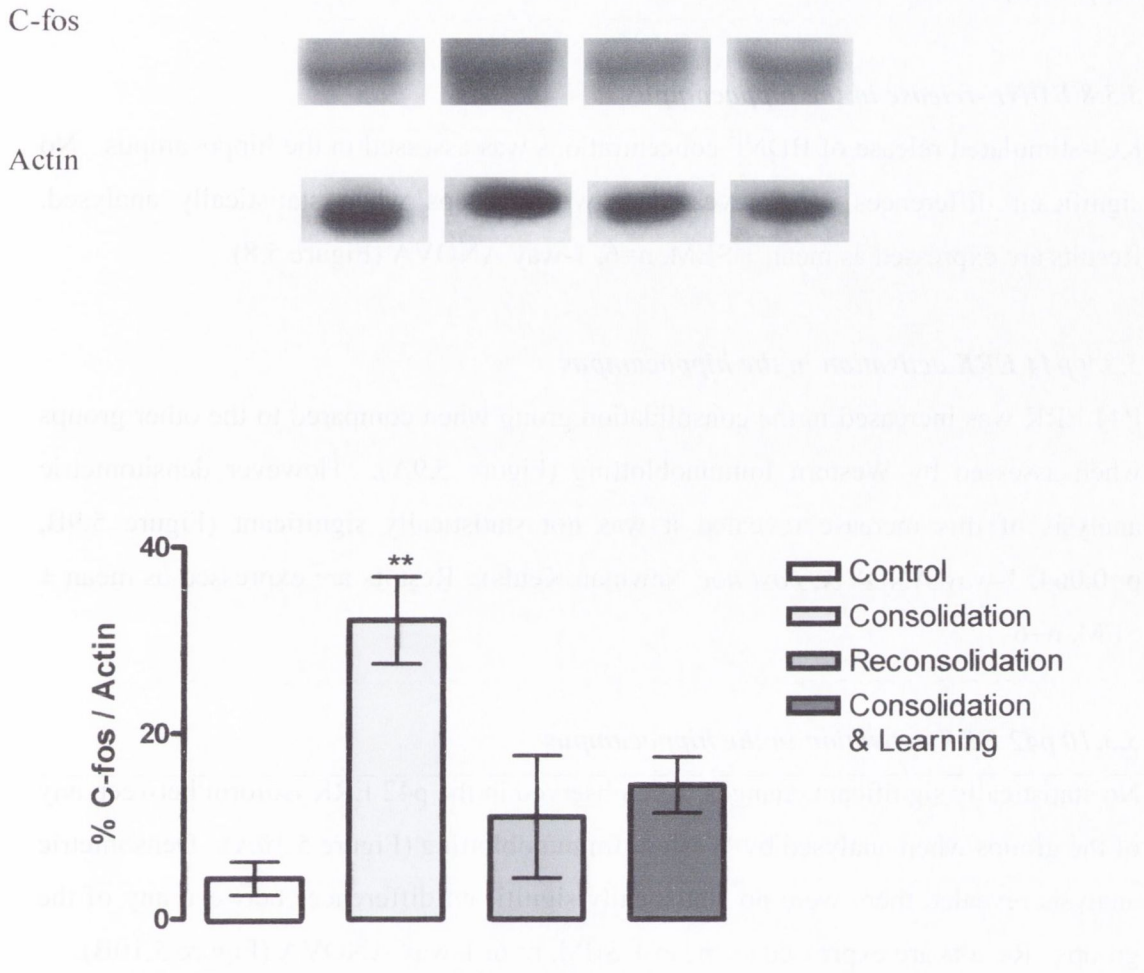
C-fos

Actin

Figure5.7 C-fos expression in the dentate gyrus

A. Sample Western Immunoblot illustrating c-fos activation in the dentate gyrus

B. The expression of c-fos as a percentage of actin was measured in the dentate gyrus of all groups (n=6 for all groups). Rats were sacrificed following completion of the experiments on day 1 and day 2. Statistical analysis revealed a significant increase in the expression of C-fos in Consolidation group (** $P<0.001$), no significant increases were observed in other groups.   Histograms represent mean values ± SEM.

Hippocampus

5.3.8 BDNF-release in the hippocampus

KCl-stimulated release of BDNF concentrations was assessed in the hippocampus. No significant differences were revealed between groups when statistically analysed. Results are expressed as mean ± SEM, n=6, 1-way ANOVA (Figure 5.8)

5.3.9 p44 ERK activation in the hippocampus

P44 ERK was increased in the consolidation group when compared to the other groups when assessed by Western Immunoblotting (Figure 5.9A). However densitometric analysis of this increase revealed it was not statistically significant (Figure 5.9B, p=0.064, 1-way ANOVA, *post hoc* Newman Keuls). Results are expressed as mean ± SEM, n=6.

5.3.10 p42 ERK activation in the hippocampus

No statistically significant changes were observed in the p42 ERK isoform between any of the groups when analysed by Western Immunoblotting (Figure 5.10A). Densometric analysis revealed there were no statistically significant differences between any of the groups. Results are expressed as mean ± SEM, n=6, 1-way ANOVA (Figure 5.10B).

5.3.11 C-fos activation in the hippocampus

C-fos activation in the hippocampus was increased in the consolidation group and the consolidation and learning group when compared to the other groups when assessed by Western Immunoblotting (Figure 5.11A). Densitometric analysis of the increase in the consolidation group and in the consolidation group revealed it was statistically significant (Figure 5.11B, * p<0.05 and **p<0.001 respectively; 1-way ANOVA, *post hoc* Newman Keuls, control: 39.01 ± 6.922%; consolidation: 74.51 ± 15.05; reconsolidation: 36.39 ± 14.75; consolidation & learning: 98.29 ± 14.79). Results are expressed as mean ± SEM, n=6.
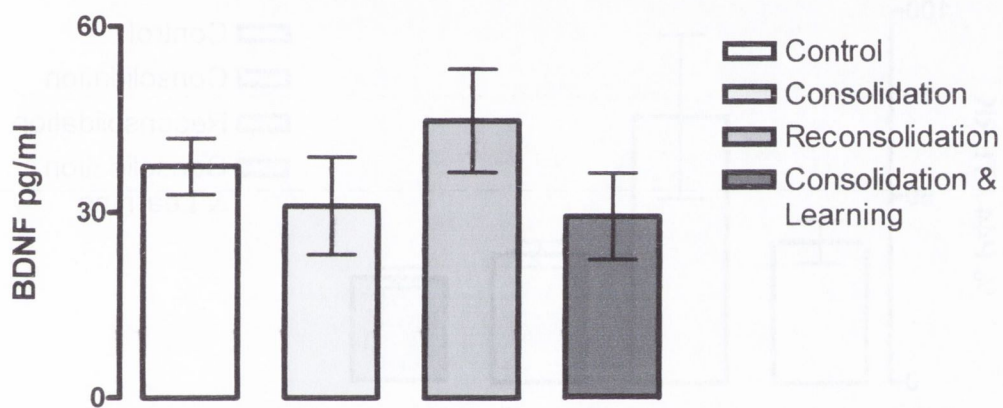
Figure 5.8 BDNF release in the Hippocampus

BDNF release was measured in the hippocampus of all groups (*n=6* for all groups), that were sacrificied on completion of experiments on day 1 and day 2. Statistical analysis revealed no significant differences between groups. Histograms represent mean BDNF concentration ± SEM in pg/ml, 1-way ANOVA.
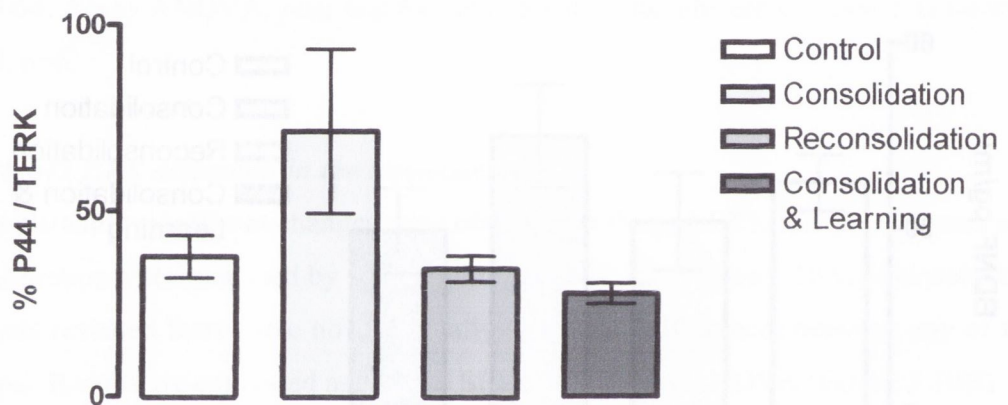
Figure5.9 p44 ERK in the hippocampus

A. Sample Western Immunoblot illustrating p44 ERK activation in the hippocampus

B. pERK was analysed in the hippocampus of all groups. The expression of the p44 isoform of pERK is given as a percentage of total ERK, *n=6* in all groups. Statistical analysis revealed no significant differences between the four groups. Histograms represent mean values ± SEM

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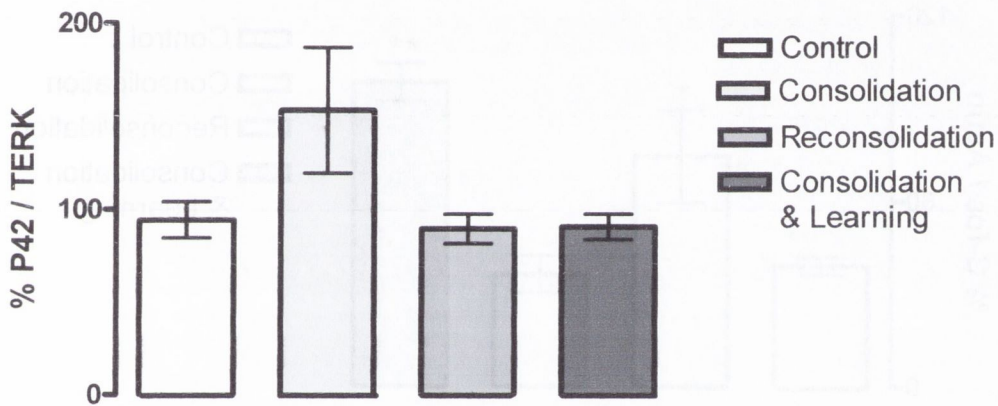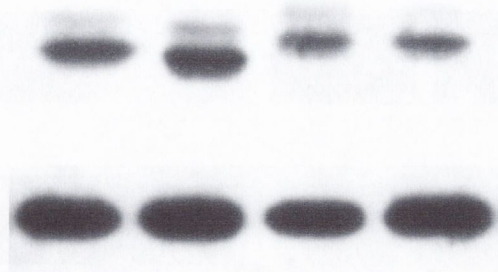
Figure5.10 p42 ERK in the Hippocampus

A. Sample Western Immunoblot illustrating p42 ERK activation in the hippocampus

B. pERK was analysed in the hippocampus in all groups. The expression of the p42 isoform of pERK is given as a percentage of total ERK, $n=6$ in all groups. Statistical analysis revealed no significant differences between the four groups. Histograms represent mean value ± SEM.

C-fos
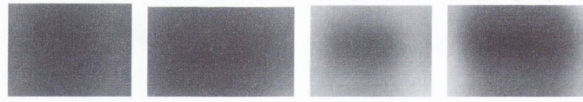
Actin
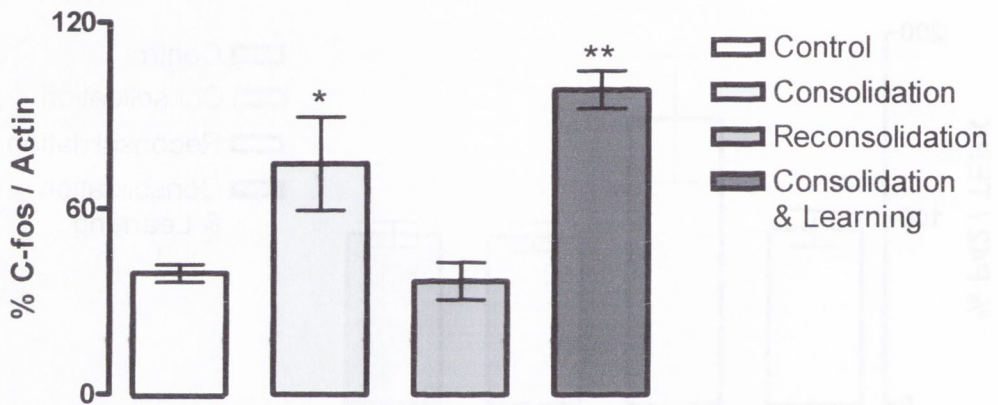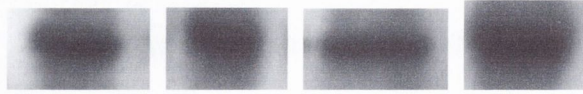
Figure 5.11 C-fos expression in the Hippocampus

A. Sample Western Immunoblot illustrating c-fos activation in the hippocampus

B. The expression of c-fos as a percentage of actin was measured in the hippocampus of all groups (n=6 for all groups). Rats were sacrificed following completion of the experiments on day 1 and day 2. Statistical analysis revealed a significant increase in the expression of c-fos in the consolidation group (* $P<0.05$) and in the consolidation and

learning group (** P<0.001), no significant increases were observed in the other groups. Histograms represent mean values ± SEM.

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Perirhinal Cortex

5.3.12 BDNF-release in the perirhinal cortex

KCl-stimulated release of BDNF was increased in the perirhinal cortex when assessed by ELISA. Analysis of the results revealed statistically significant increases in the consolidation group and the learning and consolidation group (Figure 5.12, *p<0.05 and *p<0.05 respectively; 1-way ANOVA, *post hoc* Newman Keuls, control: 0.5625 ± 0.5540pg/ml; consolidation: 27.43 ± 9.829; reconsolidation: 11.09 ± 6.389; consolidation & learning: 28.63 ± 12.07). Results are expressed as mean ± SEM, n=6.

5.3.13 p44 ERK activation in the perirhinal cortex

Increased p44 ERK expression was observed in the reconsolidation group when compared with the consolidation and learning group only when analysed by Western Immunoblotting (Figure 5.13A). Densitomeric analysis of this revealed a statistically significant increase (Figure 5.13B, *p<0.05; data; student *t*-test, reconsolidation: 10.22 ± 2.124%; consolidation & learning: 6.524 ± 1.607) Results are expressed as mean ± SEM, n=6.

5.3.14 p42 ERK activation in the perirhinal cortex

No statistically significant differences were observed between groups when analysed by Western Immunoblotting (Figure 5.14A). Densometric analysis revealed no significant differences. Results are expressed as mean ± SEM, n=6, 1-way ANOVA (Figure 5.14B)

5.3.15 C-fos activation in the perirhinal cortex

Increased c-fos expression was observed in the consolidation group and the consolidation and learning group when assessed by Western Immunoblotting (Figure 5.15A). Densitometric analysis of these increases revealed statistically significant increases in the consolidation group and in the learning & consolidation group (Figure 5.15B, **p<0.001; *p<0.05 respectively, 1-way ANOVA, *post hoc* Newman Keuls,

control: 18.89 ± 14.13%; consolidation: 59.51 ± 11.69; reconsolidation: 7.954 ± 2.844; consolidation & learning: 48.62 ± 14.35). Results are expressed mean ± SEM, n=6.

Figure 5.12 BDNF release in the perirhinal cortex

BDNF release was measured in the perirhinal cortex of all groups (*n=6* for all groups), that were sacrificied on completion of experiments on day 1 and day 2. Statistical analysis revealed a significant increase in BDNF concentrations in the Consolidation group (* P<0.05) and in the Consolidation and Learning group (* P<0.05), no significant increases were observed in the other groups. Histograms represent mean BDNF concentration ± SEM in pg/ml. 1-way ANOVA.

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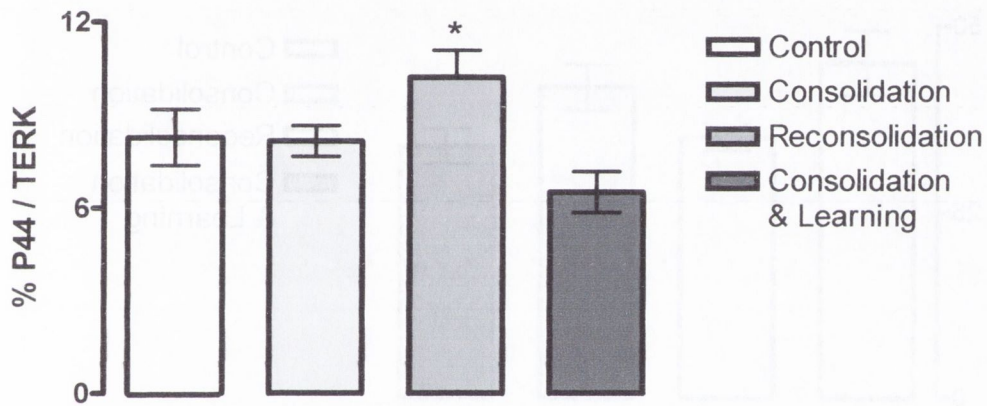
Figure5.13 p44 ERK in the perirhinal cortex

A. Sample Western Immunoblot illustrating p44 ERK activation in the perirhinal cortex

B. pERK was analysed in the perirhinal cortex in all groups. The expression of the p44 isoform of pERK is given as a percentage of total ERK, $n=6$ in all groups. Statistical analysis revealed a significant increase in the reconsolidation group (* $P<0.05$) compared to the consolidation and learning group, with no significant increase in other groups. Histograms represent mean values ± SEM.

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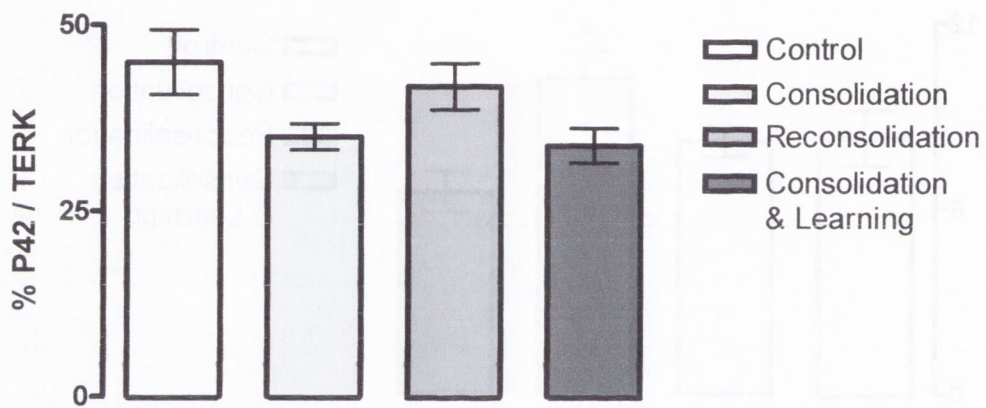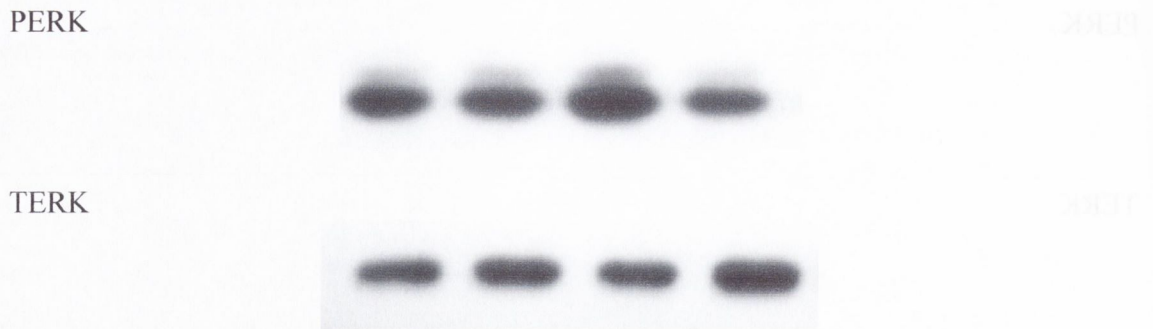
Figure5.14 p42 ERK in the perirhinal cortex

A. Sample Western Immunoblot illustrating p42 ERK activation in the perirhinal cortex

B. pERK was analysed in the perirhinal cortex of all groups. The expression of the p42 isoform of pERK is given as a percentage of total ERK, *n=6* in all groups. Statistical analysis revealed no significant differences between the four groups. Histograms represent mean values ± SEM.
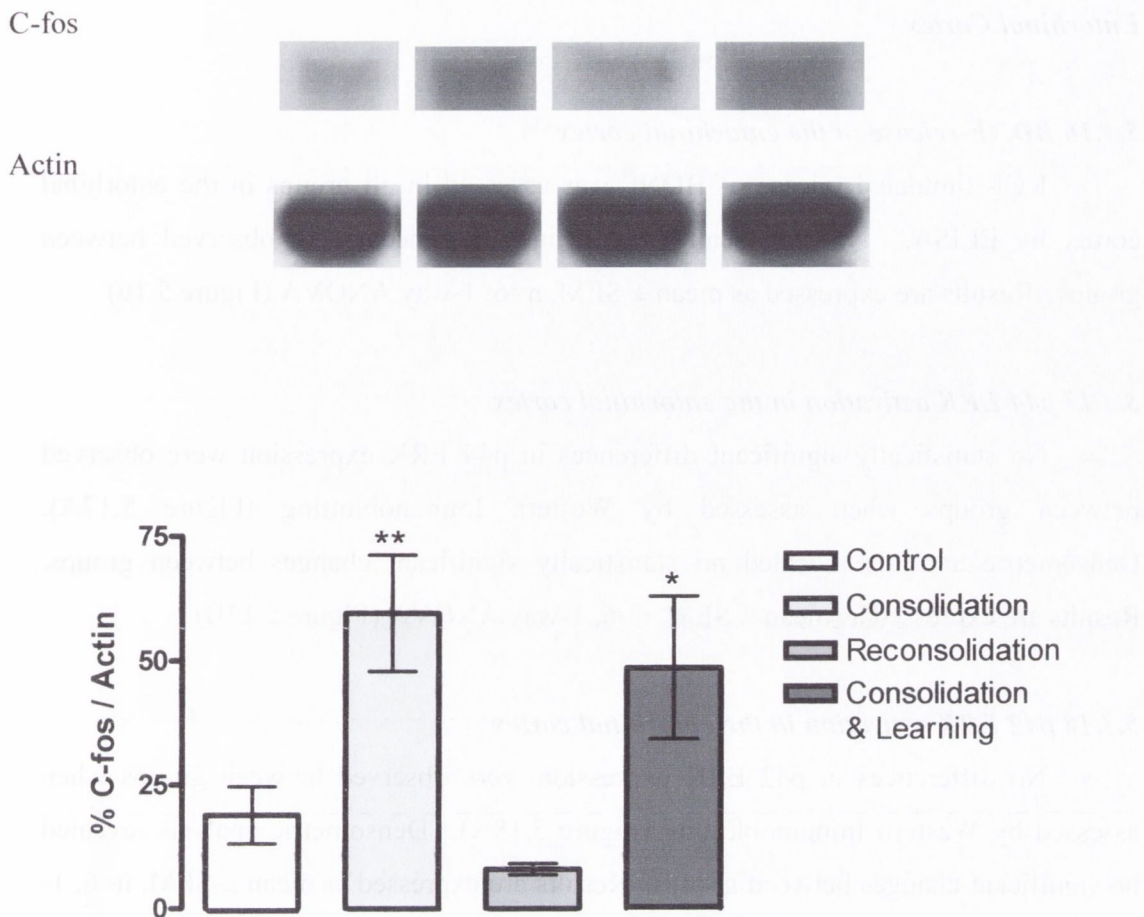
C-fos

Actin

Figure 5.15 C-fos expression in the Perirhinal cortex

A. Sample Western Immunoblot illustrating c-fos activation in the perirhinal cortex

B. The expression of c-fos as a percentage of actin was measured in the perirhinal cortex of all groups (n=6 for all groups). Rats were sacrificed following completion of the experiments on day 1 and day 2. Statistical analysis revealed a significant increase in the expression of c-fos in the consolidation group (** $P<0.001$) and in the learning and consolidation group (* $P<0.05$), no significant increases were observed in other groups. Histograms represent mean values ± SEM.

Entorhinal Cortex

5.3.16 BDNF-release in the entorhinal cortex

KCl-stimulated release of BDNF was assessed in all groups in the entorhinal cortex by ELISA. No statistically significant differences were observed between groups. Results are expressed as mean ± SEM, n=6, 1-way ANOVA (Figure 5.16)

5.3.17 p44 ERK activation in the entorhinal cortex

No statistically significant differences in p44 ERK expression were observed between groups when assessed by Western Immunoblotting (Figure 5.17A). Densometric analysis revealed no statistically significant changes between groups. Results are expressed as mean ± SEM, n=6, 1-way ANOVA (Figure 5.17B)

5.3.18 p42 ERK activation in the entorhinal cortex

No differences in p42 ERK expression were observed between groups when assessed by Western Immunoblotting (Figure 5.18A). Densometric analysis revealed no significant changes between groups. Results are expressed as mean ± SEM, n=6, 1-way ANOVA (Figure 5.18B)

5.3.19 C-fos activation in the entorhinal cortex

C-fos activation in the entorhinal cortex was increased in the consolidation group when compared with the other groups when assessed by Western Immunoblotting (Figure 5.19A). Densitometric analysis of this increase revealed it was statistically significant (Figure 5.19B, * p<0.05; 1-way ANOVA, *post hoc* Newman Keuls, control: 19.88 ± 1.669; consolidation: 48.36 ± 10.66; reconsolidation: 32.80 ± 3.165; consolidation & learning: 27.84 ± 6.311). Results are expressed as mean ± SEM, n=6.

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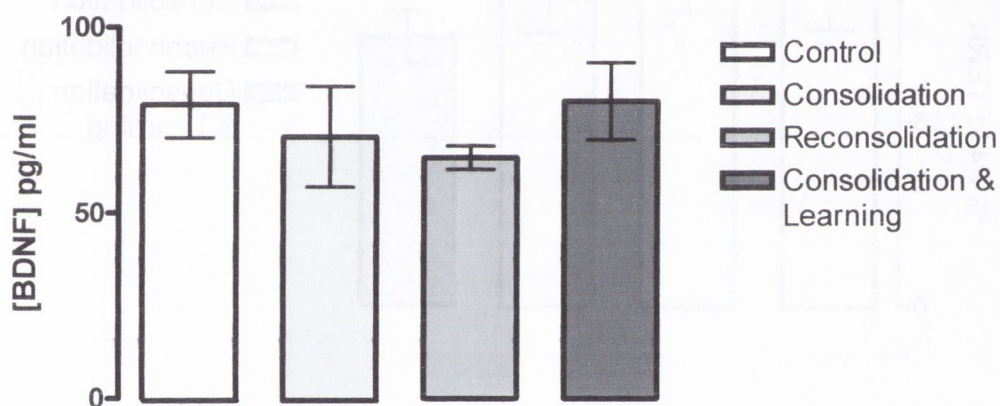
Figure 5.16 BDNF release in the entorhinal cortex

BDNF release was measured in the entorhinal cortex of all groups (*n=6* for all groups), that were sacrificied on completion of experiments on day 1 and day 2. Statistical analysis revealed no significant differences between groups. Histograms represent mean BDNF concentration ± SEM in pg/ml, 1-way ANOVA.
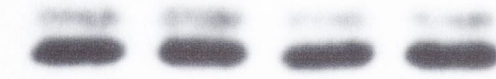
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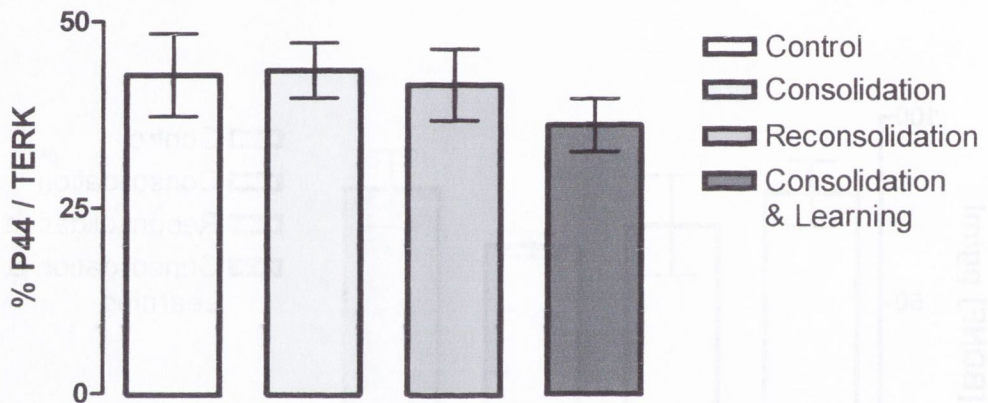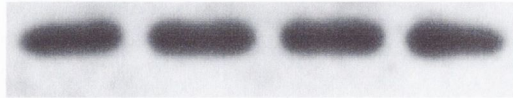
Figure5.17 p44 ERK in the entorhinal cortex

A. Sample Western Immunoblot illustrating p44 ERK activation in the entorhinal cortex

B. pERK was analysed in the entorhinal cortex of all groups. The expression of the p44 isoform of pERK is given as a percentage of total ERK, *n=6* in all groups. Statistical analysis revealed no significant differences between the four groups. Histograms represent mean values ± SEM.

PERK

TERK
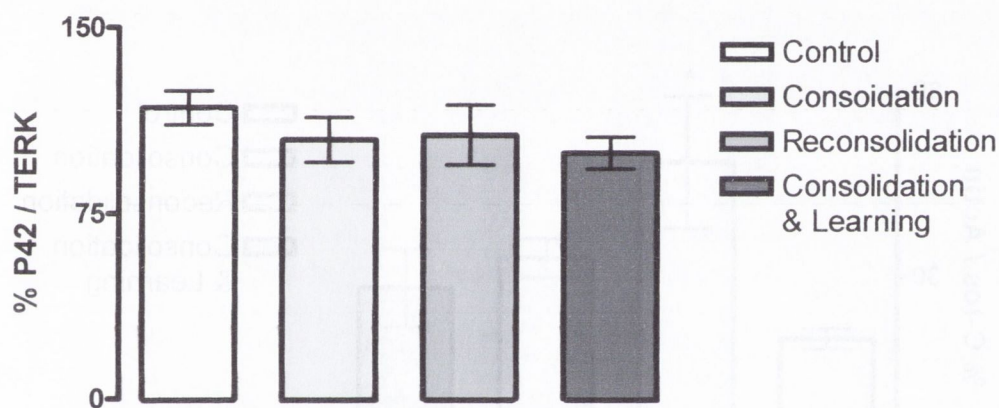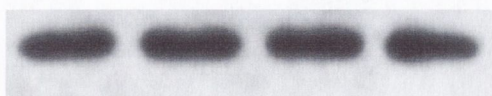
Figure 5.18 p42 ERK in the entorhinal cortex

A. Sample Western Immunoblot illustrating p42 ERK activation in the entorhinal cortex

B. pERK was analysed in the entorhinal cortex of all groups. The expression of the p42 isoform of pERK is given as a percentage of total ERK, *n=6* in all groups. Statistical analysis revealed no significant differences between the four groups. Histograms represent mean values ± SEM.
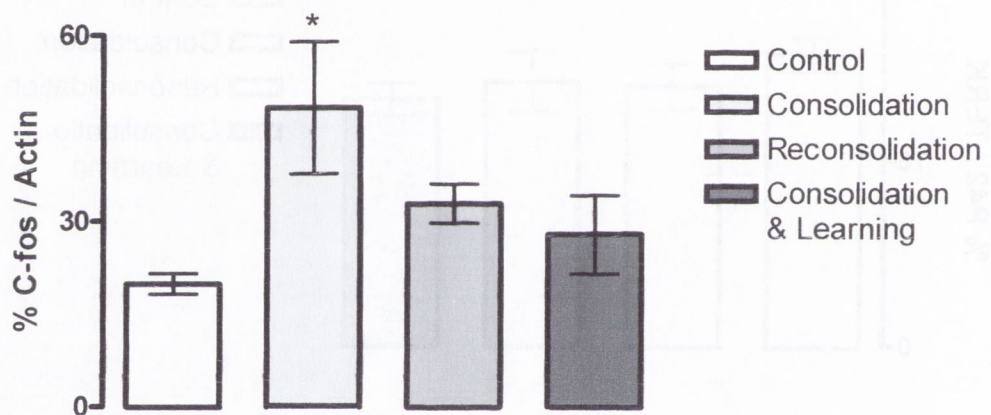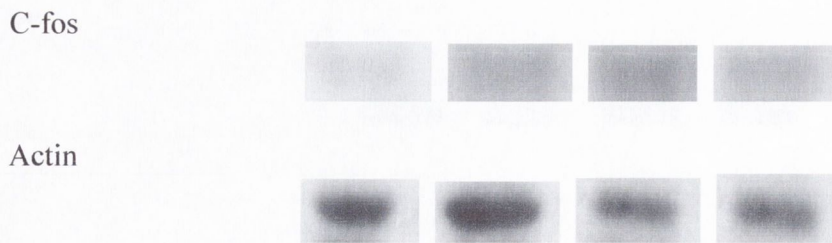
C-fos

Actin

Figure 5.19 C-fos expression in the entorhinal cortex

A. Sample Western Immunoblot illustrating c-fos activation in the entorhinal cortex

B. The expression of c-fos as a percentage of actin was measured in the entorhinal cortex of all groups (n=6 for all groups). Rats were sacrificed following completion of the experiments on day 1 and day 2. Statistical analysis revealed a significant increase in the expression of c-fos in the consolidation group (* $P<0.05$), no significant increases were observed in other groups. Histograms represent mean values ± SEM.

5.4 Discussion

Until now we have only discussed two stages of memory processing, acquisition and consolidation, and we have provided evidence to suggest that activation of Trk receptors by neurotrophins is essential to acquisition and consolidation of recognition memory. Consolidation has been typically understood as a process that happens once for each memory and, consequently, it has been assumed that after a trace is consolidated it becomes insensitive to subsequent disruption (Squire and Alvarez, 1995). However it is now understood that after retrieval of long-term memory the trace becomes vulnerable and in order to persist, the trace must undergo a protein synthesis-dependent restabilization process called reconsolidation. The primary objective of this study was to analyze the involvement of neurotrophin signaling in the consolidation and reconsolidation of long term recognition memory using the object recognition task. This task, as described earlier, has been used as a measure of hippocampal dependent learning in a number of studies (Kelly *et al*, 2003, Murray and Mishkin, 1998, Winters and Bussey, 2005).

Neurotrophin release from vesicles is believed to mediate synaptic plasticity activating intracellular proteins and transcription. BDNF release has been shown to be activity-dependent in hippocampal neurons (Hartmann *et al*., 2001) and this results in BDNF immunoreactivity through out the cell, having been found in the nucleus and surrounding cytoplasm, dendritic shafts and spines, axons, and axon terminals of the hippocampal neurons (Murer *et al*. 2001). This data supports the hypothesis that neurotrophins, or more specifically BDNF play a role in synaptic transmission and plasticity. It has also been indicated that BDNF signaling is bidirectional, acting both pre- and post-synaptically to enhance synaptic plasticity (Kohara *et al*., 2001). Neurotrophins and their downstream signaling pathways have previously been implicated in many types of memory processing and as shown in our earlier study are involved in consolidation of recognition memory. Neurotrophin-stimulated intracellular signaling is initiated upon binding to the Trk receptor (Klein *et al*., 1991b); this activation leads to activation of a number of intracellular signaling pathways including ERK/MAPK (Marsh *et al*, 1993) and Pi3K/AKT (Yao & Cooper, 1995). Numerous studies have shown that activation of the MAPK pathway is critical for memory consolidation (Atkins *et al*, 1998;

Selcher at al, 1999; Kelly *et al*, 2003) conveying signals from cell surface receptors to the nucleus where transcription factors such as the immediate early genes are activated (Davis *et al* 2000). In a similar fashion Pi3K/Akt pathway is activated at the cell membrane and also mediates signals to the nucleus (Datta *et al*., 1996, Hemmings., 1997). The immediate early gene, c-fos is also stimulated downstream of neurotrophin signaling and is often analyzed experimentally as a measure of neuronal activation. Here we analyzed more closely the role of BDNF and the intracellular signaling mechanisms it stimulates during consolidation and reconsolidation of recognition memory.

BDNF release and intracellular signaling proteins were analyzed in the dentate gyrus, the hippocampus, the perirhinal cortex and the entorhinal cortex. As previously mentioned these are all regions of the medial temporal lobe and have each been identified as central areas involved in memory processing (Broadbent *et al*, 2004, Bussey *et al*, 1999, Brown *et al*., 1999, Aggleton and Brown, 1999). Analysis of c-fos has previously been used to identify levels of neuronal activity, here it was used to compare and contrast the involvement of each of the above brain regions in this type of memory processing.

In this study we reveal that memory consolidation within the dentate gyrus sub field of the hippocampus is associated with activity-dependant BDNF release and subsequent down stream signaling by BDNF. BDNF-stimulated release was not observed in the hippocampus suggesting that the role of BDNF in this type of memory may be specific to the dentate gyrus. Other studies examining BDNF in the hippocampus without extraction of the dentate gyrus have revealed similar results. Studies examining the dentate gyrus specifically have shown that neuronal activity can induce BDNF expression both *in vivo* and *in vitro* (Lauterborn *et al*, 1996); with additional studies showing that lesions of the dentate gyrus impair spatial memory (Kesner *et al*, 2004). In most studies the hippocampus is analysed whole, without extraction of sub regions, also revealing some interesting results. One study by Mizuno and colleagues (2000) using the radial arm maze revealed significant increases in BDNF mRNA expression in the whole hippocampus. In the same study treatment of animals with antisense BDNF oligonucleotide resulted in an impairment in spatial learning. Taken together with our previous results this data further supports the hypothesis of BDNF mediated

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hippocampal-dependant learning and demonstrates for the first time a role for this neurotrophin in consolidation of recognition memory.

Analysis of ERK activation in the dentate gyrus revealed increases in phosphorylated ERK associated with memory consolidation, again in the dentate gyrus but not in the rest of the hippocampal formation. One study which supports this finding is by Wang Ying and colleagues (2002), where intrahippocampal microinfusions of BDNF trigger LTP *in vivo*, and further analysis revealed rapid phosphorylation of ERK in the dentate gyrus but not the other subregions of the hippocampus. A further study provided extensive evidence that the ERK/MAPK pathway is essential in memory consolidation and reconsolidation in the hippocampal circuitry. In the study a MEK inhibitor U0126 was used to block consolidation of object recognition memory, blocking increases in ERK activation in the dentate gyrus (Kelly *et al*, 2003). Also in the study changes in pERK were only observed in the p44 isoform in the dentate gyrus and not the p42 isoform;this finding is replicated in the present study.

Finally analysis of c-fos expression revealed increases in the dentate gyrus and in the hippocampus following consolidation, but also in the consolidation and learning group in the hippocampus. In this group, animals are re-exposed to a familiar environment and explore one novel and one familiar object. We may speculate that some reconsolidation is occurring in parrellel with new learning. Studies analyzing c-fos activation in the dentate gyrus alone have observed increases in c-fos mRNA expression. One study found that activation of NMDA receptors increased c-fos mRNA expression in the dentate gyrus (Lerea *et al*, 1992); which is interesting as BDNF release is linked to NMDA receptor activation. In contrast an alternative study using a behavior response, reward task revealed c-fos mRNA was broadly increased in the CA1 and CA3 regions of the hippocampus but not the dentate gyrus (Hess *et al*, 1995). It is possible that the CA1 and CA3 of the hippocampus are mediators of contextual memory.

The perirhinal cortex which has been linked to recognition memory in a number of studies (Barker *et al*., 2006, Aggleton and Brown, 2005, Wan *et al*., 2004) revealed increased BDNF release in the consolidation group. Previous studies have reported widespread BDNF expression in the perirhinal cortex (Hashimoto *et al*., 2000) with

increased BDNF release following induction of LTP in slices of the perirhinal cortex (Aicardi *et al.*, 2004). Interestingly analysis of ERK activation revealed no changes in the perirhinal cortex associated with memory consolidation suggesting BDNF signaling in the perirhinal cortex is not mediated by ERK, but some other protein. Although ERK analysis in memory reconsolidation revealed an increase in ERK1 activation, this area is as yet unexplored. Analysis of c-fos expression followed a similar pattern to that of BDNF in the perirhinal cortex, again with increases associated with memory consolidation. A similar study also using c-fos activation to determine neuronal activity reported increases in the perirhinal cortex in recognition memory experiments (Brown *et al*, 1999). Our results therefore support evidence from the literature indicating a role for the perirhinal cortex in reconsolidation memory.

The entorhinal cortex has previously been linked to recognition memory with increases in BDNF and Trk B mRNA in sheep 2-3hrs following consolidation of recognition memory (Broad *et al*, 2002). Data from this study did not reveal changes in BDNF release associated with memory consolidation in the entorhinal cortex, although tissue in this study was analysed at a much shorter time point of 5 minutes following consolidation. In our previous study increases in BDNF mRNA were only noticeable at 6hr following training suggesting that BDNF does not mediate synaptic excitability in this region but perhaps is involved in the later stages of synaptic remodeling of memory storage. Similarly no changes were observed in ERK activation in the entorhinal cortex, although in similar experiments Kelly and collegues (2003) reported increased ERK activation in the entorhinal cortex in consolidation of recognition memory. However c-fos activation does appear to be up regulated in the entorhinal cortex suggesting there is neuronal activity taking place during memory consolidation. Tissue analyzed after induction of LTP revealed an increase in protein synthesis and phosphorylation of CREB (Casey *et al*, 2003), which can also be used as markers of neuronal activity. Together this data suggests that there is activity taking place in the entorhinal cortex, but it is not mediated by the neurotrophins at this stage.

In this study we have produced data which indicates a strong role for neurotrophin signaling in memory consolidation however we found little evidence for neurotrophin

involvement in reconsolidation. This finding supports the work of Lee and colleagues (2004) who also found that memory reconsolidation was not dependent upon neurotrophin recruitment. Having previously identified a role for BDNF in memory consolidation, we can now see that BDNF release occurs during consolidation and this process takes place in both the dentate gyrus and the perirhinal cortex, which further supports our theory that these two brain regions are essential to modulation of recognition memory. Although we observed no changes in BDNF release in the entorhinal cortex we did observe increases in c-fos expression which suggests that there is a high level of neuronal activity in this region, which in turn supports the theory that it mediates signaling to and from the hippocampus (Insausti *et al.*, 1995, Izquierdo and Medina, 1993), perhaps in this case between the dentate gyrus and the perirhinal cortex.

The finding that BDNF is released in response to consolidation of recognition memory further supports evidence in the literature that neurotrophin release at synaptic junctions mediates synaptic plasticity and is essential to both acquisition and consolidation of memory (Hartmann *et al.*, 2001, McHugh *et al.*, 2007, Nilsson *et al.*, 2007, Wang and Salter, 1994). We have also noted increased upregulation of neurotrophin signaling protein ERK, which indicates the MAPK/ERK signaling pathway is mediating neurotrophin action during memory consolidation.

The data presented here along with our findings from out other studies indicates a positive role for neurotrophin release, expression and signaling in memory processing.

Chapter 6

Further elucidation of signalling pathways underlying neurotrophin mediation of recognition memory

6.1 Introduction

From the previous chapters, we have provided evidence to suggest an important role for the dentate gyrus and the perirhinal cortex in consolidation of recognition memory, with specific changes in release of BDNF and activation of ERK and c-fos. We have also shown that at least some similar changes accompany the process of reconsolidation. Although no significant differences in BDNF release were observed in the hippocampus and the entorhinal cortex, there were increases in c-fos expression which suggest there is high neuronal activity in these brain regions during consolidation and reconsolidation, albeit that it is not accompanied by the same signaling changes observed in the dentate gyrus and perirhinal cortex.

It has been reported that activation of ERK mediates the cellular changes associated with consolidation of recognition memory in the rat (Kelly *et al* 2003). Activation of tyrosine kinase receptors by binding of neurotrophin ligands is one of the extracellular events that leads to ERK activation. The data reported in the previous chapters is consistent with the hypothesis that this ERK activation may be stimulated by binding of neurotrophins to Trk receptors. In order to test this hypothesis, in this study we have assessed the effect of blockade of Trk receptors with tyrphostin AG879, which has previously been used to block LTP (Maguire *et al*., 1999) and which we have shown to block acquisition and consolidation of recognition memory, on activation of ERK in dentate gyrus.

We have shown in the previous chapter that consolidation is accompanied by increased BDNF release, but not ERK activation, in perirhinal cortex. Presumably BDNF may be stimulating other intracellular signaling cascades in perirhinal cortex. One strong candidate for investigation is the PI3K/Akt pathway, which has been implicated in several studies in the expression of several forms of synaptic plasticity, including LTP and spatial learning (Kelly & Lynch, 2000; Mizuno *et al*. 2003).

The overall aim of this study was to investigate if tyrphostin AG879 can, by blocking tyrosine kinase receptor activation, block the signaling changes that we have reported to accompany consolidation of recognition memory and to investigate a role for PI3K in consolidation.

6.2 Materials & Methods

Male Wistar rats aged between 2 and 4 months weighing between 250 and 350g were used in these experiments. Rats were trained in the object recognition task as described in section 2.4. Animals received an i.p. injection of tyrphostin AG879 (100μl; 1.6μg/ml) or vehicle (DMSO,10%(v/v); 5μl) one hour before commencing the task. In this experiment 5 groups were included, two consolidation groups, named vehicle-treated consolidation group and tyrphostin-treated consolidation group, these animals were sacrificed following training on day one of the task, two learning groups named vehicle-treated consolidation and learning group and tyrphostin treated consolidation and learning group, these animals were sacrificed following testing on day 2 of the task, the final group, the control group was habituated to the arena only in the absence of objects. Tissue was taken and biochemical analysis for ERK, PI3K, c-fos and BDNF was performed by western immunoblotting (section 2.13 and 2.14) and ELISA (section 2.18).

6.3.1 Intraperitioneal administration of the tyrosine kinase receptor Trk antagonist Tyrphostin inhibits recognition memory in the rat

Tyrphostin AG879 (100μl, 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl; 10% DMSO in ddH₂O) was administered intraperitionealy (i.p.) one hour prior to training in the object recognition task on day 1. Rats were tested at 24hr to test memory consolidation. Both control treated and tyrphostin treated rats explored objects equally during the training phase (Figure 3.1 A and B; Control A; 48.29 ± 5.35%, B; 51.57 ± 5.35%; tyrphostin A: 47.26 ± 2.703%, B; 52.74 ± 2.7%). The time spent exploring each object is expressed as a percentage of the total exploration time. 24hr following training object B was replaced with a novel object C and rats were placed back in the arena and allowed to explore. Vehicle treated rats successfully recognised the familiar object A, spending significantly greater time exploring the new object C (***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls, Control A: 24.89 ± 4.65%, C: 75.11 ± 4.65%). In contrast, tyrphostin-treated rats failed to identify the novel object (A: 42.57 ± 5.03%, C: 47.43 ± 5.03%).

Dentate Gyrus

6.3.2 BDNF-release in the dentate gyrus

Following complestion of the object recognition task samples from the dentate gyrus were analyzed for KCl-stimulated release of BDNF by ELISA. Statistical analysis of the results revealed a significant increase in the vehicle-treated consolidation group and tyrphostin treated consolidation and learning group (Figure 6.1; ***$p<0.0001$ and *$p<0.05$ respectively; 1-way ANOVA, *post hoc* Newman Keuls; control: 162.2 ± 14.26; tyrphostin-treated consolidation group: 109.1 ± 28.68; vehicle treated consolidation group: 224 ± 5.27; tyrphostin-treated consolidation and learning group 148.6 ± 15.31; vehicle-treated consolidation and learning group: 87.22 ± 11.88 pg/ml). Results are expressed as mean BDNF pg/ml $\pm$ SEM, n=6.

6.3.3 P44 ERK activation in the dentate gyrus

Following completion of the object recognition task samples from the dentate gyrus were analyzed for p44 ERK activation by Western Immunoblot. The representative p44ERK blot shows increase in the vehicle-treated consolidation group (Figure 6.3A). Densometric analysis of the immunoblots revealed this increase was statistically significant (Figure 6.3B; ***$p<0.0001$; 1-way ANOVA, *post hoc* Newman Keuls; control: 1.68 ± 0.33; tyrphostin-treated consolidation group: 8.133 ± 3.02; vehicle treated consolidation group: 18.42 ± 5.23; tyrphostin-treated consolidation and learning group 1.81 ± 0.29; vehicle-treated consolidation and learning group: $2.44 \pm 0.59\%$). Results are expressed as percentage p44ERK /TERK, mean $\pm$ SEM.

6.3.4 PI3 Kinase activation in the dentate gyrus

Following completion of the object recognition task samples from the dentate gyrus were analyzed for pPI3K activation by Western Immunoblot. The representative pPI3K blot shows an increase in both the vehicle-treated consolidation group and the tyrphostin treated consolidation group (Figure 6.3A). Densometric analysis of the immunoblots revealed this increase was not statistically significant (Figure 6.3B; 1-way ANOVA, *post hoc* Newman Keuls; control: 4.18 ± 1.49; tyrphostin-treated consolidation group: 8.52 ± 0.64; vehicle treated consolidation group: 9.38 ± 2.03;

tyrphostin-treated consolidation and learning group 5.35 ± 0.36; vehicle-treated consolidation and learning group: 8.40 ± 1.90%). Results are expressed as percentage pPI3K /TPI3K, mean ± SEM.

6.3.5 C-fos activation in the dentate gyrus

Following completion of the object recognition task samples from the dentate gyrus were analyzed for c-fos activation by Western Immunoblot. The representative c-fos blot shows no differences between the groups (Figure 6.4A). Densometric analysis of the immunoblots revealed no statistically significant differences (Figure 6.4B). Results are expressed as percentage c-fos /actin, mean ± SEM.

Figure 6.1 BDNF release in the dentate gyrus

Tyrphostin AG879 (100µl; 1.6µg/ml, Chemicon) or vehicle (DMSO; 100µl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. BDNF release was measured in all groups in the dentate gyrus. Statistical analysis revealed a significant increase in BDNF release in the vehicle treated consolidation group (*** P<0.001; 1-way ANOVA, *post hoc* Newman Keuls) and in the tyrphostin-treated Consolidation and Learning group (* P<0.05; 1-way ANOVA, *post hoc* Newman Keuls), no significant increases were observed in other groups. Results are expressed as mean BDNF pg/ml ± SEM, n=6.

PERK

TERK

Figure 6.2 p44 ERK in the dentate gyrus

A. Sample Western Immunoblot illustrating increased p44 ERK activation in the Dentate gyrus

B. Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH_2O) was administered i.p. one hour prior to training in the object recognition task on day 1. pERK was analysed in the dentate gyrus of all groups. P44 ERK is expressed as percentage of total ERK. Statistical analysis revealed a significant increase in the vehicle treated Consolidation group (*** $P<0.0001$; 1-way ANOVA, *post hoc* Newman Keuls), with no changes in the other groups. Histograms represent mean values ± SEM, n=6. P44 ERK is expressed as percentage of total ERK

pPI3K

TPI3K

Figure 6.3 pPI3K activation in the dentate gyrus

A. Sample Western Immunoblot illustrating pPI3K activation in the dentate gyrus

B. Tyrphostin AG879 (100µl; 1.6µg/ml, Chemicon) or vehicle (DMSO; 100µl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. pPI3K was analysed in the dentate gyrus of all groups. The expression of pPI3K is given as a percentage of total Pi3K. Statistical analysis revealed there were no significant differences between groups. Histograms represent mean values ± SEM, n=6, 1-way ANOVA.

C-fos

Actin

Figure 6.4 C-fos expression in the Dentate Gyrus

A. Sample Western Immunoblot illustrating c-fos activation in the dentate gyrus

B. Tyrphostin AG879 (100µl; 1.6µg/ml, Chemicon) or vehicle (DMSO; 100µl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. C-fos expression was measured in all groups. The expression of c-fos is expressed as a percentage of Actin. Statistical analysis revealed no significant differences were observed between groups. Histograms represent mean values ± SEM, n=6, 1-way ANOVA.

Hippocampus

6.3.6 PI3 Kinase activation in the hippocampus

Following completion of the object recognition task samples from the hippocampus were analyzed for pPI3K activation by Western Immunoblot. The representative pPI3K blot shows no changes between groups (Figure 6.5A). Densometric analysis of the immunoblots revealed this increase was not statistically significant (Figure 6.5B). Results are expressed as percentage pPI3K /TPI3K, mean ± SEM.

6.3.7 C-fos activation in the hippocampus

Following completion of the object recognition task samples from the hippocampus were analyzed for c-fos activation by Western Immunoblot. The representative c-fos blot shows an increase in the vehicle-treated consolidation and learning group (Figure 6.6A). Densometric analysis of the immunoblots revealed this increase was statistically significant (Figure 6.6B; ***$p<0.0001$; 1-way ANOVA, *post hoc* Newman Keuls; control: 98.92 ± 13.32; tyrphostin-treated consolidation group: 130.70 ± 17.89; vehicle treated consolidation group: 103.50 ± 19.59; tyrphostin-treated consolidation and learning group 100.30 ± 9.6; vehicle-treated consolidation and learning group: $195.7 \pm 0.59\%$). Results are expressed as percentage c-fos /actin, mean ± SEM.

pPI3K
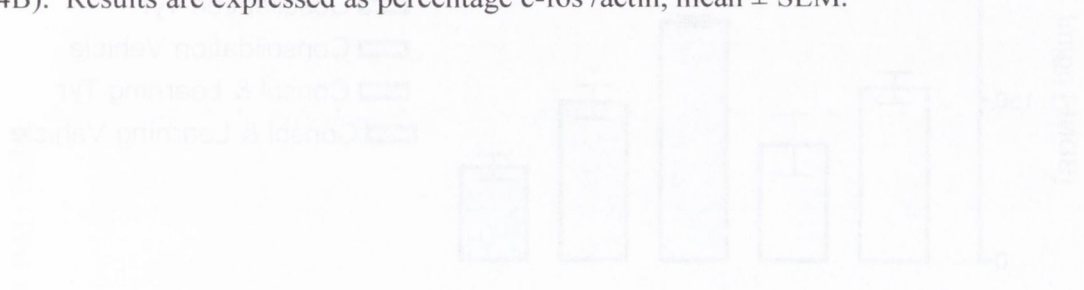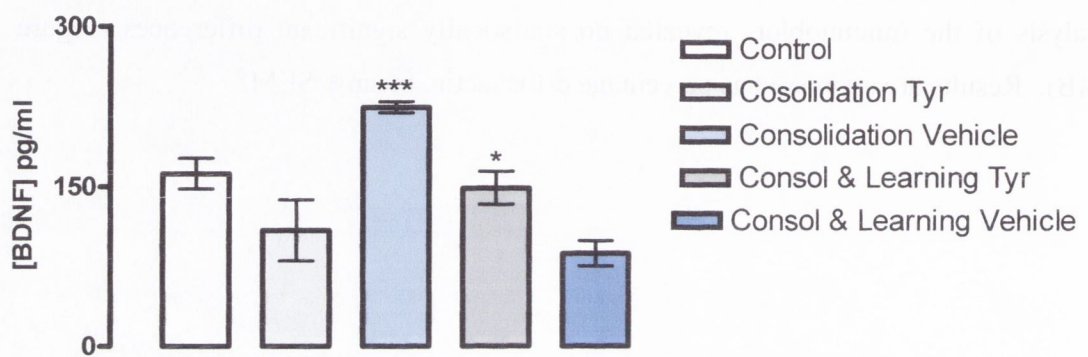
TPI3K

Figure 6.5 pPI3K activation in the hippocampus

A. Sample Western Immunoblot illustrating pPI3K activation in the hippocampus

B.   Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. pPI3K was analysed in the hippocampus of all groups. pPI3K is expressed as a percentage of total PI3K.   Statistical analysis revealed there were no significant differences between groups. Histograms represent mean values ± SEM, n=6 1-way ANOVA.

C-fos

Actin

Figure 6.6 C-fos expression in the hippocampus

A. Sample Western Immunoblot illustrating c-fos activation in the hippocampus

B. Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. C-fos was measured in the hippocampus of all groups. C-fos is expressed as a percentage of actin. Statistical analysis revealed there was a significant increase in c-fos activation in the vehicle-treated consolidation and learning group (** $P<0.01$; 1-way ANOVA, *post hoc* Newman Keuls), no significant differences were observed in the other groups. Histograms represent mean values ± SEM, n=6.

Perirhinal Cortex

6.3.8 BDNF-release in the perirhinal cortex

Following completion of the object recognition task samples from the perirhinal cortex were analyzed for KCl-stimulated release of BDNF by ELISA. Statistical analysis of the results revealed a significant increase in the vehicle-treated consolidation group (Figure 6.7; *p<0.05; 1-way ANOVA, *post hoc* Newman Keuls; control: 0.005 ± 0.001; tyrphostin-treated consolidation group: 0.0069 ± 0.002; vehicle treated consolidation group: 0.012 ± 0.003; tyrphostin-treated consolidation and learning group 0.002 ± 0.001; vehicle-treated consolidation and learning group: 0.005 ± 0.001 pg/ml). Results are expressed as mean BDNF pg/ml ± SEM, n=6.

6.3.9 PI3 Kinase activation in the perirhinal cortex

Following completion of the object recognition task samples from the perirhinal cortex were analyzed for pPI3K activation by Western Immunoblot. The representative pPI3K blot shows an increase in the vehicle-treated consolidation group (Figure 6.8A). Densometric analysis of the immunoblots revealed this increase was statistically significant (Figure 6.8B; **p<0.01; 1-way ANOVA, *post hoc* Newman Keuls; control: 6.38 ± 2.44; tyrphostin-treated consolidation group: 16.83 ± 6.10; vehicle treated consolidation group: 65.24 ± 13.86; tyrphostin-treated consolidation and learning group 6.88 ± 3.31; vehicle-treated consolidation and learning group: 30.88 ± 12.40%). Results are expressed as percentage pPI3K /TPI3K, mean ± SEM.

6.3.10 C-fos activation in the perirhinal cortex

Following completion of the object recognition task samples from the perirhinal cortex were analyzed for c-fos activation by Western Immunoblot. The representative c-fos blot shows an increase in the vehicle-treated consolidation and learning group (Figure 6.9A). Densometric analysis of the immunoblots revealed this increase was statistically significant (Figure 6.9B; *p<0.05; 1-way ANOVA, *post hoc* Newman Keuls; control: 25.04 ± 3.314; tyrphostin-treated consolidation group: 16.32 ± 1.22; vehicle treated consolidation group: 39.91 ± 7.93; tyrphostin-treated consolidation and

learning group 20.03 ± 2.01; vehicle-treated consolidation and learning group: 30.61 ± 6.15%). Results are expressed as percentage c-fos /actin, mean ± SEM.

Figure 6.7 BDNF release in the perirhinal cortex
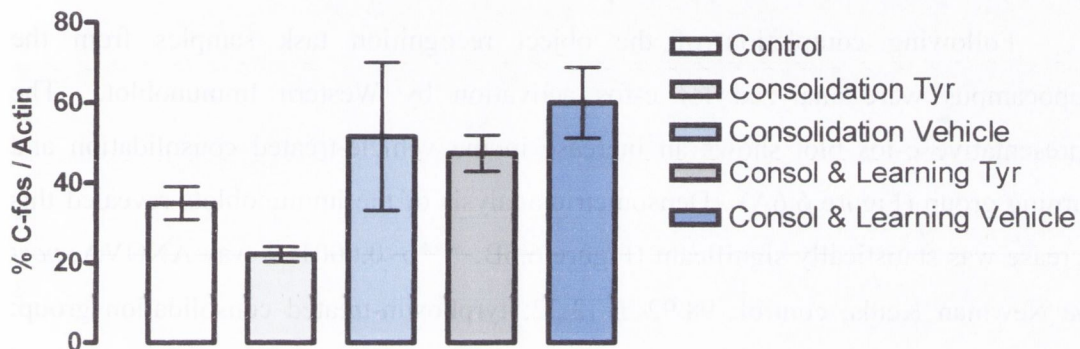
Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. BDNF release was measured in all groups in the perirhinal cortex. Statistical analysis revealed there was a significant increase in the vehicle-treated consolidation group (* $p<0.05$; 1-way ANOVA, *post hoc* Newman Keuls). No significant differences were observed in other groups. Histograms represent mean BDNF concentration ± SEM in ρg/ml, n=6.

pPI3K

TPI3K

Figure 6.8 pPI3K in the perirhinal cortex

A. Sample Western Immunoblot illustrating pPI3K activation in the perirhinal cortex

B. Tyrphostin AG879 (100µl; 1.6µg/ml, Chemicon) or vehicle (DMSO; 100µl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. pPI3K was analysed in the perirhinal cortex of all groups. pPi3K is expressed as a percentage of total PI3K. Statistical analysis revealed there was a significant increase in pPI3K activation in the vehicle treated consolidation group (** $p<0.01$; 1-way ANOVA, *post hoc* Newman Keuls). No significant differences were observed in the other groups. Histograms represent mean values $\pm$ SEM, n=6.

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C-fos

Actin

Figure 6.9 C-fos expression in the perirhinal cortex

A. Sample Western Immunoblot illustrating c-fos activation in the perirhinal cortex

B. Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. C-fos expression was measured in the perirhinal cortex of all groups. C-fos is expressed as a percentage of actin. Statistical analysis revealed there was a significant increase in c-fos activation in the vehicle treated consolidation group (* P<0.05; 1-way ANOVA, *post hoc* Newman Keuls). No significant differences were observed in the other groups. Histograms represent mean values ± SEM, n=6.

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Entorhinal Cortex

6.3.11 PI3 Kinase activation in the entorhinal cortex

Following completion of the object recognition task samples from the entorhinal cortex were analyzed for pPI3K activation by Western Immunoblot. The representative pPI3K blot shows an increase in the tyrphostin-treated consolidation group (Figure 6.10A). Densometric analysis of the immunoblots revealed this increase was statistically significant (Figure 6.10B; ***p<0.01; 1-way ANOVA, *post hoc* Newman Keuls; control: 17.17 ± 3.31; tyrphostin-treated consolidation group: 241.7 ± 65.54; vehicle treated consolidation group: 15.2 ± 1.3; tyrphostin-treated consolidation and learning group 34.07 ± 20.03; vehicle-treated consolidation and learning group: 42.46 ± 14.07%). Results are expressed as percentage pPI3K /TPI3K, mean ± SEM.

6.3.12 C-fos activation in the entorhinal cortex

Following completion of the object recognition task samples from the entorhinal cortex were analyzed for c-fos activation by Western Immunoblot. The representative c-fos blot shows no differences between the groups (Figure 6.11A). Densometric analysis of the immunoblots revealed no statistically significant differences (Figure 6.11B). Results are expressed as percentage c-fos /actin, mean ± SEM.

pPI3K

TPI3K

Figure 6.10 pPI3K activation in the entorhinal cortex

A. Sample Western Immunoblot illustrating pPI3K activation in the entorhinal cortex

B. Tyrphostin AG879 (100µl; 1.6µg/ml, Chemicon) or vehicle (DMSO; 100µl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. pPI3K was analyzed in the entorhinal cortex of all groups. pPI3K is expressed as a percentage of total PI3K. Statistical analysis revealed there was a significant increase in pPI3K in the tyrphostin-treated consolidation group (***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls) compared to other groups. Histograms represent mean values ± SEM, n=6.

C-fos

Actin

Figure 6.11 C-fos expression in the entorhinal cortex

A. Sample Western Immunoblot illustrating c-fos activation in the entorhinal cortex

B. Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. C-fos was measured in the entorhinal cortex of all groups. C-fos is expressed as a percentage of actin. Statistical analysis revealed there were no significant differences observed between the groups. Histograms represent mean values ± SEM, n=6, 1-way ANOVA.

tyrphostin-treated consolidation and learning group 5.35 ± 0.36; vehicle-treated consolidation and learning group: 8.40 ± 1.90%). Results are expressed as percentage pPI3K /TPI3K, mean ± SEM.
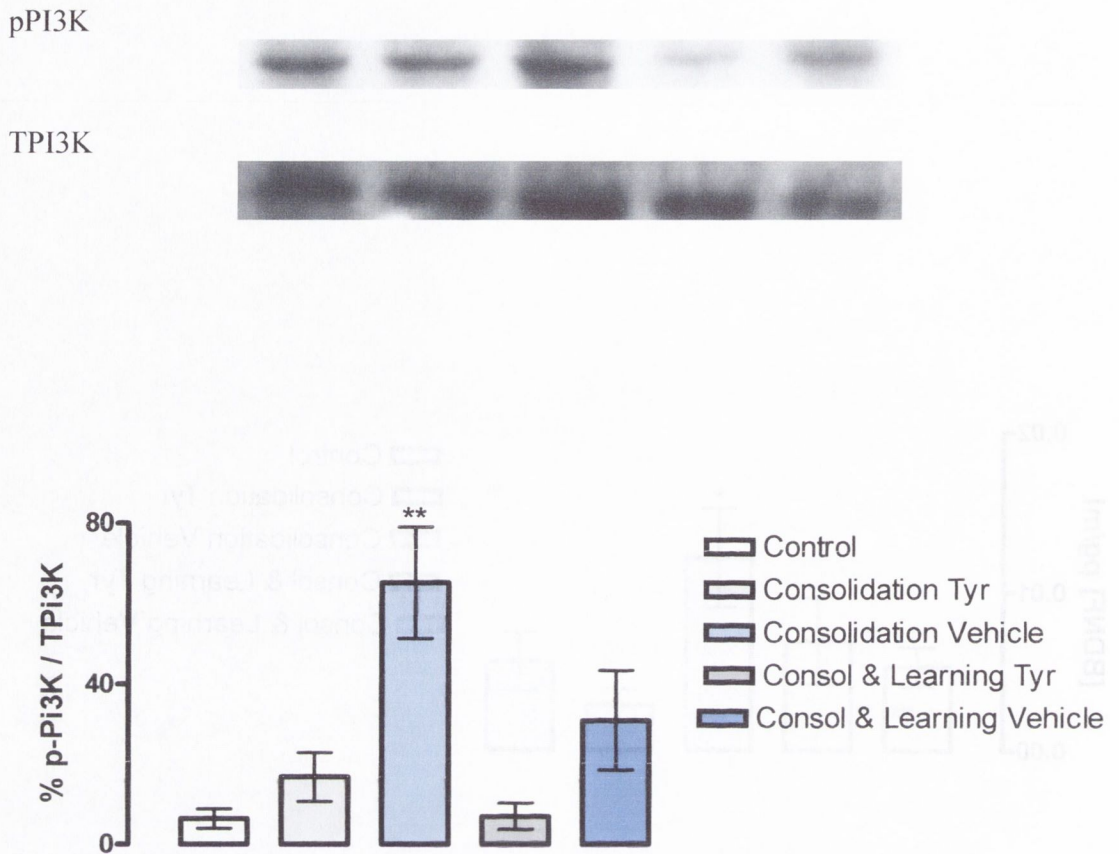
6.3.5 C-fos activation in the dentate gyrus

Following completion of the object recognition task samples from the dentate gyrus were analyzed for c-fos activation by Western Immunoblot. The representative c-fos blot shows no differences between the groups (Figure 6.4A). Densometric analysis of the immunoblots revealed no statistically significant differences (Figure 6.4B). Results are expressed as percentage c-fos /actin, mean ± SEM.

Figure 6.1 BDNF release in the dentate gyrus

Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. BDNF release was measured in all groups in the dentate gyrus. Statistical analysis revealed a significant increase in BDNF release in the vehicle treated consolidation group (*** $P<0.001$; 1-way ANOVA, *post hoc* Newman Keuls) and in the tyrphostin-treated Consolidation and Learning group (* $P<0.05$; 1-way ANOVA, *post hoc* Newman Keuls), no significant increases were observed in other groups. Results are expressed as mean BDNF pg/ml ± SEM, n=6.

PERK

TERK

Figure 6.2 p44 ERK in the dentate gyrus

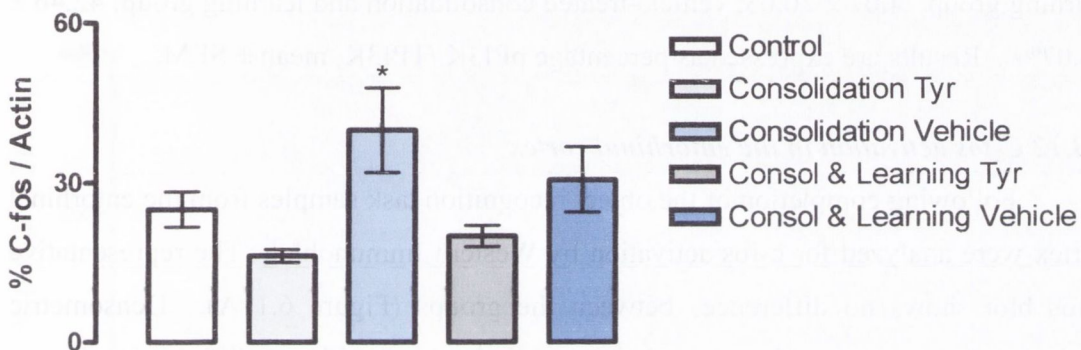A. Sample Western Immunoblot illustrating increased p44 ERK activation in the Dentate gyrus

B. Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. pERK was analysed in the dentate gyrus of all groups. P44 ERK is expressed as percentage of total ERK. Statistical analysis revealed a significant increase in the vehicle treated Consolidation group (*** P<0.0001; 1-way ANOVA, *post hoc* Newman Keuls), with no changes in the other groups. Histograms represent mean values ± SEM, n=6. P44 ERK is expressed as percentage of total ERK

pPI3K

TPI3K

Figure 6.3 pPI3K activation in the dentate gyrus

A. Sample Western Immunoblot illustrating pPI3K activation in the dentate gyrus

B.   Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. pPI3K was analysed in the dentate gyrus of all groups. The expression of pPI3K is given as a percentage of total Pi3K. Statistical analysis revealed there were no significant differences between groups. Histograms represent mean values ± SEM, n=6, 1-way ANOVA.

C-fos

Actin

Figure 6.4 C-fos expression in the Dentate Gyrus

A. Sample Western Immunoblot illustrating c-fos activation in the dentate gyrus

B. Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. C-fos expression was measured in all groups. The expression of c-fos is expressed as a percentage of Actin. Statistical analysis revealed no significant differences were observed between groups. Histograms represent mean values ± SEM, n=6, 1-way ANOVA.

Hippocampus

6.3.6 PI3 Kinase activation in the hippocampus

Following completion of the object recognition task samples from the hippocampus were analyzed for pPI3K activation by Western Immunoblot. The representative pPI3K blot shows no changes between groups (Figure 6.5A). Densometric analysis of the immunoblots revealed this increase was not statistically significant (Figure 6.5B). Results are expressed as percentage pPI3K /TPI3K, mean ± SEM.

6.3.7 C-fos activation in the hippocampus

Following completion of the object recognition task samples from the hippocampus were analyzed for c-fos activation by Western Immunoblot. The representative c-fos blot shows an increase in the vehicle-treated consolidation and learning group (Figure 6.6A). Densometric analysis of the immunoblots revealed this increase was statistically significant (Figure 6.6B; ***$p<0.0001$; 1-way ANOVA, *post hoc* Newman Keuls; control: 98.92 ± 13.32; tyrphostin-treated consolidation group: 130.70 ± 17.89; vehicle treated consolidation group: 103.50 ± 19.59; tyrphostin-treated consolidation and learning group 100.30 ± 9.6; vehicle-treated consolidation and learning group: 195.7 ± 0.59%). Results are expressed as percentage c-fos /actin, mean ± SEM.

pPI3K

TPI3K

Figure 6.5 pPI3K activation in the hippocampus

A. Sample Western Immunoblot illustrating pPI3K activation in the hippocampus

B. Tyrphostin AG879 (100µl; 1.6µg/ml, Chemicon) or vehicle (DMSO; 100µl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. pPI3K was analysed in the hippocampus of all groups. pPI3K is expressed as a percentage of total PI3K. Statistical analysis revealed there were no significant differences between groups. Histograms represent mean values ± SEM, n=6 1-way ANOVA.
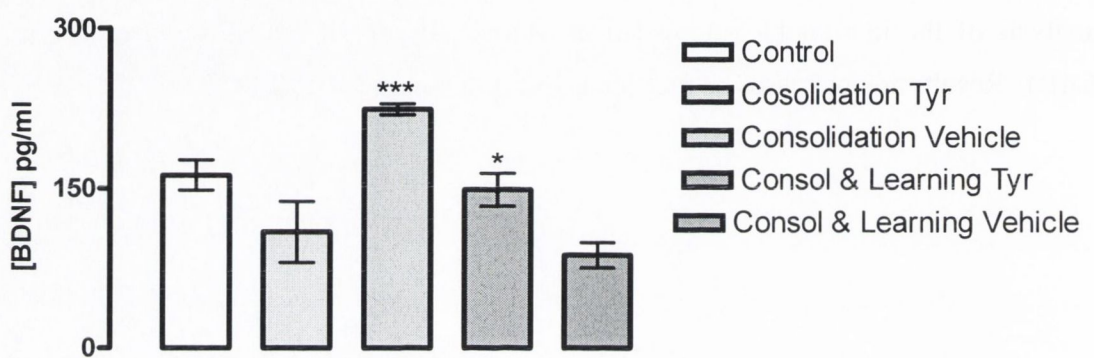
C-fos

Actin

Figure 6.6 C-fos expression in the hippocampus

A. Sample Western Immunoblot illustrating c-fos activation in the hippocampus

B. Tyrphostin AG879 (100µl; 1.6µg/ml, Chemicon) or vehicle (DMSO; 100µl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. C-fos was measured in the hippocampus of all groups. C-fos is expressed as a percentage of actin. Statistical analysis revealed there was a significant increase in c-fos activation in the vehicle-treated consolidation and learning group (** P<0.01; 1-way ANOVA, *post hoc* Newman Keuls), no significant differences were observed in the other groups. Histograms represent mean values ± SEM, n=6.

Perirhinal Cortex

6.3.8 BDNF-release in the perirhinal cortex

Following completion of the object recognition task samples from the perirhinal cortex were analyzed for KCl-stimulated release of BDNF by ELISA. Statistical analysis of the results revealed a significant increase in the vehicle-treated consolidation group (Figure 6.7; *$p<0.05$; 1-way ANOVA, *post hoc* Newman Keuls; control: 0.005 ± 0.001; tyrphostin-treated consolidation group: 0.0069 ± 0.002; vehicle treated consolidation group: 0.012 ± 0.003; tyrphostin-treated consolidation and learning group 0.002 ± 0.001; vehicle-treated consolidation and learning group: 0.005 ± 0.001 pg/ml). Results are expressed as mean BDNF pg/ml $\pm$ SEM, n=6.

6.3.9 PI3 Kinase activation in the perirhinal cortex

Following completion of the object recognition task samples from the perirhinal cortex were analyzed for pPI3K activation by Western Immunoblot. The representative pPI3K blot shows an increase in the vehicle-treated consolidation group (Figure 6.8A). Densometric analysis of the immunoblots revealed this increase was statistically significant (Figure 6.8B; **$p<0.01$; 1-way ANOVA, *post hoc* Newman Keuls; control: 6.38 ± 2.44; tyrphostin-treated consolidation group: 16.83 ± 6.10; vehicle treated consolidation group: 65.24 ± 13.86; tyrphostin-treated consolidation and learning group 6.88 ± 3.31; vehicle-treated consolidation and learning group: $30.88 \pm 12.40\%$). Results are expressed as percentage pPI3K /TPI3K, mean $\pm$ SEM.

6.3.10 C-fos activation in the perirhinal cortex

Following completion of the object recognition task samples from the perirhinal cortex were analyzed for c-fos activation by Western Immunoblot. The representative c-fos blot shows an increase in the vehicle-treated consolidation and learning group (Figure 6.9A). Densometric analysis of the immunoblots revealed this increase was statistically significant (Figure 6.9B; *$p<0.05$; 1-way ANOVA, *post hoc* Newman Keuls; control: 25.04 ± 3.314; tyrphostin-treated consolidation group: 16.32 ± 1.22; vehicle treated consolidation group: 39.91 ± 7.93; tyrphostin-treated consolidation and

learning group 20.03 ± 2.01; vehicle-treated consolidation and learning group: 30.61 ± 6.15%). Results are expressed as percentage c-fos /actin, mean ± SEM.

Figure 6.7 BDNF release in the perirhinal cortex
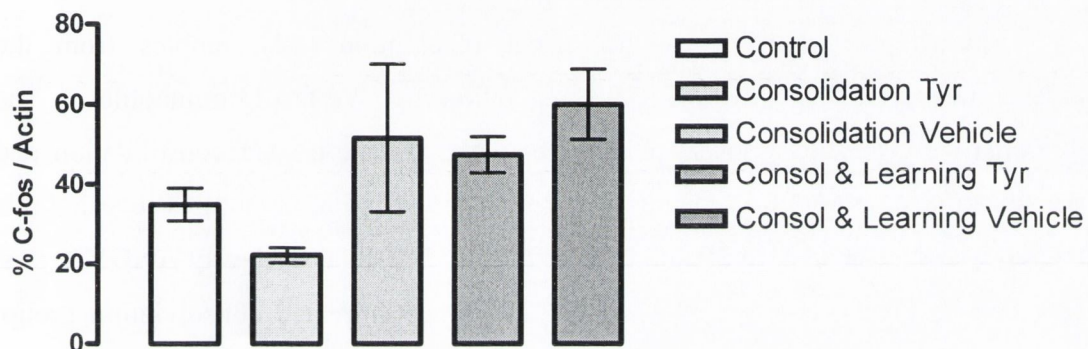
Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. BDNF release was measured in all groups in the perirhinal cortex. Statistical analysis revealed there was a significant increase in the vehicle-treated consolidation group (* $p < 0.05$; 1-way ANOVA, *post hoc* Newman Keuls). No significant differences were observed in other groups. Histograms represent mean BDNF concentration ± SEM in ρg/ml, n=6.

pPI3K

TPI3K

Figure 6.8 pPI3K in the perirhinal cortex

A. Sample Western Immunoblot illustrating pPI3K activation in the perirhinal cortex

B. Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. pPI3K was analysed in the perirhinal cortex of all groups. pPi3K is expressed as a percentage of total PI3K. Statistical analysis revealed there was a significant increase in pPI3K activation in the vehicle treated consolidation group (** $p<0.01$; 1-way ANOVA, *post hoc* Newman Keuls). No significant differences were observed in the other groups. Histograms represent mean values ± SEM, n=6.

C-fos

Actin

Figure 6.9 C-fos expression in the perirhinal cortex

A. Sample Western Immunoblot illustrating c-fos activation in the perirhinal cortex

B. Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. C-fos expression was measured in the perirhinal cortex of all groups. C-fos is expressed as a percentage of actin. Statistical analysis revealed there was a significant increase in c-fos activation in the vehicle treated consolidation group (* $P<0.05$; 1-way ANOVA, *post hoc* Newman Keuls). No significant differences were observed in the other groups. Histograms represent mean values ± SEM, n=6.

Entorhinal Cortex

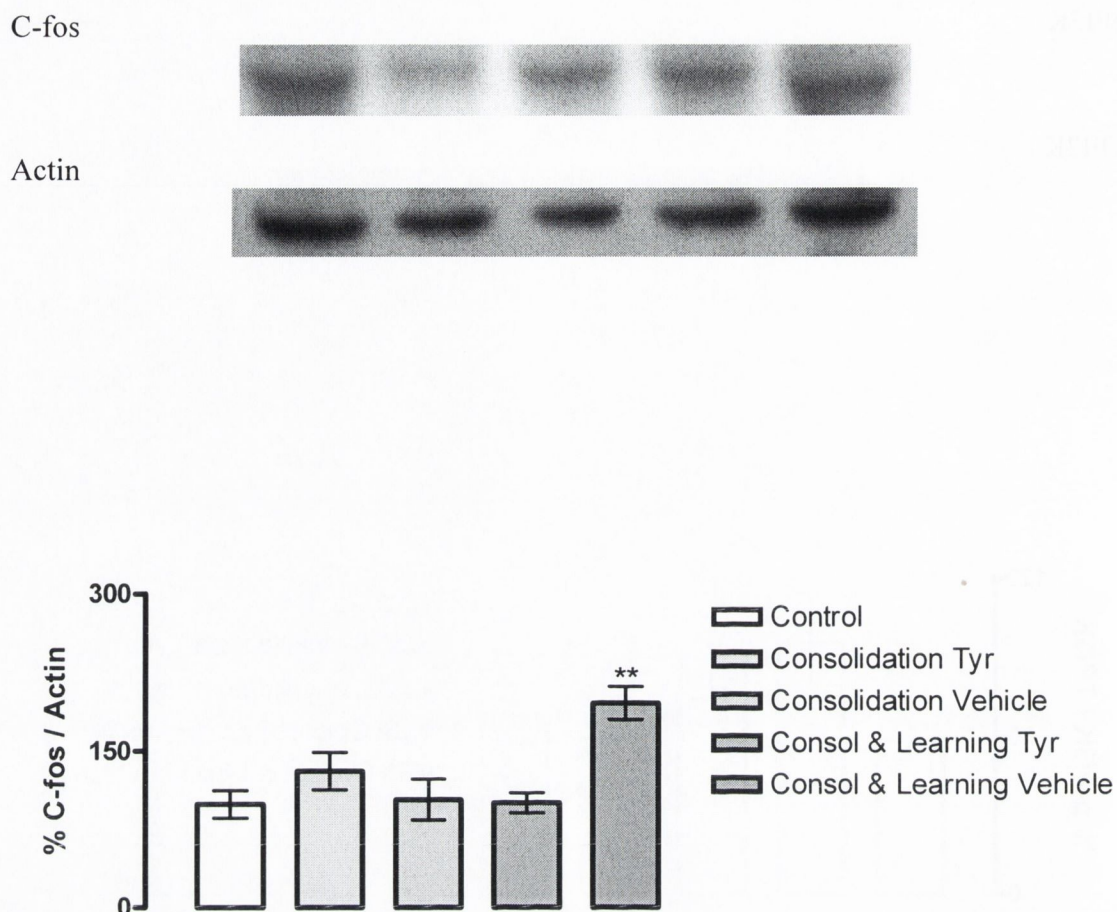### 6.3.11 PI3 Kinase activation in the entorhinal cortex

Following completion of the object recognition task samples from the entorhinal cortex were analyzed for pPI3K activation by Western Immunoblot. The representative pPI3K blot shows an increase in the tyrphostin-treated consolidation group (Figure 6.10A). Densometric analysis of the immunoblots revealed this increase was statistically significant (Figure 6.10B; ***$p<0.01$; 1-way ANOVA, *post hoc* Newman Keuls; control: 17.17 ± 3.31; tyrphostin-treated consolidation group: 241.7 ± 65.54; vehicle treated consolidation group: 15.2 ± 1.3; tyrphostin-treated consolidation and learning group 34.07 ± 20.03; vehicle-treated consolidation and learning group: $42.46 \pm 14.07\%$). Results are expressed as percentage pPI3K /TPI3K, mean $\pm$ SEM.

6.3.12 C-fos activation in the entorhinal cortex

Following completion of the object recognition task samples from the entorhinal cortex were analyzed for c-fos activation by Western Immunoblot. The representative c-fos blot shows no differences between the groups (Figure 6.11A). Densometric analysis of the immunoblots revealed no statistically significant differences (Figure 6.11B). Results are expressed as percentage c-fos /actin, mean $\pm$ SEM.

pPI3K

TPI3K

Figure 6.10 pPI3K activation in the entorhinal cortex

A. Sample Western Immunoblot illustrating pPI3K activation in the entorhinal cortex

B. Tyrphostin AG879 (100µl; 1.6µg/ml, Chemicon) or vehicle (DMSO; 100µl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. pPI3K was analyzed in the entorhinal cortex of all groups. pPI3K is expressed as a percentage of total PI3K. Statistical analysis revealed there was a significant increase in pPI3K in the tyrphostin-treated consolidation group (***p<0.0001; 1-way ANOVA, *post hoc* Newman Keuls) compared to other groups. Histograms represent mean values ± SEM, n=6.

C-fos

Actin

Figure 6.11 C-fos expression in the entorhinal cortex

A. Sample Western Immunoblot illustrating c-fos activation in the entorhinal cortex

B. Tyrphostin AG879 (100μl; 1.6μg/ml, Chemicon) or vehicle (DMSO; 100μl: 10% DMSO in ddH$_2$O) was administered i.p. one hour prior to training in the object recognition task on day 1. C-fos was measured in the entorhinal cortex of all groups. C-fos is expressed as a percentage of actin. Statistical analysis revealed there were no significant differences observed between the groups. Histograms represent mean values ± SEM, n=6, 1-way ANOVA.

6.4 Discussion

Having established a role for neurotrophins and Trk receptor activation in memory consolidation and tracked the intracellular signaling pathway, through which neurotrophins mediate synaptic plasticity, the aim of this study was to reinforce our findings from the previous chapters and test the hypothesis that ERK activation is in fact a mediator of Trk receptor signaling during consolidation of recognition memory. It has been reported that activation of ERK mediates the cellular changes associated with consolidation of recognition memory in the rat (Kelly *et al* 2003) and the data presented here further support this theory. In order to test this hypothesis, in this study we have assessed the effect of blockade of Trk receptors with tyrphostin AG879, which has previously been used to block LTP (Maguire *et al.*, 1999) and which we have shown to block acquisition and consolidation of recognition memory, on activation of ERK in dentate gyrus.

Although very few changes were observed in the hippocampus and entorhinal cortex compared with the changes we found in the dentate gyrus and the perirhinal cortex in the previous study, we still observed increases in c-fos activation which suggests that there is neuronal activity taking place in these regions that are not mediated via ERK/MAPK activation. We decided to investigate this further and analyze PI3K, which is also known as a mediator of neurotrophin signaling. PI3K has previously been shown to be involved in LTP and spatial learning paradigms (Kelly & Lynch, 2000; Mizuno *et al.*, 2003). PI3K is believed to contribute to multiple events important in synaptic plasticity and memory formation including AMPA receptor insertion to the postsynaptic membrane (Man *et al*, 2003), partial activation of the ERK/MAPK pathway (Perkinton *et al*, 2002; Kelly & Lynch, 2000), and protein synthesis (Cammalleri *et al*, 2003). One study investigating the role of PI3K activity in contextual memory reported increased PI3K activity in the hippocampus whole (Chen *et al*, 2005).

From our previous studies we have shown that tyrphostin AG879, the tyrosine kinase inhibitor, blocked both acquisition and consolidation of recognition memory. Tyrphostins have been shown to inhibit autophosphorylation of protein tyrosine kinases (Lyall *et al.*, 1989) and more specifically tyrphostin AG879 has been shown to block Trk

A signaling indicating that AG879 competes for the substrate binding site, preventing signaling, which renders tyrphostins useful inhibitors for investigating possible roles and signaling mechanisms of the Trk receptors (Ohmichi *et al.*, 1993, Rende *et al.*, 2000). In this study tyrphostin was used to inhibit memory consolidation with the hope that we would see a down regulation of the neurotrophin intracellular signaling proteins, ERK and PI3K; as in our previous study we also looked at BDNF release to investigate the changes that tyrphostin AG879 may have on neurotrophin release at the synapse. We identified that i.p. administration of tyrphostin AG879 was sufficient to block consolidation of recognition memory, presumeably by inactivation of the neurotrophin receptors Trk A, Trk B and Trk C. Having blocked consolidation we then investigated the effects of tyrphostin on intracellular signaling.

When analyzing BDNF release in the dentate gyrus we found a significant increase in BDNF release in response to memory consolidation and this effect was blocked by tyrphostin AG879. This evidence suggests that tyrphostin upon blocking of the Trk receptors blocks regulated release of the neurotrophins, this further supports the theory that neurotrophins can regulate their own release, perhaps via modulation of NMDA receptors and neurotransmitters (Lindholm *et al.*, 1994, Nilsson *et al.*, 2007). However we found no changes in BDNF release in the hippocampus as in our previous study which further supports our theory that the dentate gyrus is possibly playing a more important role than the rest of the hippocampal formation in this type of memory processing.

In the dentate gyrus we found an increase in ERK activation as before and this increase was blocked by administration of tyrphostin, again with no changes in the hippocampus as previously found. However analysis of PI3K revealed no changes within the dentate gyrus or the hippocampus suggesting the ERK/MAPK signaling pathway is more important in mediating neurotrophic effects in the dentate gyrus.

From our previous studies we have identified the perirhinal cortex as a central structure involved in consolidation of recognition memory. Here in this study we demonstrate that BDNF release is increased in the perirhinal cortex during memory

consolidation and this is blocked by tyrphostin as in the dentate gyrus. This evidence is again supporting our previous findings and hypothesis that neurotrophins are mediators of memory consolidation upon activation of the Trk receptors.

On analyzing ERK and PI3K activation in the perirhinal cortex we found small increases in ERK activation but quite significant increases of PI3K activation in memory consolidation. Although these proteins have not been examined to any great degree in the perirhinal cortex there was one study which analyzed an IEG *zif*268 in visual pair-association in the perirhinal cortex, to find its expression was increased after the task (Tokuyama *et al*, 2002), and this gene has been reported to be activated by the ERK/MAPK pathway following LTP induction (Davis *et al*, 2000). From these results we can only suggest that perhaps PI3K is a more important mediator of neurotrophin activity than ERK in the perirhinal cortex, at least in this form of learning.

The entorhinal cortex is interconnected reciprocally with the perirhinal cortex which serves as a gateway for information flow to hippocampal structure (Witter & Amaral, 1991) meaning it too plays an important role in memory formation. In our experiments we have not observed any great changes in neurotrophins, their receptors or their signaling proteins in the entorhinal cortex. In this study we found an increase PI3K activation in the tyrphostin treated consolidation group, in the entorhinal cortex. There is no evidence in the literature of PI3K changes in response to tyrphostin treatment or in the entorhinal cortex. This surprising result may be interpreted as suggesting that blockade of Trk receptors results in increased activation of PI3K via a pathway which is independent of Trk activation. The mechanism underlying this change has not been investigated; further experiments are required to confirm the effect and to elucidate the underlying mechanism.

This study further supports our data from previous chapters indicating neurotrophin activation of the Trk receptors mediates acquisition and consolidation of recognition memory. This evidence supports a role for neurotrophin action in both the early and late stages of memory processing. Tyrphostin blockade of the Trk receptor-stimulated signaling that accompanies consolidation supports data from the literature that

ERK and PI3K mediate neurotrophin-induced intracellular signaling responsible for mediating some forms of synaptic plasticity.

Chapter 7

General Discussion

7.1 General Discussion

Despite extensive research effort there is still little understanding of the intrinsic mechanisms behind memory storage and processing. For this thesis it was decided to investigate the possible brain regions involved and the proteins mediating the intracellular signaling cascades underlying a specific form of memory, namely object recognition memory. Molecular cascades are believed to be triggered by new experiences that induce synaptic and cell-wide alterations, among them learning-induced synthesis of new proteins, modulation of gene expression and morphological synaptic remodeling which are ascribed to play a pivotal role in the consolidation of memory (Flood *et al.*, 1973, Goelet *et al.*, 1986). There are many different types of memory such as emotional, fear, contextual, taste, odor, recognition, episodic and spatial memory to name but a few. It is possible that for each different type of memory, different proteins are involved in different stages of memory formation. Here we decided to focus on recognition memory in the rat, as the paradigm allows examination of different stages of memory. This learning paradigm assesses the ability of animals to recognize the presence of a novel object and it is suitable as a task to target both acquisition and consolidation of information as it allows a series of novel objects to be explored by the rat at time points corresponding to phases of acquisition and consolidation. The main benefit of the object recognition task is that it can be considered as 'pure' memory paradigm, as there are no incentives for the rat to explore, as the usual positive or negative reinforcers (such as food rewards and foot shocks) are absent from the test, it relies completely on the animal's propensity to explore its surrounding environment.

Schovile assessment of H.M. identified the essential role of the hippocampus in memory formation and sparked a new interest in ascribing distinct functions to different regions of the brain. Since then investigation of the medial temporal lobe lead to discoveries such as that by Horel and Misantone in 1976, who found that disruption of the connection between the anterior temporal lobe with dorsal and medial brain regions resulted in monkeys being unable to complete a visual discrimination task. The regions of the medial temporal lobe we choose to focus on were the dentate gyrus, the hippocampus, the perirhinal cortex and the entorhinal cortex. In most studies the hippocampus is analyzed whole but in this case we decided to dissect out the dentate

gyrus, a sub region of the hippocampus and analyze it separately. From the literature the dentate gyrus appears to be an important subfield of the hippocampus for memory processing, particularly of spatial learning. The perirhinal cortex and the entorhinal cortex were also chosen as they are two major sources of input to the hippocampus. The perirhinal cortex has been investigated extensively and has been revealed to be a central region to recognition memory processing (Brown *et al.*, 1999).

Learning is believed to induce synthesis of new proteins which are ascribed to play a pivotal role in the consolidation process (Bekinschtein *et al.*, 2007). Both early and late stages of memory consolidation and LTP have been shown to be dependent upon protein synthesis (Lu *et al.*, Commarota *et al.*, 2007, Herandez *et al.*, 2002, Scharf *et al.*, 2002). During memory formation, protein synthesis is thought to be required to transform newly learned information into stable synaptic modifications. The functional significance of adult neurogenesis remains one of the key unsolved issues in neuroscience (Fuchs and Gould, 2000; Kempermann et al., 2004b). Although there may be a correlation between neurogenesis and memory, the relationship remains controversial (Gould et al., 1999b; Lu and Chang, 2005). Activities known to enhance learning and memory, such as exercise or living in an enriched environment, promotes neurogenesis in the adult hippocampus (Kempermann et al., 1997; Nilsson et al., 1999; van Praag et al., 1999; Cotman and Berchtold, 2002). Learning itself has also been shown to enhance the survival of newly generated neurons in the dentate (Gould et al., 1999). The most direct evidence so far was demonstrated in an experiment in which inhibition of neurogenesis using a mitotic inhibitor impaired hippocampal-dependent, but not hippocampal-independent memory (Shors et al., 2001). Neurotrophins have been extensively implicated in synaptic plasticity and neuronal differentiation and have been demonstrated to be essential for the development of protein synthesis which is essential to LTP and memory processing (Patterson *et al.*, 1996, Tyler *et al.*, 2002, Morris *et al.*, 2003). Of particular interest to this study consolidation and reconsolidation of recognition memory has been shown to be protein synthesis dependent, a process which may depend on ERK-mediated signaling, Kelly and colleagues successfully blocked memory by use of an ERK inhibitor (2003). Protein synthesis is believed to modulate

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synapse formation and dendritic spine growth, which are morphological processes thought to underlie memory storage (Bamji *et al.*, 2006, Tyler and Pozzo-Miller, 2003). Neurotrophins were the proteins of choice to analyze in their role in memory and learning because of their known involvement in stimulating ERK activity and protein synthesis.

As mentioned previously we chose to use the object recognition task to analyze learning and because of the flexibility of the task allowing examination of different stages of memory so that both acquisition and consolidation of memory could be tested. The problem we had to address was the lack of pharmacological agents available to block the Trk receptors individually without cross reactivity with the other receptors as Trk receptors share 80% homology of their structural arrangements. We choose to use the tyrosine kinase inhibitor, tyrphostin AG879, which has previously been used to block LTP and spatial memory by inhibition of the Trk receptors non-specifically (Maguire *et al.*, 1999, Gooney *et al.*, 2002). It has never been used before in assessment of acquisition and consolidation of recognition memory as in this study. Tyrphostins have been shown to inhibit autophosphorylation of protein tyrosine kinases (Lyall *et al.*, 1989) and more specifically tyrphostin AG879 has been shown to block Trk A signaling indicating that AG879 competes for the substrate binding site, preventing signaling, which renders tyrphostins useful inhibitors for investigating possible roles and signaling mechanisms of the Trk receptors (Ohmichi *et al.*, 1993, Rende *et al.*, 2000). As we could not target the neurotrophin receptors individually we decided to block the neurotrophins themselves. We used neutralizing antibodies to target the neurotrophins individually. It was considered that antibody binding would prevent the binding of neurotrophins to their preferred receptors. The antibodies chosen have less that 1% cross-reactivity with the other neurotrophins, according to the manufacturer, lending confidence in the specificity of their effects. This strategy also allowed us to discriminate between the actions of BDNF and NT4/5, which are ligands at the same receptor, Trk B.

The next issue we decided to address was neurotrophin signaling which is activated upon binding of the Trk receptors. Neurotrophins bind to the Trk receptor, activating a cascade of signaling events which are also believed to be important in

expression of various forms of synaptic plasticity. The ERK/MAPK signaling pathway is believed to form the major signal transduction pathway mediating extracellular stimuli, among which is the binding of neurotrophins to their preferred Trk receptor, to transcription in the nucleus (Schaeffer and Weber, 1999). ERKs were originally discovered as regulators of cell division and differentiation but, like neurotrophins, were soon reported to play a major role in synaptic plasticity, learning and memory (Atkins *et al.*, 1998; Sweatt, 2001, 2004; Mazzucchelli *et al.*, 2002). ERK, which is activated by neurotrophin binding of the Trk receptors, has been shown to be involved in other types of hippocampal-dependent memory. Blum and colleagues in 1999 displayed evidence showing a role for ERK in long term spatial memory. Another study using specific inhibitor of the kinase, UO126, injected rats with the inhibitor and found the animals were unable to distinguish the familiar object from the novel object. The authors found increased ERK phosphorylation in various structures of the medial temporal lobe implicating the ERK/MAPK pathway in the signaling cascades activates during memory consolidation (Kelly *et al.*, 2003). In the same study the authors also established a possible role for the ERK/MAPK pathway in reconsolidation of long term recognition memory, finding that the inhibitor UO126 effectively blocks reconsolidation of recognition memory, and this was shown to be dependent on reactivation of the memory trace by brief re-exposure to the objects. In addition to activation of certain kinases measurement of immediate early gene (IEG) activation such as c-fos across brain regions provides a means of visualizing the pattern of neural activation resulting from specific behaviors in animal models. Previous studies have shown increased c-fos synthesis following place and response learning in the hippocampus and the dorsal striatum (Gill *et al.*, 2007). Measurement of ERK activation in this study was to reinforce the findings of others like Kelly and colleagues by replicating their data, supporting the role of ERK in memory processing.

Having found the greatest changes in response to learning and memory with BDNF, and given that most other studies in the literature support this, we made it the main focus of this study especially when analyzing neurotrophin release. Neurotrophin release from synaptic vesicles is believed to mediate synaptic plasticity activating intracellular proteins and transcription. More specifically, BDNF release has been shown

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to be activity-dependent in hippocampal neurons (Hartmann *et al.*, 2001) and this results in BDNF immunoreactivity through out the cell, having been found in the nucleus and surrounding cytoplasm, dendritic shafts and spines, axons, and axon terminals of the hippocampal neurons (Murer *et al.* 2001). This data supports the hypothesis that neurotrophins or more specifically BDNF mediates synaptic transmission and plasticity. It has also been indicated that BDNF signaling is bidirectional, acting both pre- and post-synaptically to enhance synaptic plasticity (Kohara *et al.*, 2001), therefore by analyzing BDNF release we could see how it responds at the synaptic junction in response to cognition. We also replicated previous studies which showed upregulation of ERK activation in association with consolidation (Kelly *et al.*, 2003). This leads us to suggest that the ERK activation associated with consolidation is stimulated by BDNF activation of Trk B following its activity dependent release.

Having found that consolidation is accompanied by increased BDNF release, but not ERK activation, in perirhinal cortex, we choose to analyze PI3K, another signaling molecule believed that is believed to be involved in memory processing and is activated downstream of Trk receptor signaling. We demonstrated that during early consolidation PI3K is activated simultaneously with BDNF release in the perirhinal cortex, indicating a role for this signaling pathway in information storage in this brain region.

In this study we identified that both i.p. and i.c.v. administration of tyrphostin AG879 blocks both acquisition and consolidation of recognition memory by inactivation of the neurotrophin receptors Trk A, Trk B and Trk C. This data suggests that both acquisition and consolidation of recognition memory is dependent upon Trk receptor activation by neurotrophins, however we cannot discount the possibility that tyrphostin AG879 may be exerting non-specific effects on signaling via inhibition of other tyrosine kinases. When we consider this result in the context of our findings that inhibition of neurotrophins blocks memory consolidation we can speculate that inhibition of memory in this case is as a result of Trk receptor inhibition. The greatest limitation within this study was our lack of ability to analyze the Trk receptor activation directly. Ideally we would have liked to analyze Trk receptor activation and not just expression to confirm that our observed changes were as a result of neurotrophin signaling. Analysis of Trk

receptor phosphorylation would hopefully demonstrate which receptors were activated to the greatest extent in which brain regions and at which stages of memory consolidation were they most important. Again the problem here was with Trk receptor homology and the lack of antibodies available to selectively bind to the receptor subtypes individually.

Once we had identified neurotrophins and Trk receptors as important proteins involved in memory processing we wanted to investigate the sequence of events which underlie acquisition and consolidation of recognition memory. We decided to design a time-course experiment to analyze the role of neurotrophin and Trk receptor expression over a 24hr period where memory is consolidated following training in the object recognition task. The times of 0 min, 2hr, 6hr and 24hr were chosen in the context of the published literature but the gaps in between were found to be too large to detect changes in protein following expression of mRNA. Having observed increases in neurotrophin mRNA expression we still do not know the source of this expression whether it is from neuronal tissue or glial cells. We can only speculate that it is most likely in neuronal tissue, given the activity-dependence of neurotrophin release and the paucity of data indicating a role for glia in memory consolidation although we cannot rule out the possibility that these changes may occur in glial cells. We have identified a role for BDNF, NGF and NT4/5 in recognition memory but as yet we have not distinguished which neurotrophin is the most important as regards acquisition and consolidation of memory. Having focused on BDNF in our latter studies we demonstrated a possible role for BDNF in acquisition with its release in response to training in the object recognition task along with showing its involvement in consolidation. This information is supported by other findings in the literature as BDNF is the most extensively investigated neurotrophin although this does not mean it is the most important.

In spite of these issues we have succeeded in identifying a role for BDNF, NGF and NT4/5 in consolidation of recognition memory and in establishing Trk receptor activation as an essential mediator of acquisition and consolidation of recognition memory. BDNF appears to play a particularly important role in the hippocampus, an area associated with a number of types of memory formation and synaptic plasticity; indeed

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this region has the highest neuroanatomical expression of BDNF and its Trk B receptor in the mammalian brain (Murer *et al.* 2001). BDNF has previously been shown to be involved in short and long term memory formation of a hippocampal-dependent one-trial fear-motivated learning task in rats (Alonso., 2002). Consistent with these data, in this study we identified a role for BDNF in consolidation of recognition memory. Similarly we report a positive role for NT4/5 in memory consolidation but to date there are few studies in the literature focused on this neurotrophin Numerous studies have indicated a role for NGF in various forms of synaptic plasticity in a similar fashion to BDNF. Kelly and colleagues (1998) displayed a role for NGF in LTP of the perforant path-grannule cell synapse using an inbred strain of rats deficient in NGF, this deficiency was accompanied by an impairment in LTP. In addition, i.c.v. injection of NGF reversed this impairment. We support this evidence with our findings that NGF is necessary for consolidation of recognition memory in the rat. Most studies have reported insignificant changes in NT3 or Trk C expression following learning (Kang and Schuman, 1995, Bramham *et al.*, 1996, Chen *et al.*, 1999). In broad agreement with this we report that inhibition of NT3 had no effect on acquisition or consolidation of recognition memory, with anti-NT3 treated animals successfully identifying the novel objects when tested. For this reason NT3 appears to be of lesser importance than the other neurotrophins in its possible role of mediation of synaptic plasticity. It appears that the greatest changes in neurotrophin expression were observed in the dentate gyrus and the perirhinal cortex, both are brain regions which have been largely associated with recognition memory in previous studies (Kelly *et al.*, 2003, Cowell *et al.*, 2006, Furtk *et al.*, 2007). We also observed that the most notable changes in the neurotrophins were immediate or in the early hours following memory consolidation with BDNF displaying the most prevalent modifications and NT3 may in-fact be decreased in a response to novelty. In response to this we speculated that the immediate effects of neurotrophin release and signaling are most important in mediating protein synthesis which is essential to synaptic plasticity. The finding that BDNF is released in response to consolidation of recognition memory further supports evidence in the literature that neurotrophin release at synaptic junctions mediates synaptic plasticity and is essential to both acquisition and consolidation of memory (Hartmann *et al.*, 2001, McHugh *et al.*, 2007, Nilsson *et al.*, 2007, Wang and

Salter, 1994). We have also noted increased upregulation of neurotrophin signaling protein ERK, which indicates the MAPK/ERK signaling pathway is mediating neurotrophin action during memory consolidation, this data further supports evidence in the literature for the involvement of the MAPK signaling cascade in memory processing (Kelly et al., 2003). Having focused on BDNF we demonstrate that BDNF is released in response to training in the object recognition task and have confirmed its effects are mediated through activation of the ERK/MAPK pathway and the PI3K/AKT pathway and that these signaling cascades are interrupted when Trk receptor action is blocked by a tyrosine kinase inhibitor tyrphostin AG879.

The objective of this study was to identify the roles of neurotrophins and the Trk receptors in learning and memory using the object recognition task. This study succeeded in identifying a role for BDNF, NGF and NT4/5 in consolidation of recognition memory and in establishing Trk receptor activation and its down stream signaling events as an essential mediator of acquisition and consolidation of recognition memory.

In contrast to our findings regarding acquisition, we have shown that simultaneous activity of all neurotrophins, with the exception of NT3, is required to allow consolidation to occur. This leads us to suggest that activation of both Trk A (by NGF) and Trk B (by both BDNF and NT4/5) and stimulation of subsequent intracellular signaling cascades, underpins consolidation of recognition memory.

8.1 Future Studies

1. Having identified a role for neurotrophins in consolidation of recognition memory it would be of benefit to investigate Trk receptor activation and expression through out the medial temporal lobe and to identify which cells are expressing the Trk receptors to identify which neurotrophins are most important in which brain regions. Immunocytochemistry techniques would allow investigation of localization of expression of these proteins.

2. In this study we focused on acquisition and consolidation of recognition memory and only briefly touched on memory reconsolidation. It would be interesting to fully investigate the role of neurotrophins in this process and to attempt to identify other proteins involved.

3. It would also be of interest to take a closer look at neurotrophin regulated transcription and assess which proteins are expressed that mediate the morphological changes believed to be crucial to memory storage. One possible experiment would be to conduct a similar protocol as used in chapter 4 of this thesis where a timepoint experiment was conducted using the object recognition task and assess changes in neurogenesis. To analyse this, BrdU (Bromodeoxyuridine) staining is one option. BrdU is a synthetic thymidine analog that gets incorporated into a cell's DNA when the cell is dividing (during the S-phase of the cell cycle). Antibodies against BrdU that are conjugated to fluorescent markers can be used to label these cells, thereby providing visual evidence of cell division and neurogenesis. Another way to investigate neurogenesis is a new mechanism described by Chen and collegues (2006) using MRI to count neurons and their dendritic outgrowths. This method would require the animals to be perfused following completion of the object recognition task and the brain fixed in paraformaldehyde before being scanned.

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VII Appendix

Solutions Used

Carbonate Coating Buffer pH 9.7
Sodium hydrogen carbonate	0.025M
Sodium carbonate	0.025M
Distilled water	

Electrode Running Buffer
Tris Base	25mM
Glycine	200mM
SDS	17mM

Krebs Solution
NaCl	136mM
KCl	2.5mM
KH_2PO_4	1.18mM
$MgSO_4$	1.18mM
$NaHCO_3$	16mM
Glucose	10mM
Containing $CaCl_2$	2mM

Phosphate-buffered Saline (PBS) pH 7.4
Na_2HPO_4	80mM
NaH_2PO_4	20mM
NaCl	100mM

PBS-Tween (PBS-T)
0.1% Tween-20 solution in PBS

Sample Buffer
Tris-HCL	0.5M, pH 6.8
Glycerol	10% v/v
SDS	0.05% w/v
B-mercaptoethanol	5% w/v
Bromophenol blue	0.05% w/v

Separating Gel
Bisacrylamide	33% w/v
Tris-HCL	1.5M, pH 8.8
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, pH 6.8
    SDS   1% w/v
    Ammonium persulphate 0.5% w/v
    Distilled water
    TEMED   0.1% w/v

Transfer Buffer pH 8.3
    Tris Base   25mM
    Glycine   192mM
    Methanol   20% v/v
    SDS   0.05% w/v
    Distilled water

TBS-Tween Wash Buffer
    Tris-HCL (pH 7.6)   20mM
    NaCl   150mM
    Tween-20   0.05% v/v
    Distilled water

VIII Publications

Paper

Blockade of NMDA receptors pre-training, but not post-training impairs object displacement learning in the rat. Larkin, A.E., Fahey,B., Gobbo, O., Callaghan, C.K., Cahill, E., O'Mara, S., Kelly, A., 2008, Brain Research 126-132

Abstracts

Recognition memory in the rat is associated with increased BDNF and ERK and Pi3K activation in the dentate gyrus and the perirhinal cortex. Callaghan, C.K., Kelly, A., 2007, SFN

The acquisition and consolidation of recognition memory are neurotrophin dependent. Callaghan, C.K., Kelly, A., 2007, BNA and Neuroscience Ireland

A Role for Neurotrophins in Recognition Memory. Callaghan, C.K., Kelly, A., 2007, ECNP

Recognition memory in the rat is associated with increased BDNF release and ERK activation in the dentate gyrus. Callaghan, C.K., Kelly, A., 2006, ECNP

Recognition memory in the rat is associated with increased brain-derived neurotrophin release and erk activation in the dentate gyrus. Callaghan, C.K., Losher, C.J., Kelly, A., 2005, BNA