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An Investigation into the Role of Neurotrophins in the Effect of Exercise on Cognitive Function in Humans and Rats

by

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

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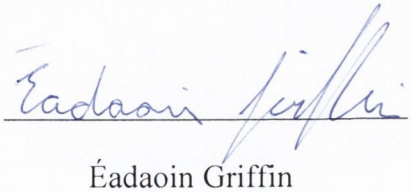

Éadaoin Griffin

II Summary

Physical exercise is associated with maintenance of cognitive function, including a decreased risk of Alzheimer's disease and other forms of dementia. Candidate mediators of the effect of exercise on cognitive function are the neurotrophins. These secreted peptides promote the growth and differentiation of developing neurons, support maintenance and survival of adult mature neurons and modulate neuronal plasticity. Exercise can alter neurotrophin expression; serum brain-derived neurotrophin factor (BDNF) expression has been repeatedly shown to increase with exercise in young men and exercise-induced increases in brain BDNF have also been demonstrated in rats. The objective of this study was to investigate the effects of exercise on cognitive function in humans and rats and to further elucidate the roles of neurotrophins in the link between physical exercise and improved cognition.

The effect of physical exercise on cognitive function in sedentary young men was investigated using both an acute and a chronic exercise protocol. The acute exercise bout involved an incremental exercise test to exhaustion, which selectively improved medial temporal lobe-dependent learning as assessed by a face-name matching task. This exercise-induced cognitive enhancement was associated with a concomitant increase in serum BDNF concentration. Furthermore, 5 weeks of aerobic training increased aerobic fitness and improved face-name task performance, but not response accuracy in the Stroop and N-back tasks, indicating that the aerobic training-induced cognitive enhancement was also selective for the medial temporal lobe-dependent task.

The effect of a 7-day forced exercise protocol on cognitive function in the male Wistar rat was investigated. One week of treadmill running enhanced recognition memory in the object substitution task, while exercise had no effect on spatial memory in the object displacement task. The exercise-induced cognitive enhancement was associated with increased expression of BDNF in the serum and muscle of exercised rats. A parallel increase was observed in BDNF protein concentrations in the dentate gyrus, hippocampus and perirhinal cortex, while no alteration to BDNF mRNA expression was observed.

Having established a correlative link between exercise-induced cognitive enhancement and concomitant increases in BDNF concentration, an assessment of the effect of acute intracerebroventricular infusion of exogenous BDNF was made. A single bolus infusion of recombinant BDNF mimicked the effect of the forced exercise protocol on non-spatial recognition memory at a dosage based on the exercise-induced increase in hippocampal-BDNF previously measured, providing strong evidence for the role of BDNF in mediating exercise-induced cognitive enhancement. This finding was supported by the infusion of a function blocking BDNF antibody in conjunction with exercise. Taken together, these data are consistent with the hypothesis that physical exercise enhances cognitive function through a neurotrophin-mediated mechanism. BDNF appears to mediate both acute and chronic exercise-induced enhancements in learning and memory.

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IV Acknowledgements

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V List of Abbreviations

AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleprorionic acid
ANOVA	Analysis of variance
Arc	Activity-regulated cytoskeletal-associated protein
BBB	Blood-brain-barrier
BDNF	Brain derived neurotrophic factor
bpm	beats per minute
BrdU	5'-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CA1	Cornu Ammonis 1
CA2	Cornu Ammonis 2
CA3	Cornu Ammonis 3
CNS	Central nervous system
CRE	Ca^{2+} response element
CREB	c-AMP response element-binding protein
CSF	Cerebrospinal fluid
DAB	Diaminobenidine
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase
GABA	γ-aminobutyric acid
HFS	High frequency stimulation
HPA axis	Hypothalamic-pituitary-adrenal axis
HRP	Horseradish peroxidase conjugate
icv	Intracerebroventricular
IGF-1	Insulin-like growth factor 1
ip	Intraperitoneal
iv	Intravenous
KCL	Potassium chloride
LTP	Long-term potentiation

LTM	Long-term memory
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
met	Methionine
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MTL	Medial temporal lobe
MW	Molecular weight
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5
OD	Object displacement
OD_{260}	Optical density at 260nm
OS	Object substitution
$p75^{NTR}$	p75 neurotrophin receptor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphotidylinositol-3 kinase
PKC	Protein kinase C
PLC-γ	Phospholipase C-γ
PNS	Peripheral nervous system
pro-BDNF	proneurotrophin BDNF
pro-NGF	proneurotrophin NGF
RNA	Ribonucleic acid
RSK	Ribosomal S6 kinase
RT-PCR	Real-time polymerase chain reaction
SDS	Sodium dodecylsulphate
SEM	Standard error of the mean
SOS	son of Sevenless
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween

TMB	3,3', 5,5'-Tetramethyl-benzidine
tPA	Tissue plasminogen activator
Trk	Tropomyosin-related kinase
Trk A	Tropomyosin-related kinase receptor tyrosine kinase A
Trk B	Tropomyosin-related kinase receptor tyrosine kinase B
Trk C	Tropomyosin-related kinase receptor tyrosine kinase C
val	valine
$val^{66}met$	amino acid substitution, valine to methionine at codon 66
VO_2 max	Maximal oxygen consumption rate

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Chapter 1
Introduction

Chapter 1: Introduction

1.1 General Introduction

Exercise is known to be important for the maintenance of physical fitness and general good health. In addition, a significant relationship exists between exercise and cognitive function (Colcombe & Kramer, 2003; Kramer *et al.*, 2006). While this concept of 'a healthy mind in a healthy body' is a familiar one, the link between physical activity and cognitive function is not well understood. Furthermore, the problem of age-related cognitive decline is becoming ever more prevalent in our society, as a result of current demographic trends towards an increasingly elderly population. However, physically-active older people are more likely to maintain cognitive function, and have a decreased risk of Alzheimer's disease and other forms of dementia, relative to their sedentary counterparts (Barnes *et al.*, 2003; Larson *et al.*, 2006). Hence, there is potential for the development of exercise training programmes to protect against age-related cognitive decline.

Neurotrophins are candidate mediators of the effect of exercise on cognitive function. This family of secreted peptides act as growth factors for the phenotypic development of neuronal populations in the developing nervous system, are involved in the maintenance of neurons in the adult nervous system and modulate neuronal plasticity. It has been suggested that alterations in neurotrophin levels contribute to the characteristic neurodegeneration seen in Alzheimer's disease and Parkinson's disease (Siegel & Chauhan, 2000). Furthermore, exercise can alter neurotrophin expression. Most notably, expression of brain-derived neurotrophic factor (BDNF) in the serum has been repeatedly shown to increase with exercise in young men (Gold *et al.*, 2003; Rojas Vega *et al.*, 2006; Winter *et al.*, 2007; Tang *et al.*, 2008) and increases in brain BDNF have also been demonstrated in rats (O'Callaghan *et al.*, 2007; Soya *et al.*, 2007; Lou *et al.*, 2008). Although evidence supports the role of neurotrophins in mediating of the effects of exercise on cognitive function, the mechanism behind this is not well understood.

1.2 Cognitive function

In the 1950's the case of a severely epileptic patient brought new understanding to the study of memory. In a seminal paper, Scoville and Milner described the consequences of a radical surgery, which was performed in an attempt to alleviate the patient's debilitating seizures. This surgery involved a bilateral medial lobe resection, including the hippocampal formation, amygdala and entorhinal cortex. The patient, known by the initials H.M., had profound amnesia for all events that occurred following the surgery (Scoville & Milner, 1957). This amnesia manifest as impairments in acquisition of both episodic memory (memory for events that have a spatial and temporal context) and semantic memory (general knowledge about the world), whereas his non-declarative memory, such as motor learning ability was spared. H.M.'s lexical and semantic memories existing prior to the surgery were also undisturbed (Corkin, 2002). This data provided the first evidence for the importance of the medial temporal lobe in normal memory function. More recent literature has provided further evidence for the central role of the hippocampus in activity-dependent learning and memory (Lynch, 2004).

1.2.1 Hippocampus

The hippocampus is located in the medial temporal lobe (MTL), and consists of four main regions: the dentate gyrus, the hippocampus proper (including cornu ammonis 1, 2 and 3: CA1, CA2 and CA3), the entorhinal cortex and the subiculum. The hippocampus proper and dentate gyrus, together with the cingulate gyrus, were termed the *archicortex*, due to the trilaminar structure of these areas, which includes the subgranular zone, the granular cell layer and the pyramidal cell layer. The adjacent *neocortex* region consists of 6 layers and includes the parahippocampal, entorhinal and perirhinal cortices and the fusiform region. The hippocampus receives input to the dentate gyrus via the perforant path, from pyramidal cells in the entorhinal cortex. Mossy fibres of the dentate granule cells synapse with pyramidal cells of the CA3 region, which in turn synapse with CA1 neurons to form the Schaffer collateral pathway (see Figure 1.1), which synapses with the subiculum. The subiculum lies between the hippocampus proper and the entorhinal and other cortices and may provide an interface between the hippocampus and the neocortex during consolidation of long-term memories (Craig & Commins, 2006).

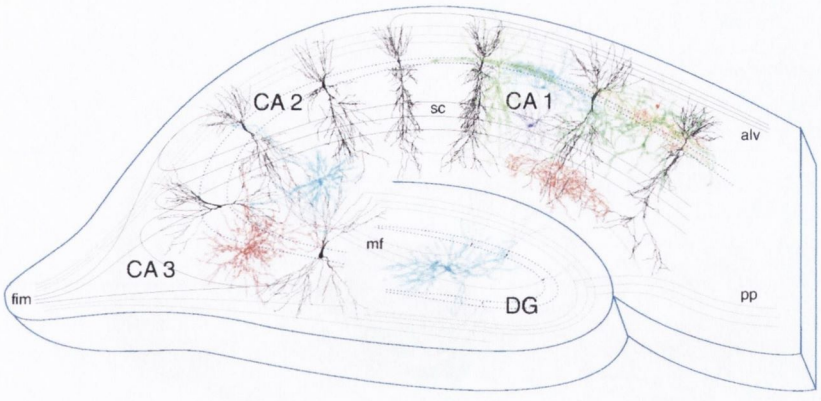


Figure 1.1: Schematic diagram of the signalling networks of the rat hippocampus

(Adapted from: www.stanford.edu/group/maciverlab/) The hippocampus receives input to the dentate gyrus (DG) via the perforant path (pp). The mossy fibres (mf) of the dentate granule cells synapse with pyramidal cells of the CA3 region. The CA3 pyramidal cells synapse with CA1 neurons to form the Schaffer collateral pathway (sc). Also shown is the alveus (alv) part of the fimbria (fim) pathway that connects to the fornix.

The hippocampus forms a part of the limbic system (see Figure 1.2), which is a collection of closely associated cortical regions (including the hippocampal gyrus, cingulate gyrus and entorhinal cortex), subcortical nuclei (including the amygdala and hypothalamus) and fibre tracts (including the fornix). Much of the limbic system appears to be functional in emotional and motivational processes, while the hippocampus is known to be vital for learning and memory. However, there are some forms of memory, including recognition memory, that are not always affected by selective hippocampal lesions (Buckley, 2005). Regional functional distinctions have been reported within the MTL between the hippocampal region and the surrounding cortical areas (Kirwan & Stark, 2004). It has been suggested that the perirhinal cortex is specialised for processing object identity, including roles in both memory and perception, whereas the hippocampus may be specialised for processing spatial and temporal associations (Buckley, 2005). Although the individual structures in the MTL appear to have different roles, it is a highly interconnected and dynamic system and therefore the division is not absolute (Kirwan & Stark, 2004).

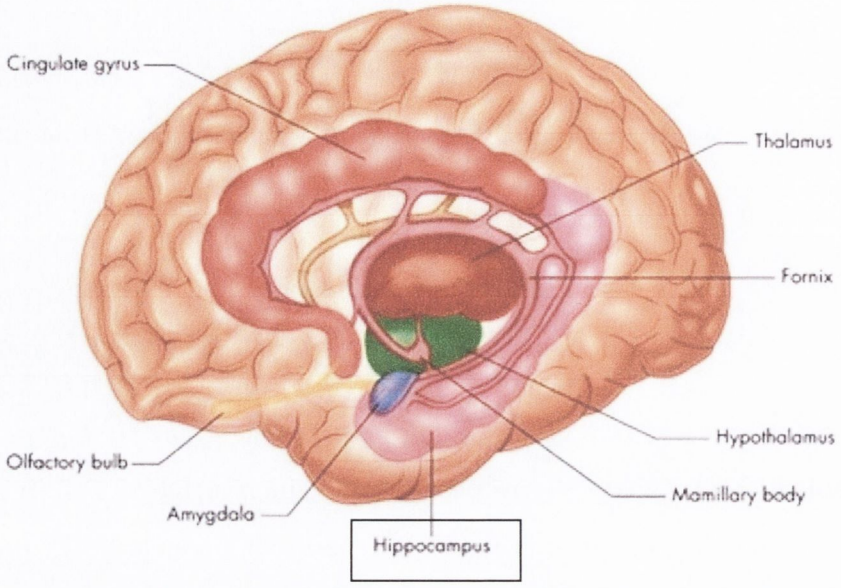


Figure 1.2: Structural layout of the human brain, displaying the limbic system

(Image taken from www.abieducation.com/binder/English/chap1.html)

1.2.2 Age-related cognitive decline

One of the risk factors associated with aging is cognitive decline, including an increased risk of developing Alzheimer's disease (AD), a degenerative condition resulting in profound amnesia. Age-related cognitive decline is characterised by decrements in aspects of memory, attention and perception (McAuley *et al.*, 2004). The hippocampus is known to be particularly susceptible to aging and age-related impairments in associative learning and spatial memory are attributed to degenerative changes in this area (Burke & Barnes, 2006). This occurs as a result of neuronal and neuropil shrinkage, lowered rate of neurogenesis, impaired angiogenesis, or an accumulation of deleterious compounds in the brain (Trejo *et al.*, 2004), such as β-amyloid. Even older people who are considered healthy may suffer from mild cognitive impairment. High-resolution magnetic resonance imaging (MRI) scans have been used to demonstrate a decline in tissue densities in the frontal, parietal, and temporal cortices as a function of age. However, losses in these areas were reduced substantially as a function of cardiovascular fitness in older people (Colcombe *et al.*, 2003). Moreover, executive control (including working memory, interference control and task coordination) processes are known to decline substantially with age but aerobic fitness-training interventions particularly improve performance in this type of cognitive task (McAuley *et al.*, 2004). Taken together, evidence from the literature supports a role for exercise in maintaining cognitive function, although the mechanism has yet to be

elucidated. Given that physical exercise could potentially be used as a preventative measure against the damaging effects of aging on the brain, it is important to study this link between physical activity and cognitive function.

1.2.3 Assessing cognitive function

Having highlighted the importance of the hippocampus in learning and memory, it is important to use cognitive tasks that activate this region of the brain when assessing the effect of exercise on cognition. Evidence from patients with right amygdalo-hippocampectomy demonstrated that face recognition recruits the right medial temporal lobe. Face memory retention was impaired in these patients, but not face perception. Deficits in facial perception are associated with more posterior damage to the right temporal lobe, consistent with the fusiform face area. The relevant damaged structures for facial memory retention are the hippocampus proper and the entorhinal, perirhinal and parahippocampal cortices (Crane & Milner, 2002). Further evidence, using high-resolution functional MRI acquisition and analysis methods, demonstrated hippocampal engagement during a face-name association task. The face-name association task involved viewing a series of unfamiliar face that are paired with names. Following a short delay the faces were presented again in randomised order and the participant was asked to attempt to recall the names. CA2, CA3 and the dentate gyrus were activated during encoding, as evidenced by a large increase in cerebral blood flow, which correlates with activity. Retrieval resulted in decreasing activation of the hippocampus, as performance became practised, indicating that the hippocampal formation was more involved in learning processes. The subiculum was more active during retrieval and relatively less active during encoding (Zeineh *et al.*, 2003).

Further evidence, from a different task assessing associative memory, suggested activation of the hippocampus and parahippocampal region during both encoding and retrieval of novel picture pairs. Meanwhile, the transentorhinal portion of the perirhinal cortex was activated during encoding but not retrieval. Interestingly, this region is the site of the earliest neuropathalogical changes in AD (Pihlajamaki *et al.*, 2003). Evidence from chronic cannabis users indicated impairments in performance of a face-name association task associated with the high density of endocannabinoid receptors in the hippocampus. However, some recovery of function was attributed to a compensatory mechanism resulting in the relative hyperactivity of the parahippocampal

cortex (Nestor *et al.*, 2008). Furthermore, patient H.M., who had the majority of his hippocampus removed, was able to remember the address and floor plan of a home he moved to after the onset of his anterograde amnesia. This was attributed to the preservation of his perirhinal and parahippocampal cortices (Corkin, 2002). This evidence indicates a significant role for the cortical regions closely associated with the hippocampus, such as the parahippocampal and perirhinal cortex, in memory function. Therefore, it may be more appropriate to consider the effect of exercise on medial-temporal lobe-dependent memory, rather than hippocampal-dependent memory.

Having established a method of assessing the effect of exercise on medial-temporal lobe-dependent learning and memory, it is important to consider what effect exercise may have in other regions of the brain. The Stroop task (Stroop, 1935) exploits the conflict between an automatic behaviour and a decision rule that requires this behaviour to be inhibited (Leung *et al.*, 2000). The classic Stroop task involves coloured word stimuli, in which congruent stimuli (coloured word stimuli presented in the font the word represents) are presented frequently and incongruent stimuli are infrequent, in order to maximise the cognitive interference characteristic of this task. The selective attention required activates the anterior cingulate, insula, premotor and inferior frontal regions of the brain (Leung *et al.*, 2000; Ferris *et al.*, 2007). The N-back task assesses working memory and primarily recruits the dorsolateral prefrontal cortex. This task also deactivates the medial frontal, middle temporal, and parahippocampal areas (Meyer-Lindenberg *et al.*, 2001). Therefore, attempting this task prior to completion of a medial temporal lobe-dependent task could impair performance in the medial temporal lobe-dependent task. Working memory is an executive control process known to decline with age (McAuley *et al.*, 2004). A fundamental characteristic of working memory is that its capacity to handle information is limited. Hence, the N-back task involves increasing cognitive load and ultimately decreasing task performance (Callicott *et al.*, 1999).

Testing cognitive function in humans may be the most relevant method of assessing the potential therapeutic implications of physical activity in ameliorating cognitive decline, however, with the use of an animal model it is also possible to assess any changes at the molecular and cellular levels. This additional information may give some indication of the mechanisms involved. There are many tasks designed to test cognitive function in

animal models. Among the many tasks that have been developed to assess cognitive function in rodents, the classic novel-object recognition task may be the most informative in the present context. It tests a form of cognitive performance related to episodic memory in humans (Li *et al.*, 2008). It is based on the spontaneous exploratory behaviour of rats and therefore is comparable to memory tests used in man, as it does not require any positive or negative reinforcements such as food or electric shocks (Ennaceur & Delacour, 1988). The discrimination between novel and familiar objects is central to this task. Furthermore, a variation of this task, the object displacement task can be used to test spatial recognition memory. The perirhinal cortex is an important region for the novel-object recognition task but the hippocampus is also involved, particularly in spatial aspects of the task (Kelly *et al.*, 2003). Evidence suggests that the hippocampus processes information related to the familiarity of arrangements of items, while the perirhinal cortex is associated with the recognition of the familiarity of individual items, in both rats and humans (Wan *et al.*, 1999; Pihlajamaki *et al.*, 2004), although part of the hippocampus was also activated by the identification of novel objects in humans (Pihlajamaki *et al.*, 2004).

1.2.4 Exercise and cognitive function

In addition to the ability of sustained physical activity and increased aerobic fitness to enhance cognitive function, acute bouts of exercise have also been shown to enhance information processing in humans. A positive effect of acute physical exercise on cognitive function has repeatedly been reported in trained subjects, for exercise lasting more than 20min but less than an hour. When exercise lasted more than an hour, the appearance of fatigue symptoms were common (Grego *et al.*, 2004). Extended periods of steady state exercise that lead to dehydration and depletion of energy stores compromised information processing and memory function. However, intense anaerobic exercise may facilitate aspects of cognitive function, depending on an individual's fitness level (Tomporowski, 2003). This indicates that the effect of acute bouts of physical exercise on cognitive function in humans may be dependent on the participant's cardiovascular fitness level.

Evidence suggests that the intensity of the exercise bout is also a factor. The ability to learn new words was reportedly accelerated by 20% following intense exercise, but lexical-learning was not altered by more moderate exercise (Winter *et al.*, 2007). In

contrast, the effect of different exercise intensities on cognitive function was also investigated using the Stroop task. Cognitive function was reportedly enhanced following a graded exercise test and both high and low intensity 30min endurance cycles (Ferris *et al.*, 2007). Given the variability in protocols used and the often contradictory results reported, the four methodological factors which should be considered when studying the physiological effects of exercise on cognitive function in humans are: 1; the fitness of the subjects, 2; the intensity and duration of the exercise, 3; the nature of the psychological task and 4; the time at which the psychological task was presented relative to the period of exercise (Grego *et al.*, 2004).

Similarly, animal models have been used to demonstrate exercise-induced cognitive enhancements in a variety of tasks, such as navigation of the radial arm maze, escape latency in the Morris water maze, novel object recognition and passive avoidance (Anderson *et al.*, 2000; Albeck *et al.*, 2006; O'Callaghan *et al.*, 2007; Chen *et al.*, 2008). However, there is conflicting data in the literature regarding exercise-induced cognitive enhancement, which is likely to be the result of the large variety in the intensity, duration and modality of exercise used in both voluntary and forced exercise protocols. Enhancements in spatial memory have been frequently reported, with improvements in Morris water maze performance seen following both three weeks and one week of voluntary wheel running in young rats (Adlard *et al.*, 2004; Vaynman *et al.*, 2004). However, 10-weeks of treadmill running in older rats (Barnes *et al.*, 1991), 40 days of voluntary wheel running in mice (Rhodes *et al.*, 2003) and 1 week of treadmill exercise in young rats (O'Callaghan *et al.*, 2007) had no effect on this test of spatial memory, although novel object recognition was improved following 7 days of forced exercise (O'Callaghan *et al.*, 2007). Therefore, when studying the effect of exercise on cognitive function in rodents, the intensity, duration and modality of the exercise should be considered, along with the nature of the behavioural task. The mechanism behind these exercise-induced cognitive enhancements is yet to be fully elucidated. However, neurotrophic factors have been proposed as mediators of this effect.

1.3 Neurotrophic factors

Rita Levi-Montalcini and Viktor Hamburger first discovered the neurotrophins in 1951, when they implanted a mouse sarcoma tumour close to the spinal cord of a developing

chicken *in ovo*. They discovered a secreted soluble factor, which was subsequently named nerve growth factor (NGF) induced hypertrophy and outgrowth of the dorsal root ganglion (Levi-Montalcini & Hamburger, 1951). This discovery was followed in 1982 by Barde and colleagues, who isolated BDNF from pig brain (Barde *et al.*, 1982). Since then, neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5) have been discovered (Hohn *et al.*, 1990; Hallbook *et al.*, 1991), both of which are also expressed in the mammalian brain. Two more neurotrophins, neurotrophin 6 and neurotrophin 7, have also been identified but only in fish (Gotz *et al.*, 1994; Lai *et al.*, 1998). Neurotrophins have been characterised for their involvement in development of the nervous system and maintenance of neurons in the adult brain but more recently a possible role for neurotrophins in exercise-induced cognitive enhancement has come to light.

1.3.1 Neurotrophin synthesis, storage and release

Neurotrophins are synthesised as larger precursor proteins, pro-neurotrophins, which require further processing prior to secretion as mature proteins. Each neurotrophin contains a signal peptide following the initiation codon, a pro-region, and the mature sequence (Binder, 2007). The signal peptide directs the synthesis of the emerging protein to the ribosomes of the rough endoplasmic reticulum (ER), where the newly formed polypeptide chain is sequestered inside, and the signal peptide is cleaved off immediately after sequestration. The remaining pro-neurotrophin spontaneously forms non-covalently-linked homodimers, which transit to the Golgi apparatus and then accumulate in the membrane stacks of the trans-Golgi network (Lessmann *et al.*, 2003) (see Figure 1.3). The pro-neurotrophins (MW~30kDa) are cleaved intracellularly by prohormone convertases such as furin, to release the mature proteins (MW~14kDa) (Mowla *et al.*, 2001). Mature neurotrophins form non-covalently linked homodimers (MW~28kDa) and dimerization appears to be essential for neurotrophin receptor activation (Binder, 2007).

The mature neurotrophins are secreted via either the constitutive secretory pathway or the regulated secretory pathway, which is calcium-dependent. Regulated neurotrophin secretion has been demonstrated for NGF and BDNF in the central nervous system (CNS) but not NT-3 or NT-4 (Lessmann *et al.*, 2003). Constitutive secretion of NGF is confined to the neuronal soma and the very proximal parts of dendrites. In contrast, the activity-dependent secretion of NGF occurs all along the neuronal processes,

particularly in the dendrites (Blochl & Thoenen, 1996), indicating some spatial disparity between these secretory pathways. Evidence suggests that the activity-dependent neurotrophin synthesis is mediated by the excitatory neurotransmitters glutamate and acetylcholine, which up-regulate synthesis, while the inhibitory neurotransmitter γ-aminobutyric acid (GABA) down-regulates neurotrophin production (Lindholm *et al.*, 1994).

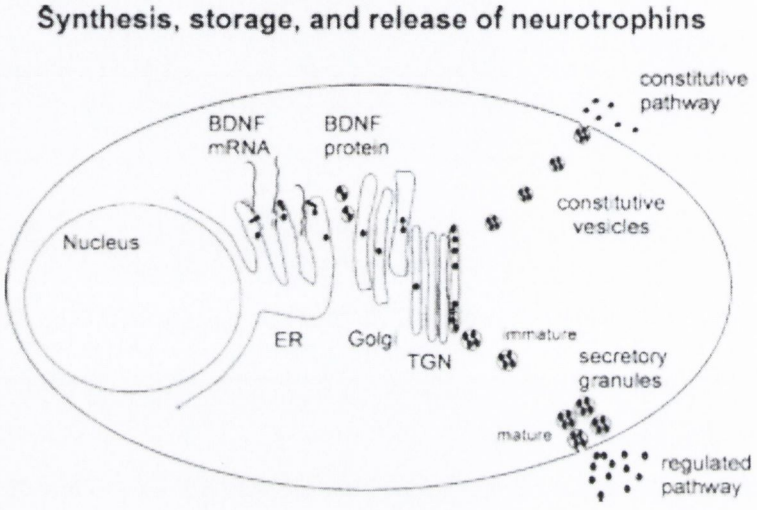


Figure 1.3: The route of BDNF from synthesis to secretion (Lessmann *et al.*, 2003).

Neurotransmitters are chemical messengers in the nervous system. Upon release from the pre-synaptic cleft they bind to specific receptors on the post-synaptic neuron, resulting in a transient alteration to the electrical properties of the membrane. Excitatory neurotransmitters trigger depolarization of the post-synaptic membrane, which in turn activates various intracellular signalling cascades, resulting in the up-regulation of neurotrophin release. This occurs via post-translational protein modifications and differential gene expression (Blochl & Thoenen, 1996). Furthermore, neurotrophin secretion is also subject to positive feedback regulation, in which neurotrophins act in an autocrine manner to trigger neurotrophin secretion (Schinder & Poo, 2000).

Stimulation of NGF-release has been demonstrated via glutamate receptor activation or high potassium concentrations. This regulated NGF secretion was shown to be independent of extra-cellular calcium, but dependent on calcium-release from intracellular stores and was mediated by sodium influx via voltage-gated sodium

channels and non-NMDA glutamate receptors (Blochl & Thoenen, 1996). Furthermore, an increase in intracellular calcium concentration activates calmodulin, resulting in phosphorylation of c-AMP response element-binding protein (CREB), which binds to the Ca^{2+} response element (CRE) within the BDNF gene to regulate BDNF gene transcription (Tao *et al.*, 1998). In addition, activity-dependent secretion of BDNF occurs under conditions that induce long-term potentiation (LTP), a cellular analogue for learning and memory, suggesting a causal link between neurotrophin secretion and activity-dependent synaptic plasticity (Schinder & Poo, 2000). NT-3 and NT-4 secretion does not appear to be modulated by neuronal activity.

Alternatively, evidence suggests that pro-BDNF is secreted from hippocampal neurons in an activity dependent manner and the extracellular serine protease, plasmin, cleaves pro-BDNF to mature BDNF (Pang *et al.*, 2004). However, the processes of activity-dependent secretion and extracellular cleavage of pro-BDNF may not be functionally relevant *in vivo*. Neurons have a limited capacity to process pro-BDNF, which is exhausted when neurons are transfected with BDNF cDNAs. Moreover, pro-NGF but not pro-BDNF has been detected in adult rodent brains following injury, possibly as a result of inefficient processing of pro-neurotrophins by inflammatory cells. It is mature BDNF, not pro-BDNF, that accumulates in neurons and is secreted upon stimulation (Matsumoto *et al.*, 2008).

1.3.2 Neurotrophin receptors and signalling

There are two types of receptor to which the neurotrophins bind, the tropomyosin-related kinase (Trk) receptor tyrosine kinases and the p75 neurotrophin receptor ($p75^{NTR}$), related to the tumour necrosis factor receptor superfamily. The neurotrophins bind with high affinity to their respective Trk receptor tyrosine kinases; Trk A primarily binds NGF, Trk B has a high affinity for BDNF and NT-4/5, while NT-3 is the primary ligand for Trk C but can bind to all three Trk receptors (Huang *et al.*, 1999) (see Figure 1.4, A). The $p75^{NTR}$ binds each of the neurotrophins with approximately equal affinity but binds the pro-form of the neurotrophins with higher affinity than the mature form (Madani *et al.*, 1999).

Trk receptors are widespread throughout the CNS and peripheral nervous system, with differential distribution of the Trk receptor subtypes. In the CNS, Trk A is highly

expressed in cholinergic neurons of the basal forebrain, while the highest concentrations of Trk B are found in the granular cell layer of the dentate gyrus and the pyramidal cell layer of the hippocampus. Trk C expression demonstrates significant overlap with Trk B in the CNS, but Trk C is not expressed in the choroid plexus and ependymal cell layer of the cerebral ventricles, where high levels of the non-catalytic, truncated Trk B receptors are expressed. Truncated Trk B receptors have the same extracellular and transmembrane domain as full-length Trk B, but have a very short cytoplasmic domain that lacks the catalytic kinase region (Barbacid, 1994). Signalling through the Trk receptors supports cell survival, differentiation and synaptic plasticity (Zhang *et al.*, 2000) while activation of the $p75^{NTR}$ induces apoptosis (Dechant & Barde, 1997) via activation of the Jun cascade and ceramide turnover (Xu *et al.*, 2000).

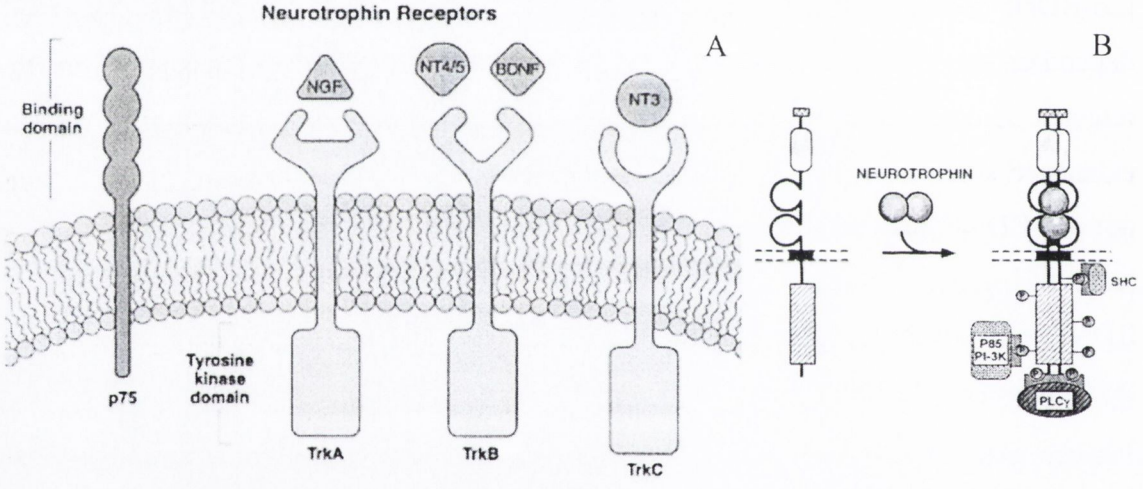


Figure 1.4: Neurotrophins receptors (Siegel & Chauhan, 2000) & Trk receptor ligand-binding (Barbacid, 1994)

(A) The neurotrophin receptors and their respective high affinity ligands. **(B)** Neurotrophin binding to a Trk receptor results in receptor dimerization and autophosphorylation of their cytoplasmic tyrosine residues. The resulting phosph-tyrosines are recognised by the SH2 domains of downstream signalling elements such as the enzyme PLC-γ, the p85 regulatory subunit of PI3K and the adaptor protein Shc.

Trk tyrosine kinase receptors are transmembrane proteins with highly conserved intracellular domains which mediate well defined signal transduction pathways (Thoenen & Sendtner, 2002). Neurotrophin binding induces dimerization in the Trk

receptors and kinase activation. Subsequently, receptor autophosphorylation on multiple tyrosine residues within the intracellular domains, provides protein interaction sites for Shc, phospholipase C-γ (PLC-γ) and phosphatidylinositol-3 kinase (PI3K) (Binder, 2007) (see Figure 1.4, B), leading to the activation of three major signalling pathways, promoting cell survival and differentiation (Xu *et al.*, 2000). Neurotrophin signal transduction through these signalling cascades involves both the propagation and amplification of the signal, by the protein kinases and adaptor proteins involved (Kaplan & Stephens, 1994).

Trk ligand binding induces tyrosine phosphorylation of Shc, which in turn triggers interaction of the adaptor proteins Shc/Grb2/Sos leading to transient Ras activation. Ras activation promotes neuronal survival through either the p38 MAPK pathway or PI3K activation. Transient Ras activation induces a series of phosphorylations including Raf, MEK 1 & 2 and extracellular-regulated protein kinases (ERK1/2) (Alonso *et al.*, 2002), leading to activation of ribosomal S6 kinase (RSK) and phosphorylation of the transcription factor CREB (see Figure 1.5) (Patapoutian & Reichardt, 2001). More sustained Ras activation leads to prolonged activation of ERK signalling, involving the small G protein Rap-1, which regulates neuronal differentiation and requires endocytosis of the neurotrophin-receptor complex (Patapoutian & Reichardt, 2001).

Trk receptor activation can activate PI3K through at least two different pathways. Ras-dependent activation of PI3K is the most important pathway through which neurotrophins promote cell survival. PI3K is also activated through 3 adaptor proteins, Shc/Grb2/Gab-1 (Patapoutian & Reichardt, 2001), which results in the translocation of this cytoplasmic protein to the membrane where it has access to its substrates, the phosphotidylinositol phospholipids (Kaplan & Stephens, 1994). The putative effector of PI3K is the serine and threonine kinase Akt, which phosphorylates IκB, leading to activation of the transcription factor NFκB (see Figure 1.5), ultimately leading to the promotion of neuronal survival (Patapoutian & Reichardt, 2001; Alonso *et al.*, 2002).

Trk ligand binding activates PLC-γ, which hydrolyzes phosphotidylinositol-4,5-bisphosphate to generate inositoltrisphosphate (IP3) and diacylglycerol (DAG) (see Figure 1.5). IP3 induces release of Ca^{2+} from intracellular stores, which increases the

concentration of cytosolic Ca^{2+}, a common second messenger. DAG-induced activation of protein kinase C (PKC) is required for activation of the ERK cascade, leading to neurite outgrowth. Release of Ca^{2+} from intracellular stores, activates calcium/calmodulin-dependent protein kinase-2 (CaMKII) and the p38 subfamily of mitogen-activated protein (MAP) kinases in hippocampal neurons (Patapoutian & Reichardt, 2001; Alonso *et al.*, 2002).

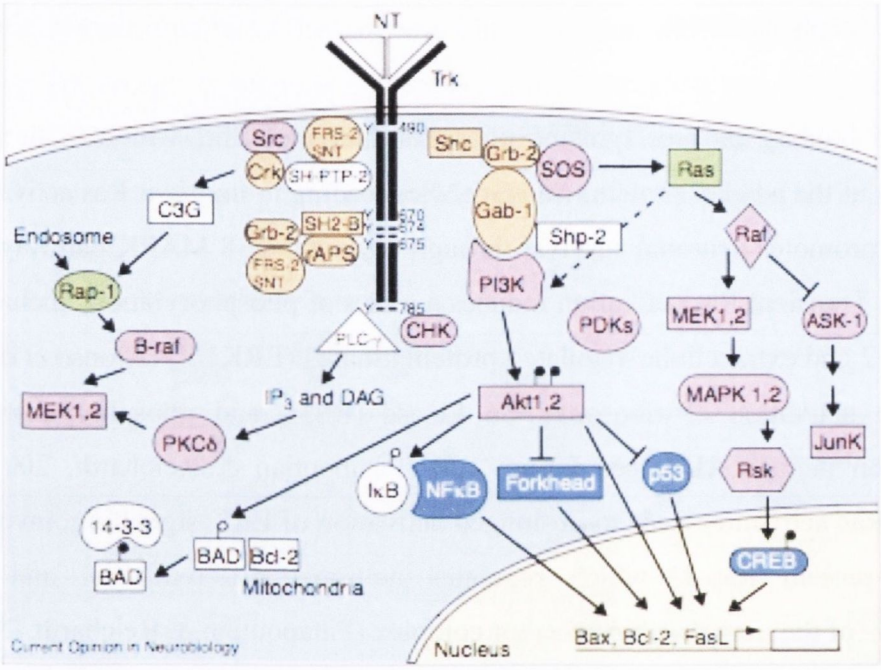


Figure 1.5:Neurotrophin signal transduction pathways mediated by Trk receptors (Patapoutian & Reichardt, 2001)

Adapter proteins are coloured orange, kinases pink, small G proteins green and transcription factors blue.

Neurotrophin binding to the $p75^{NTR}$ activates intracellular signalling cascades involving NFκB, Jun kinase and acidic sphingomyelinase. As discussed, activation of the transcription factor NFκB is involved in the promotion of neuronal survival. In contrast, the Jun kinase pathway promotes apoptosis and sphingomyelin hydrolysis generates ceramide, which has been shown to inhibit PI3K and Akt. Neurotrophin signalling through the $p75^{NTR}$ refines the ligand specificity of the Trk receptors, regulates axonal growth, mediates retrograde transport of the neurotrophins and helps regulate survival and apoptosis of neuronal populations. In particular the $p75^{NTR}$ promotes apoptosis of neurons that lack Trk receptor signalling. Furthermore, Trk

receptor ligand binding suppresses activation of the Jun cascade, via Ras activation (see Figure 1.5), preventing activation of the apoptotic pathway. Trk signalling also suppresses sphingomyelin hydrolysis but not induction of the NFκB cascade (Patapoutian & Reichardt, 2001). Although the $p75^{NTR}$ binds mature neurotrophins, pro-neurotrophins are high affinity ligands for $p75^{NTR}$ and it has been suggested that local secretion of pro-neurotrophins, along with the regulated activity of plasmin involved in the cleavage of pro-neurotrophins to a mature form, may define the pro-apoptotic or pro-survival effects of the neurotrophins (Lee *et al.*, 2001).

1.3.3 Neurotrophins and synaptic plasticity

Synaptic plasticity is the term used to describe activity-dependent alterations in synaptic strength. Long-term potentiation (LTP) is a frequently studied model of synaptic plasticity that has been proposed as a cellular analogue of learning and memory. LTP is a lasting enhancement of synaptic transmission, typically induced by high-frequency stimulation (HFS) of excitatory inputs into the hippocampus, causing a rapid elevation of intracellular calcium concentration via activation of N-methyl-D-aspartate (NMDA) glutamate receptors, leading to calcium influx through voltage gated calcium channels in conjunction with the release of calcium from intracellular stores. LTP maintenance is less well characterised. The first 1-3 hours post-HFS is considered early phase LTP and reportedly requires covalent modification of existing proteins and protein trafficking at synapses, while late-phase LTP can last weeks *in vivo* and is dependent upon protein synthesis (Lynch, 2004; Bramham & Messaoudi, 2005). Evidence in support of the hypothesis that LTP may be a biological substrate of memory includes the fact that LTP is most easily demonstrable in the hippocampus, an area of the brain known to be fundamentally important in memory acquisition. Furthermore, HFS trains which mimic naturally-occurring theta rhythm recorded in the hippocampus during exploratory behavior, readily induce LTP and inhibitors of hippocampal LTP also block hippocampal learning and memory retention (Lynch, 2004). Hence, neurotrophins have been implicated as mediators of learning and memory, partly due to the evidence for their roles in synaptic plasticity.

Neurotrophins enhance the efficacy of synaptic transmission in an activity-dependent manner, with evidence to suggest increased secretion of NGF and BDNF as a result of neuronal excitement, resulting in enhanced release of excitatory neurotransmitters and

elevated intracellular calcium concentrations (McAllister *et al.*, 1999). Neurotrophins can be secreted both pre and post-synaptically and locally secreted neurotrophins can act retrogradely at pre-synaptic terminals to induce long-lasting synaptic modifications. In addition, neurotrophins can function anterogradely, released by pre-synaptic cells to regulate post-synaptic target cells (Schinder & Poo, 2000). Neurotrophins reportedly play both instructive and permissive roles in activity-dependent synaptic modification. Instructive roles of neurotrophins include modification of pre-synaptic neurotransmitter release, post-synaptic sensitivity or synaptic morphology leading to persistent synaptic modification (Schinder & Poo, 2000), with a permissive role for BDNF demonstrated by the impairment in LTP in BDNF knockout mice (McAllister *et al.*, 1999). Furthermore, intrahippocampal infusion of BDNF has been shown to induce LTP in the hippocampus (Messaoudi *et al.*, 2002) and exercise has been shown to enhance LTP and improve spatial learning in rats, in conjunction with increased BDNF concentrations in the brain (van Praag *et al.*, 1999; O'Callaghan *et al.*, 2007). In addition, intracerebroventricular (icv) injections of an anti-BDNF antibody have been shown to impair spatial memory (Mu *et al.*, 1999). Taken together, this evidence suggests an integral role for neurotrophins in synaptic plasticity. In particular, BDNF appears to be involved in processes of neuronal plasticity linked to learning and memory.

1.3.4 NGF

Nerve Growth factor (NGF) was the first of the neurotrophins to be discovered. It was characterised via its trophic effects on sensory and sympathetic neurons (Levi-Montalcini & Hamburger, 1951). β-NGF is a 12.5kDa protein (Edwards *et al.*, 1988) which supports survival of cholinergic neurons in the CNS and is synthesised primarily in the brain by cholinergic target tissues such as the cortex, hippocampal pyramidal layer and striatum (Binder, 2007). Expression of Trk A, the high affinity receptor for NGF, in the CNS is primarily on the axons of NGF-dependent cholinergic neurons (Cellerino, 1996). NGF is also required for the differentiation and survival of sympathetic and some sensory neurons in the peripheral nervous system (Kaplan & Stephens, 1994). Basal concentrations of NGF protein in the hippocampus are very low (Narisawa-Saito & Nawa, 1996) and basal levels of Trk A mRNA in the hippocampus are also low (Cellerino, 1996), indicating that NGF may have a lesser role in hippocampal-dependent learning and memory, relative to BDNF for instance. However,

activity dependent secretion of NGF has been demonstrated (Blochl & Thoenen, 1996; McAllister *et al.*, 1999) and a role for NGF-Trk A signalling in the consolidation of contextual memory has been reported (Woolf *et al.*, 2001). Furthermore, icv infusions of NGF have been shown to partly reverse cholinergic cell body atrophy and improve retention of spatial memory tasks in aged rats displaying behavioural impairments (Fischer *et al.*, 1987) and it has been suggested that deficits in NGF release and subsequent signalling in the dentate gyrus contributes to age-related impairments in LTP (Kelly *et al.*, 2000).

More recently, research has indicated that stress-related events are characterised by alterations in NGF synthesis and utilization in both humans and animal models. Moreover, NGF is implicated in the activation of the hypothalamic-pituitary-adrenal (HPA) axis, indicating a link to immune responses. Mast cells, eosinophils, and T and B lymphocytes produce NGF and express Trk A receptors, indicating that NGF can act as an autocrine/ paracrine factor in the development and regulation of immune cells. Evidence also suggests that both circulating and brain NGF concentrations are altered by stressful events, such as aggressive behaviour, fear, forced physical activity, sudden environmental changes and social isolation. Furthermore, chronic stress seems to be a key factor leading to loss of neurons in the CNS and a reduction in the size of the hippocampus. This indicates that NGF may play a functional role in stress coping responses (Aloe *et al.*, 2002).

NGF concentrations were measured in serum samples from humans and were found to display considerable physiological variance. Although serum NGF concentrations were not normally distributed, intra-individual serum concentrations remained stable over time, and a slight age-related decrease was reported (Lang *et al.*, 2003), which may have implications for age-related cognitive decline due to a lack of trophic support for cholinergic neurons. It has been suggested that alterations in neurotrophin levels due to age, genetic background or other factors contribute to the characteristic neurodegeneration seen in AD. However, increases in NGF expression in surviving neurons of the hippocampus and certain neocortical regions and decreases in Trk A in the cortex and nucleus basalis have been reported with AD (Siegel & Chauhan, 2000), suggesting the involvement of altered NGF signalling in the pathogenesis of this disease.

1.3.5 BDNF

Brain-derived neurotrophic factor (BDNF) is synthesised as a 32kDa precursor, which is cleaved to the 14kDa protein, mature BDNF that forms non-covalently linked homodimers to give a 28kDa polypeptide (Mowla *et al.*, 2001). Despite their size, evidence suggests that certain polypeptides can cross the blood-brain barrier (Pan & Kastin, 2004). Specifically, BDNF has been shown to cross the blood-brain barrier in both directions (Poduslo & Curran, 1996) with BDNF influx into the brain occurring via a saturable, unidirectional transport system and BDNF efflux occurring via bulk flow, in association with the reabsorption of cerebrospinal fluid (Pan *et al.*, 1998). BDNF is expressed along with its receptor tyrosine kinase Trk B in both central and peripheral nervous tissue, as well as non-neuronal tissue, such as heart, muscle and vascular tissue. The binding of this ligand to its receptor promotes cell survival and differentiation, including neurogenesis in the dentate gyrus of the hippocampus (Lee *et al.*, 2002) and angiogenesis in vascular endothelial cells (Kim *et al.*, 2004; Kermani & Hempstead, 2007). In the periphery, platelets appear to bind, store and release BDNF upon activation at the site of traumatic injury, to facilitate repair of peripheral nerves or other tissues that contain Trk B receptors (Fujimura *et al.*, 2002). Potential sources of BDNF in the periphery include vascular endothelial and smooth muscle cells, activated macrophages and lymphocytes (Lommatzsch *et al.*, 2005). In the CNS, BDNF distribution is widespread and BDNF co-localizes with Trk B at glutamatergic synapses (Bramham & Messaoudi, 2005). Furthermore, BDNF and Trk B expression is highest in the cerebellum, hippocampus and cerebral cortex, regions known to be involved in developmental and adult synaptic plasticity (McAllister *et al.*, 1999).

Evidence suggests that neuronal secretion of BDNF occurs in an activity-dependent manner, as a result of neuronal excitation. Moreover, secreted BDNF can act in an autocrine and paracrine manner to further stimulate its own secretion. Furthermore, activity-dependent secretion of BDNF potentiates synaptic transmission in glutamatergic synapses, by acting both pre- and post-synaptically to enhance neurotransmitter release and membrane excitability (Schinder & Poo, 2000). BDNF has been shown to modulate LTP in the CA1 region of the hippocampus by binding to pre-synaptic Trk B, altering the ability of the pre-synaptic neurons to generate repetitive exocytotic events in response to tetanic stimulation (Xu *et al.*, 2000). BDNF exerts a

role in long-term memory formation *in vivo* in the CA1 region, via activation of the transcription factor CREB, through the ERK1/2 signalling pathway (Alonso *et al.*, 2002). Recognition memory formation has been shown to increase BDNF mRNA expression in regions associated with visual recognition such as the inferior medial temporal cortex, the CA1 region of the hippocampus and the basolateral amygdala in sheep, but not in the dentate gyrus. Trk B mRNA also increases in the medial temporal cortex (Broad *et al.*, 2002).

The binding of BDNF to pre-synaptic Trk B increases synapsin-1 phosphorylation, via activation of the MAP kinases (Jovanovic *et al.*, 2000). The synapsins are neuron-specific phosphoproteins, crucial for exocytosis in the pre-synaptic nerve terminal. They tether small synaptic vesicles to the actin cytoskeleton in a phosphorylation-dependent manner and regulate the proportion of vesicles in the nerve terminals that are available for release (Hilfiker *et al.*, 1999). Hence, binding of BDNF to pre-synaptic Trk B potentiates neurotransmitter release (Jovanovic *et al.*, 2000), leading to the facilitation of LTP in the post-synaptic neuron (Xu *et al.*, 2000) and also reducing synaptic fatigue (Bramham & Messaoudi, 2005). BDNF is also a potent neuroexcitant at the post-synaptic membrane, inducing extremely rapid depolarisations, leading to modulation of calcium influx through voltage-dependent calcium channels and NMDA receptors (Kafitz *et al.*, 1999; Kovalchuk *et al.*, 2004; Bramham & Messaoudi, 2005).

There is a frequent single-nucleotide polymorphism in the targeting region of the human BDNF gene that produces a non-conservative amino acid substitution (valine to methionine), at codon 66 (val^{66}met) (Egan *et al.*, 2003; Hariri *et al.*, 2003). This variant is located in the sequence encoding for pro-BDNF. Hence, it does not alter the biological activity of mature BDNF; rather, the polymorphism alters intracellular trafficking and packaging of pro-BDNF and thus, the regulated secretion of mature BDNF (Hariri *et al.*, 2003). Secretory granules containing val BDNF accumulate at the synapses, while met BDNF aggregates remain in the cell body. This indicates that met BDNF protein cannot be secreted at the synapses, leading to impairments in activity-dependent BDNF secretion but not tonic secretion in met carriers (Egan *et al.*, 2003). This results in impairments in hippocampal function in humans, such that met carriers do not perform as well in episodic memory task as val/val homozygotes, with the lowest scores occurring in the met/met homozygote group associated with disrupted

hippocampal disengagement during the N-back task (Egan *et al.*, 2003), supporting the notion that BDNF plays a prominent role in memory sub-processes that engage the medial temporal lobe (Goldberg *et al.*, 2008).

1.3.5.1 Exercise and BDNF

A study investigating the effects of physical exercise on multiple sclerosis (MS) patients and age-matched controls was the first to suggest that moderate exercise induces increases in serum BDNF concentrations in humans. A 30min standardised exercise test at 60% of the maximal oxygen consumption rate (VO_2 max) transiently increased serum BDNF levels in the healthy controls. The acute exercise-induced BDNF increase had returned to baseline at 30min post-exercise (Gold *et al.*, 2003). This finding was supported by Rojas Vega and colleagues, who reported transiently increased serum BDNF concentrations following a short duration incremental exercise test to exhaustion in young male athletes, but not after a 10min moderate exercise bout, which preceded the incremental exercise (Rojas Vega *et al.*, 2006). Similarly, exercise-induced increases in serum BDNF were found to be intensity-dependent, with transient increases in serum BDNF reported after a graded exercise test and a high-intensity endurance cycle but not a low-intensity endurance cycle. In addition, serum BDNF concentrations were found to correlate with blood lactate concentrations after the graded exercise test (Ferris *et al.*, 2007).

Winter and colleagues (2007), also investigated the effect of different exercise intensities on serum BDNF concentrations in young male sports students. All participants completed intense anaerobic sprints, moderate aerobic running and a sedentary rest period. Intense running induced increases in circulating BDNF. However, BDNF baseline values differed for the 3 conditions of exercise, hence exercise-induced increases were reported as slope differences in BDNF concentrations, with a significantly greater slope difference in the intense versus moderate exercise, and moderate versus sedentary conditions of exercise (Winter *et al.*, 2007). More recently, 15 minutes of step-exercise was also shown to induce a transient increase in serum BDNF levels in humans. Moreover, the authors reported a large degree of inter-individual variation in serum BDNF concentration but relatively stable intra-individual values (Tang *et al.*, 2008), which may explain the variability in the BDNF data from the study by Winter *et al.* Furthermore, acute exercise was also shown to enhance cognitive

function as evidenced by improved performance in the Stroop task and accelerated lexical learning (Ferris *et al.*, 2007; Winter *et al.*, 2007), indicating a role for exercise-induced increases in BDNF in cognitive enhancement.

This proposed role for BDNF in exercise-induced cognitive enhancement is further supported by evidence from animal studies. Forced exercise, in the form of treadmill running, has been shown to increase brain BDNF concentrations in rats (O'Callaghan *et al.*, 2007; Soya *et al.*, 2007; Lou *et al.*, 2008). In addition, forced exercise can recover diminished BDNF expression as a result of LPS-infusion and age-related decline (Wu *et al.*, 2007; Wu *et al.*, 2008). Furthermore, treadmill exercise enhanced spatial memory performance in the Morris water maze in aged rats (Albeck *et al.*, 2006; Wu *et al.*, 2007) and improved novel object recognition memory in young rats (O'Callaghan *et al.*, 2007). Similarly, voluntary wheel running was shown to increase BDNF concentrations and enhance Morris water maze performance. However, the exercise-induced improvements in cognitive function were blocked by infusion of a specific immunoadhesion chimera that mimics Trk B and selectively binds BDNF (Vaynman *et al.*, 2004), further supporting a role for BDNF in exercise-induced cognitive enhancement.

1.3.6 NT-3 & NT-4/5

NT-3 was first described in 1990 (Hohn *et al.*, 1990). It binds preferentially to Trk C but can also bind to Trk A and Trk B and its mRNA and protein are widely distributed in the CNS (Binder, 2007). It is the first neurotrophin to be expressed in the peripheral nervous system during embryogenesis and during perinatal development NT-3 promotes the survival and the differentiation of sensory neurons, many of which become dependent on NGF during later stages of postnatal development (Lessmann *et al.*, 2003). NT-3 is an important autocrine factor supporting Schwann cell survival and differentiation and NT-$3^{+/-}$ heterozygote knockouts display defects in nerve regeneration and mylination (Sahenk *et al.*, 2008). Evidence suggests that NT-3 is involved in synaptic transmission and neuronal excitability; in particular, it enhances synaptic strength at Schaffer collateral-CA1 synapses and enhances paired-pulse facilitation in the perforant path-dentate gyrus pathway. Furthermore, similar to BDNF, it inhibits GABA-ergic inhibition and enhances survival and differentiation of neural progenitor cells (Binder, 2007). Moreover, Trk B and Trk C receptors are often co-

expressed on neurons in the CNS, while the truncated non-catalytic forms of these receptors are found on both neurons and glia (McAllister *et al.*, 1999).

NT-4/5 is the fourth member of the neurotrophin family (Hallbook *et al.*, 1991) and is a high affinity ligand for the Trk B receptor. It has very low baseline levels in the brain, with the expression of NT-4/5 most prominent in the postnatal hippocampus, neocortex, cerebellum and in thalamic nuclei, this expression continues into adulthood (Lessmann *et al.*, 2003). A unique feature of NT-4/5 is that is appears to require binding to the low-affinity receptor $p75^{NTR}$ for efficient signalling and retrograde transport in neurons (Ibanez, 1996). Furthermore, in contrast to other neurotrophin knockouts, NT-$4/5^{-/-}$ knockout mice are normal and long-lived with no obvious neurological deficits. However, application of NT-4/5 to hippocampal neurons in culture enhances excitatory synaptic transmission (Binder, 2007), indicating a potential functional role for NT-4/5 in mediating plasticity.

1.3.7 IGF-1

Insulin-like growth factor 1 (IGF-1) is a trophic factor that circulates at high levels in the blood stream. The principal source of circulating IGF-1 is the liver (Sjogren *et al.*, 1999) and its physiological function was thought to be as a mediator of the effects of growth hormone in the body (Jones & Clemmons, 1995). However, it was discovered that liver IGF-1 is not required for post-natal body growth (Yakar *et al.*, 1999), prompting questions about its physiological significance. IGF-1 can cross the blood-brain-barrier; it enters the brain via the blood-cerebrospinal fluid (CSF) pathway, indicated by heavy IGF-1 staining of choroid plexus cells after intracarotid injection of IGF-1 (Carro *et al.*, 2000). Furthermore, there are data to indicate that circulating IGF-I is involved in cognitive performance through its trophic effects on central glutamatergic synapses. Mice with low-serum IGF-1 levels have impaired performance in the Morris water maze and disrupted hippocampal LTP. These deficits are ameliorated by prolonged systemic administration of IGF-I (Trejo *et al.*, 2007), indicating a role for this growth factor in cognitive function. Evidence also suggests that IGF-1 is a neuroprotective agent, assisting in the clearance of β-amyloid and increasing neurogenesis and angiogenesis in the brain. Diminished serum IGF-1 during aging has been proposed as a factor that contributes to age-associated cognitive decline (Trejo *et al.*, 2004; Trejo *et al.*, 2007).

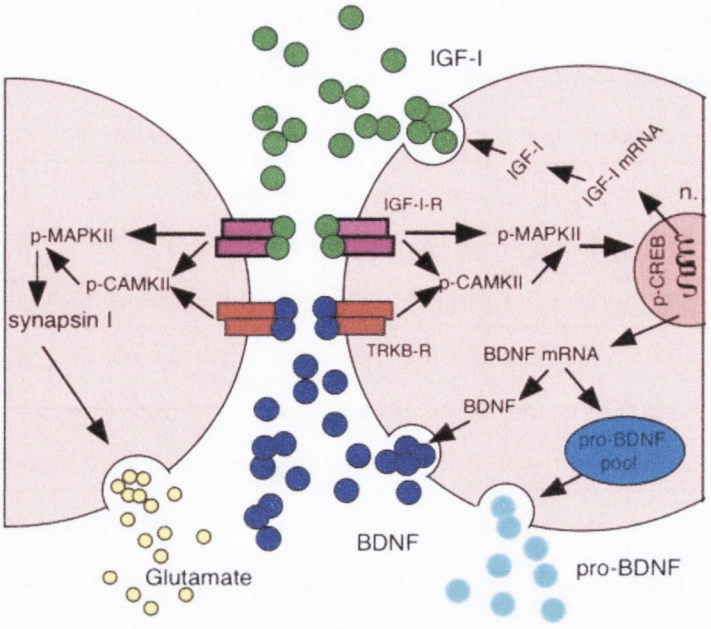


Figure 1.6: Hypothetical mechanism for IGF-1 modulation of BDNF-mediated synaptic plasticity in the hippocampus during exercise (Ding et al., 2006)

Exercise can induce IGF-I production in the hippocampus and IGF-I and BDNF are shown to have similar downstream signalling mechanisms, incorporating both p-CAMKII and p-MAPKII signalling cascades, resulting in CREB phosphorylation and up-regulation of both IGF-1 and BDNF mRNA (Ding *et al.*, 2006).

Exercise has activational effects on the brain, triggering hippocampal BDNF increases, neuronal accumulation of IGF-1 and increased hippocampal neurogenesis. These activational effects of exercise on the brain are mimicked by systemic injection of IGF-1, while blocking IGF-1 uptake by brain cells blocks c-Fos expression in the brain (Carro *et al.*, 2000), a marker of neuronal activity. IGF-1 had been shown to activate similar downstream pathways to BDNF and has abundant receptors in the hippocampus, indicating a potential role in nerve growth and differentiation and neurotransmitter synthesis and release (see Figure 1.6). Exercise has been shown to significantly increase pro-BDNF levels in the rat brain. This effect was reversed by blockade of the IGF-1 receptor, suggesting that exercise may increase BDNF levels by modulating pro-BDNF through the IGF-1 receptor. Blocking the IGF-1 receptor in the brain reversed the exercise-induced increase in IGF-1 mRNA, BDNF mRNA, BDNF protein and pro-BDNF (Ding *et al.*, 2006). Furthermore, blocking the IGF-1 receptor altered the amount of $BrdU^+$ cells in the brain, indicating that IGF-1 uptake to the brain

is necessary for exercised-induced increases in hippocampal neurogenesis (Trejo *et al.*, 2001).

1.3.8 Neurotrophins and the blood-brain barrier

The blood-brain-barrier (BBB) consists of a series of tight junctions between microvascular endothelial cells that restrict the movement of molecules across it. As a result of this barrier, there are considerably different ionic and molecular compositions in the blood and the CSF. Hence, ionic gradients exist across the blood brain barrier, including a nearly 10-fold gradient for glucose entry into the brain, which is maintained to assist in the supply of the brain's main energy substrate (Marchi *et al.*, 2004). However, evidence suggests that certain polypeptides can cross the BBB (Pan & Kastin, 2004). Indeed, the BBB is considered a relative solute barrier that contains specific carrier proteins to selectively move proteins across it in order to maintain CNS homeostasis. Evidence suggests that the BBB is differentially permeable to the neurotrophins, being most permeable to BDNF, NT-3 and NGF respectively, as evidenced by plasma washout times following intravenous injection (Poduslo & Curran, 1996). Furthermore, BDNF influx into the brain has been demonstrated; increased BDNF associated with the brain parenchyma rather than endothelial cells indicated complete passage of this neurotrophin across the BBB (Pan *et al.*, 1998).

In addition, evidence for a unidirectional transport system for BDNF was reported, with inhibition of labelled BDNF entry into the brain occurring after a bolus iv infusion of unlabelled BDNF, indicating that this transport system is saturable. It was proposed that BDNF efflux occurs via bulk flow, in association with the reabsorption of cerebrospinal fluid, as no evidence for self-inhibition was found following bolus icv infusions of BDNF (Pan *et al.*, 1998). Furthermore, there is evidence to suggest that exercise can induce a transient increase in the permeability of the BBB, as demonstrated by increased Evan blue albumin extravasation into the cerebellum and cerebral cortex following 30min forced swim exercise (Sharma *et al.*, 1991). However, a similar effect was demonstrated following eight hours of immobilization stress in young rats (Sharma & Dey, 1986), indicating that increased blood-brain barrier permeability may not be an exercise-related effect, rather it may occur as a result of stress.

1.4 Neurogenesis

Neurogenesis is a term used to describe the proliferation, differentiation and survival of new neurons. Following the discovery that neurogenesis persists into adulthood (Altman & Das, 1965), adult neurogenesis has been demonstrated in the hippocampus of all mammalian species, including humans (Eriksson *et al.*, 1998). The subgranular zone of the dentate gyrus gives rise to new cells that migrate to the granular cell layer, where they differentiate into neuronal cells and extend projections to the CA3 region (Markakis & Gage, 1999). Adult-generated neurons are integrated into neural networks and become functional *in vivo* (van Praag *et al.*, 2002). Furthermore, use of an antimitotic agent to reduce the number of new neurons in the hippocampus has been shown to impair specific forms of cognitive function, such as trace fear conditioning (Shors *et al.*, 2002). However, considering the length of time it takes neurons to mature and become integrated into a network, any potential functional consequences of adult neurogenesis occur as long-term adaptations rather than acute benefits (Kempermann *et al.*, 2004).

BrdU labelling is the "gold standard" in measuring neurogenesis. 5'-bromo-2'-deoxyuridine (BrdU) is a synthetic analogue to thymidine, which is incorporated into newly synthesised DNA during the S-phase of the cell cycle (Markakis & Gage, 1999). The advent of BrdU labelling led to the discovery that adult-generated granule cells are incorporated into hippocampal circuitry, indicating that they are functionally significant. BrdU can cross the blood-brain barrier and therefore, can be administered by intraperitoneal injection (Kitamura & Sugiyama, 2006). Once incorporated into the DNA, BrdU can be labelled by immunohistochemical techniques using monoclonal antibodies. However, there are some drawbacks to the technique. Firstly, BrdU labelling is a marker of DNA synthesis, not cell division. Hence, it is also incorporated into damaged DNA undergoing repair. Also, the technique requires tissue fixation and DNA denaturation; therefore, it cannot be used for the detection of neurogenesis in live tissue. Furthermore, the bioavailability of BrdU is between 30min and 120min. Considering that the cell cycle of a progenitor cell is approximately 25hrs, repeated injections of BrdU are required to quantitatively label cells (Taupin, 2007). Ki67 is an endogenous marker of cellular proliferation, present at all active-stages of the cell cycle. It is absent from quiescent cell in the G_0 and early G_1 phases of the cell cycle. Unlike BrdU, which is a marker of both neurogenesis and cellular repair, Ki67 is solely

a marker of cell proliferation (Scholzen & Gerdes, 2000; Wojtowicz & Kee, 2006). Hence, the most appropriate measure of neurogenesis may be an assessment of both BrdU labelling and Ki67 expression, in conjunction with a marker for neuronal phenotype such as NeuN.

The most characteristic feature of the neurotrophins is their ability to stimulate the growth and differentiation of neurons and to maintain neuronal survival. Therefore, it is not surprising that neurotrophins are also implicated in mediating the rate of adult neurogenesis. BDNF is highly expressed in granule cells of the dentate gyrus, but $BDNF^{+/-}$ heterozygous knockout mice have decreased levels of BDNF in the hippocampus and demonstrate decreased labelling of BrdU positive cells in the dentate gyrus, indicating that BDNF is important for the proliferation of neural stem cells (Lee *et al.*, 2002). Furthermore, two weeks of intrahippocampal BDNF infusions has been shown to increase the rate of neurogenesis in adult rats, as evidenced by increased $BrdU^{+}$/ $NeuN^{+}$ double labelling in the granule cell layer (Scharfman *et al.*, 2005).

1.5 Angiogenesis

Angiogenesis is the term used to describe the growth of new blood vessels. Endothelial cells of the vasculature are the main regulators of vascular homeostasis and angiogenesis. Vascular endothelial growth factor (VEGF) is a secreted protein that interacts with receptor tyrosine kinases on endothelial cells to promote angiogenesis. In addition, evidence suggests that BDNF-Trk B signalling in endothelial cells also promotes angiogenesis (Kermani & Hempstead, 2007). Furthermore, considering that neurogenesis occurs in close proximity to blood vessels in the adult rat sub-granular zone of the dentate gyrus, where VEGF expression is high and angiogenesis is ongoing, it is possible that neurogenesis and angiogenesis might be mechanistically linked, via VEGF (Palmer *et al.*, 2000). Indeed, more recent evidence indicates that VEGF also has neurotrophic and neuroprotective effects and can actually stimulate neurogenesis *in vivo* (Jin *et al.*, 2002). Moreover, this coupling between angiogenesis and neurogenesis has been used to identify an *in vivo* correlate of exercise-induced neurogenesis. MRI measurements of exercise-induced increases in cerebral blood volume have recently been shown to correlate with post-mortem measurements of neurogenesis in mice. In addition, cerebral blood volume changes in the dentate gyrus of humans were shown to correlate with aerobic fitness and cognitive function (Pereira *et al.*, 2007).

1.6 Exercise and neurogenesis

The rate of neurogenesis is known to decline as a function of age. However, physical exercise increased neural stem cell proliferation and the number of immature neurons and promoted the maturation and differentiation of immature neurons in middle-aged mice (Wu *et al.*, 2008). Furthermore, exercise restored the diminished rate of neurogenesis caused by LPS injection (Wu *et al.*, 2007), while a lack of exercise, as a result of two weeks of hindlimb suspension, impaired neurogenesis in young rats (Yasuhara *et al.*, 2007). Voluntary wheel running for 3 weeks increased the rate of neurogenesis, as assessed by BrdU labelling (Kitamura *et al.*, 2003). However, voluntary wheel running also enhanced cell death in pre-existing neurons of the dentate gyrus, resulting in no overall alteration in dentate gyrus volume, but an enhanced rate of neuronal turnover, which may be functionally important as young granule cells differ in both active and passive membrane properties, resulting in an easier induction of LTP in young neurons (Kitamura & Sugiyama, 2006). This evidence supports a role for physical exercise in modulating the rate of adult neurogenesis.

In addition, exercise-induced increases in the rate of adult neurogenesis have been shown to be exercise intensity-dependent. Low-intensity forced treadmill running was shown to be a more beneficial form of exercise to increase neurogenesis in the dentate gyrus of the hippocampus, relative to moderate intensity exercise. Moderate forced exercise increased NMDA receptor mRNA levels in the hippocampus, but mild exercise increased both NMDA receptor mRNA and BDNF mRNA (Lou *et al.*, 2008), indicating that exercise-induced increases in neurogenesis involves both NMDA type glutamate receptors and BDNF. Furthermore, 3 weeks of voluntary wheel running increased the rate of neurogenesis and increased the concentration of BDNF in the hippocampus of wild type but not NMDA ε1 knockout mice (Kitamura *et al.*, 2003), indicating that exercise activates NDMA receptors, which in turn enhance BDNF production and stimulate neurogenesis. This evidence, together with the data showing that direct BDNF infusion can increase neurogenesis (Scharfman *et al.*, 2005) and that decreased endogenous BDNF in $BDNF^{+/-}$ knockout mice is associated with decreased neurogenesis (Lee *et al.*, 2002), suggests a role for BDNF in mediating the rate of adult neurogenesis, which may have long-term functional implications (Kempermann *et al.*, 2004).

1.7 Objectives

The main objective of this study was to gain a better understanding of the link between physical activity and cognitive function. The aim of the human research was firstly; to assess the effect of an acute bout of maximal physical exercise on cognitive function, secondly; to investigate the effect of chronic exercise in the form of an aerobic training programme on cognitive function in healthy young sedentary men. Cognitive function was assessed using three different memory tasks which are know to activate different regions of the brain, in order to investigate whether any exercise-related changes in performance were global or specific to particular brain regions. Thirdly, the effect of an acute bout of exercise and the effect of chronic exercise paradigms on serum neurotrophin concentrations were assessed, to examine the role of neurotrophins in any exercise-related changes in cognitive function observed.

In order to investigate the role of neurotrophins in the link between exercise and improved cognition on a cellular and molecular level, a rodent model was used. The aim was to establish the effect of treadmill exercise on recognition memory in the male Wistar rat. Both spatial and non-spatial recognition memory were assessed following the forced exercise protocol, to investigate whether exercise selectively enhances particular types of memory. It is proposed that forced exercise enhances cognitive function through a neurotrophin-mediated mechanism; therefore, the expression of BDNF, NGF, NT-4/5 and IGF-1 protein was analysed in the serum, muscle and specific brain regions including the perirhinal cortex, which is important for novel object recognition memory. An assessment of the effect of the exercise protocol on neurogenesis was also made. Finally, exogenous BDNF and anti-BDNF infusion studies were completed in an effort to confirm the emerging role of BDNF in exercise-induced enhancement in cognitive function.

Chapter 2
Materials and Methods

Chapter 2: Materials & Methods

2.1. Materials

2.1.1. Human blood collection

Cannulas (20G)	B D, Oxford, England.
Posiflow	B D, Oxford, England.
0.9% Sodium Chloride Injection (Sterile)	B|Braun, Dublin, Ireland.
Syringes (sterile, 10ml)	B D, Oxford, England.
Vacutainer serum tubes	B D, Oxford, England.

2.1.2. Animals

Wistar Rats (3 month old males)	Bioresources, TCD.
Laboratory rat diet	RedMills, Kilkenny, Ireland.

2.1.3. Animal cannulation and treatments

Anti-BDNF neutralising antibody	Chemicon, Cork, Ireland.
Cytochrome C	Sigma, Wicklow, Ireland.
Dummy cannula (to fit 2.6mm guide cannula)	Plastics 1, Virginia, USA.
Guide cannulas (2.6mm, 20G)	Plastics 1, Virginia, USA.
Internal cannulas (1mm projection)	Plastics 1, Virginia, USA.
Human recombinant BDNF	R&D Systems Europe, Oxon, UK.
Sheep serum	Santa Cruz, Heidelberg, Germany.
Syringe (10μl)	Hamilton, Bonaduz, Switzerland.

2.1.4. ELISA Kits

Human BDNF ELISA kit	Promega, Madison, USA.
Human BDNF Duoset ELISA kit	R&D Systems Europe, Oxon, UK.
Mouse IGF-1 Duoset ELISA kit	R&D Systems Europe, Oxon, UK.
Rat β-NGF Duoset ELISA kit	R&D Systems Europe, Oxon, UK.
Human NT4 Duoset ELISA kit	R&D Systems Europe, Oxon, UK.
Substrate Solution	R&D Systems Europe, Oxon, UK.

2.1.5. General Laboratory Chemicals

Acetone	Sigma, Wicklow, Ireland.
Acrylamide electrophoresis reagent	Invitrogen, Dublin, Ireland.
Ammonium persulphate	Sigma, Wicklow, Ireland.
Bio-Rad dye reagent concentrate	Bio-Rad, California, USA.
Bovine serum albumin (BSA)	Sigma, Wicklow, Ireland.
5-Bromo-2-deoxyuridine (BrdU)	Sigma, Wicklow, Ireland.
Bromophenol blue	Sigma, Wicklow, Ireland.
Calcium chloride	Lennox, Dublin, Ireland.
DAB Chromogen tablets	Dako, Glostrup, Denmark.
Diethyl Pyrocarbonate (DEPC)	Sigma, Wicklow, Ireland.
Dimethyl sulphoxide	Sigma, Wicklow, Ireland.
Di-Sodium hydrogen orthophosphate (Na_2HPO_4)	Sigma, Wicklow, Ireland.
DL-Dithiothreitol (DTT)	Sigma, Wicklow, Ireland.
Ethanol	Lennox, Dublin, Ireland.
Glucose	Lennox, Dublin, Ireland.
Glycerol	Sigma, Wicklow, Ireland.
Glycine	Sigma, Wicklow, Ireland.
Halothane	BioResources, TCD.
Heparin (Mucous) Injection	Leo Laboratories, Dublin, Ireland.
Hydrochloric acid	Lennox, Dublin, Ireland.
Hydrogen peroxide	Sigma, Wicklow, Ireland.
Leupeptin	Sigma, Wicklow, Ireland.
Magnesium sulphate	Sigma, Wicklow, Ireland.
Magnesium Chloride	Sigma, Wicklow, Ireland.
β-Mercaptoethanol	Sigma, Wicklow, Ireland.
Methanol (MeOH)	Lennox, Dublin, Ireland.
Nitrocellulose membrane	Amersham, Stockholm, Sweden.
N'N' Methylenebisacrylamide	Sigma, Wicklow, Ireland.
Normal Horse serum	Vector, Peterborough, UK.
OCT	Tissue Tek, Zoeterwoude, The Netherlands.
Paraformaldehyde	Sigma, Wicklow, Ireland.
Pepstatin A	Sigma, Wicklow, Ireland.

Phenylmethylsulfonyl fluoride (PMSF)	Sigma, Wicklow, Ireland.
Potassium chloride	Sigma, Wicklow, Ireland.
Potassium dihydrogen orthophosphate (KH_2PO_4)	Sigma, Wicklow, Ireland.
Potassium hydroxide	Sigma, Wicklow, Ireland.
Potassium phosphate	Sigma, Wicklow, Ireland.
2-Propanol	Sigma, Wicklow, Ireland.
Sodium carbonate (Na_2CO_3)	Sigma, Wicklow, Ireland.
Sodium bicarbonate($NaHCO_3$)	Sigma, Wicklow, Ireland.
Sodium chloride	Sigma, Wicklow, Ireland.
Sodium dodecylsulphate (SDS)	Sigma, Wicklow, Ireland.
Sodium hydrogen carbonate	Lennox, Dublin, Ireland.
Sodium hydroxide	Lennox, Dublin, Ireland.
Sodium phosphate (monobasic)	Sigma, Wicklow, Ireland.
Sodium phosphate (dibasic)	Sigma, Wicklow, Ireland.
Sucrose	Sigma, Wicklow, Ireland.
Sulfuric acid	Sigma, Wicklow, Ireland.
Tetramethylbenzidine (TMB)	R&D Systems Europe, Oxon, UK.
Tris-base	Sigma, Wicklow, Ireland.
Tris-HCl	Sigma, Wicklow, Ireland.
Triton X-100	Sigma, Wicklow, Ireland.
Trypan blue	Sigma, Wicklow, Ireland.
Tween-20	Sigma, Wicklow, Ireland.
Vectastain ABC kit	Vector Labs, Peterborough, UK.

2.1.6. General Laboratory Products & Plastics

Cork discs	Tissue Tek, Zoeterwoude, The Netherlands.
Cover glasses (22 x 60mm)	Lennox, Dublin, Ireland.
Hyperfilm	Pierce, Rockford, USA.
Microtest 96-well flat bottomed plates	Sarstedt, Nümbrecht, Germany.
Microtubes	Sarstedt, Nümbrecht, Germany.
96 Microwell Nunc ELISA plates	eBiosciences, Hatfield, UK.
Microscope slides (single frosted)	Lennox, Dublin, Ireland.
Microvette CB300	Sarstedt, Nümbrecht, Germany.

Needles 26G, 3/8"	BD Microlance, Oxford, England.
Parafilm, laboratory roll	Lennox, Dublin, Ireland.
Petri dish, square	Sarstedt, Nümbrecht, Germany.
Pipette tips	Sarstedt, Nümbrecht, Germany.
Pipette tips (Gilson, 10ml)	Anachem, Bedfordshire, UK.
Plastic transfer pipettes	Sarstedt, Nümbrecht, Germany.
Scalpels (disposable)	Swann-Morton, Sheffield, UK.
Standard grade No.1 filter paper	Whatman, England.
Syringes (sterile, 1ml)	BD Plastipak, Oxford, England.
Syringes (sterile, 10ml)	BD Plastipak, Oxford, England.
Syringes (sterile, 50ml)	BD Plastipak, Oxford, England.
Tubes (50ml)	Sarstedt, Nümbrecht, Germany.
Tubes (15ml)	Sarstedt, Nümbrecht, Germany.

2.1.7. Molecular Reagents & materials

Absolute ethanol	Sigma, Wicklow, Ireland.
Agarose	Invitrogen, Dublin, Ireland.
Biosphere filter pipette tips	Sarstedt, Nümbrecht, Germany.
Ethidium Bromide	Sigma, Wicklow, Ireland.
High Capacity cDNA Reverse transcription kit	Applied Biosystems, Warrington, UK.
Loading Dye (6X)	Promega, Madison, WI, USA.
Molecular Grade Water	Sigma, Wicklow, Ireland.
Optical adhesive covers	Applied Biosystems, Warrington, UK.
PCR tubes	Sarstedt, Nümbrecht, Germany.
RNA*later*™	Ambion, Warrington, UK.
RNase away	Invitrogen, Dublin, Ireland.
RNase-free microtubes	Ambion, Warrington, UK.
RNaseZap® wipes	Ambion, Warrington, UK.
Nucleospin RNA II isolation kit, MacheryNagel	Technopath, Limerick, Ireland.
Taqman gene expression assays	Applied Biosystems, Warrington, UK.

Taqman universal PCR master mix	Applied Biosystems Warrington, UK.
10 X TBE buffer	Invitrogen, Dublin, Ireland.
96-well optical reaction plates	Applied Biosystems, Warrington, UK.
μStripPro	Sarstedt, Nümbrecht, Germany.

2.1.8. Western Blotting Reagents and Antibodies

Anti-BrdU Mouse monoclonal antibody	Millipore, Cork, Ireland.
Anti-mouse (Goat) IgG Peroxidase conjugate	Sigma, Wicklow, Ireland.
Anti-phospho-CaMKII rabbit polyclonal antibody	Chemicon, (Millipore) Cork, Ireland.
Anti-rabbit (Goat) IgG HRP Peroxidase conjugate	Sigma, Wicklow, Ireland.
Anti-synapsin I rabbit polyclonal antibody	Chemicon, (Millipore) Cork, Ireland.
Anti-Trk B Rabbit Polyclonal IgG	Upstate Cell Signalling, (Millipore) Cork, Ireland.
ß-Actin Mouse Monoclonal antibody	Sigma, Wicklow, Ireland.
Cl-XPosure Film (5 x 7")	Pierce, Rockford, USA.
ERK2 Mouse Monoclonal IgG	Santa Cruz, Heidelberg, Germany.
Hybond-C Extra Nitrocellulose membrane	Amersham, Buckinghamshire, UK.
pERK Mouse monoclonal antibody	Santa Cruz, Heidelberg, Germany.
Precision Plus Protein Standards (Dual Colour)	Bio-Rad, California, USA.
Propanol	Sigma, Wicklow, Ireland.
ReBlot Plus strong antibody stripping solution	Chemicon, Cork, Ireland
SuperSignal®	Pierce, Rockford, USA.
Standard grade No. 3 filter paper	Whatman, England.
Tetramethyl-diamine (TEMED)	Sigma, Wicklow, Ireland.

2.2. Human Studies

2.2.1. Participants

Participants were recruited through poster advertisements, mainly from the student population of Trinity College Dublin. All were male, sedentary, non-smokers and none were involved in any regular physical training prior to commencement of the study. The Ethical Committee for Research Involving Human Participants, Faculty of Health Sciences, Trinity College Dublin approved the experimental protocol for this study. Exercising participants were required to complete a medical questionnaire, received a routine medical examination and gave written informed consent in accordance with the Declaration of Helsinki.

2.2.2. Experimental Protocol

Participants were allocated to an exercise group (EX) or a sedentary control group (CON) for the acute study (testing session 1). The acute-exercise protocol used was an incremental exercise test to volitional exhaustion (see section 2.2.4). Cognitive tests were completed before and after this acute-exercise bout, in order assess the effect of exhaustive exercise on cognitive function. Blood samples were collected from the EX group throughout the testing session, in order to assess the effect of acute exercise on serum growth factor concentrations (see Figure 2.1, "Testing Session 1").

Two chronic exercise protocols were used to assess the effects of both a 3-week and a 5-week training intervention on cognition and on serum concentrations of BDNF and IGF-1. These aerobic training programmes were identical in all aspects except for duration. Following testing session 1 the EX group was split into the subgroups: C-EX3, C-EX5, A-EX3 & A-EX5. The chronic-exercise subgroups, C-EX3 and C-EX5, completed 3 and 5 weeks of aerobic training respectively. The acute-exercise subgroups, A-EX3 and A-EX5, remained sedentary for the corresponding 3 and 5-week intervals (Figure 2.1). All subgroups repeated the testing session following the appropriate 3- or 5-week interval. The CON group also remained sedentary prior to completion of testing session 2, after either a 3-week or 5-week interval. Results from this group were pooled.

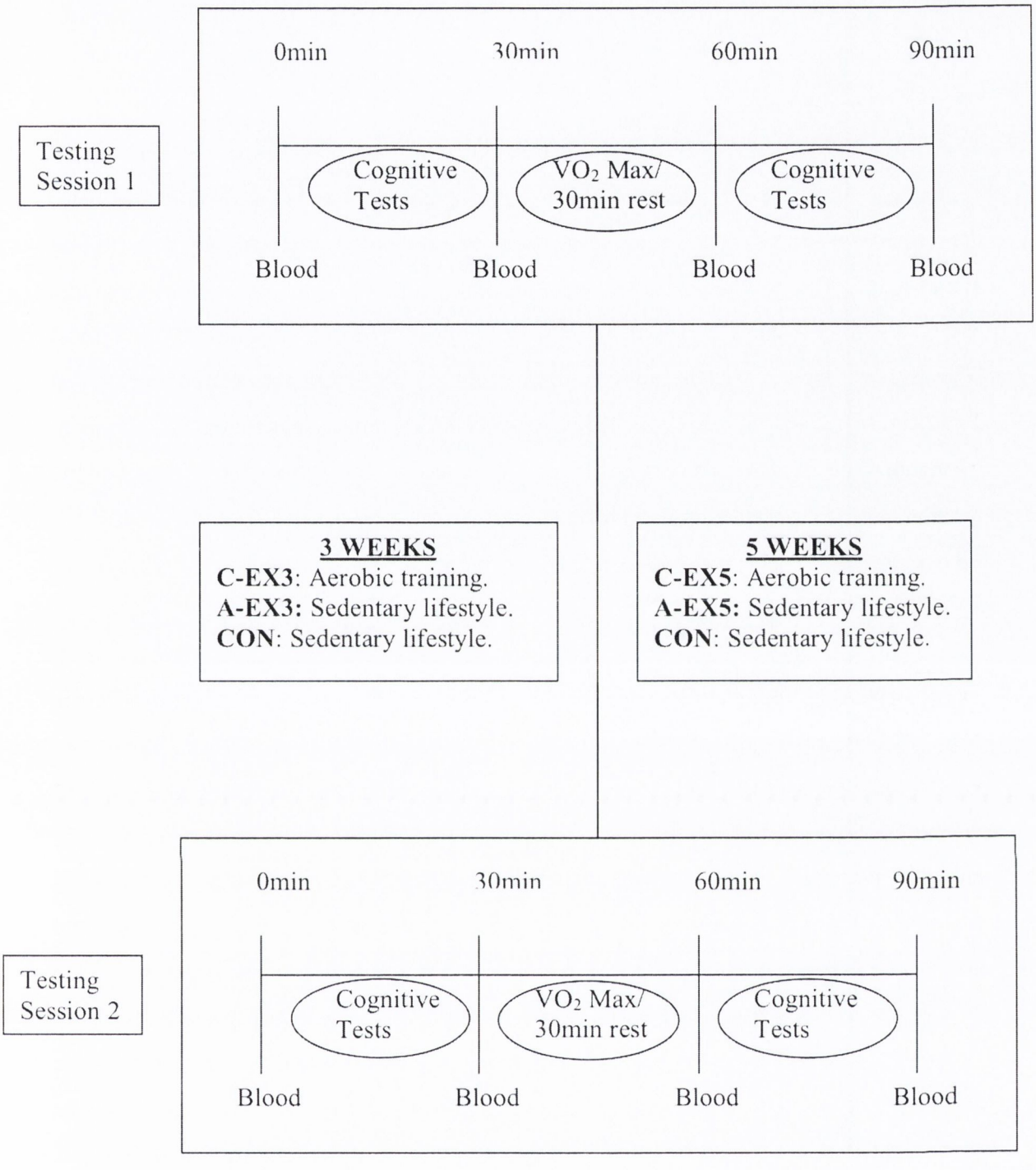


Figure 2.1: Overview of the experimental protocol for the human studies

The acute protocol is illustrated in the 'Testing Session 1' section. A baseline blood sample was taken at 0min, followed by the first set of cognitive tests. Another blood sample was taken at 30min before the VO_2 max test (30min rest for the CON group) was completed. A third, post-acute bout, blood sample was taken at 60min, the second set of cognitive tests was completed and a final blood sample was drawn at 90min. The efficacy of the 2 training programmes was assessed in 'Testing Session 2'. This was completed 3 weeks after testing session 1 by the C-EX3 & A-EX3 subgroups and after a 5-week interval by the C-EX5 & A-EX5 subgroups.

2.2.3. Blood sampling

Blood samples were taken into 6ml plain vaccutainer evacuated specimen tubes, which were free from anti-coagulant. Samples were taken through an in-dwelling catheter placed in the anticubital vein by a qualified phlebotomist. The catheter was flushed with a 0.9% sterile saline solution after each sample, to prevent formation of clots that may have blocked the tube. Four samples were taken per session, at time 0, 30, 60 and 90min (see Figure 2.1). The blood samples were incubated at room temperature for at least 20min to allow clotting and then placed on ice until centrifuged for 20min at 5000rpm. The supernatant was removed and stored at -80°C for later analysis of the serum concentration of IGF-1 and BDNF.

2.2.4. Acute exercise

The acute exercise consisted of an incremental exercise test to volitional exhaustion. It was performed on a stationary cycle ergometer (Lode Excalibur, Groningen, Netherlands) to establish maximal oxygen consumption rate (VO_2 max). The programme consisted of an incrementally increasing workload beginning at 75W. The workload increased by 50W every three minutes until 9min, and subsequently by 25W each minute until volitional exhaustion was reached. The subject wore a facemask throughout the test in order to collect expired air, which was analysed for volume and content using an online system (Metalyser, Cortex Biophysik). Participants wore a heart rate monitor (Polar) throughout. Approximate maximal heart rates were taken as 220 beats per minute (bpm) minus the participant's age (in years). Participants were verbally encouraged to continue cycling until their heart rate was close to the theoretical maximum calculated. This acute exercise bout was completed by the EX group during testing session 1 and by the EX subgroups, A-EX3, A-EX5, C-EX3 & C-EX5 during testing session 2. The CON group rested for a corresponding 30min period during testing session 1 and 2.

2.2.5. Cognitive testing

2.2.5.1. Face-name task

The face-name task is a cognitive function test involving encoding and retrieval processes that recruit the hippocampus and related cortical areas. The E Prime suite of applications was used to run this task (Psychology Software Tools Inc., Pittsburgh, USA). A series of four encoding blocks were interleaved with a distraction task and four recall blocks (see Figure 2.2 B). The encoding block ("Learn") consisted of a series of ten unfamiliar faces, which were paired with names (see Figure 2.2, A). Each of the face-name pairs appeared on the screen for 3500ms with a 500ms blank between stimuli. The subject attempted to commit the face-name associations to memory.

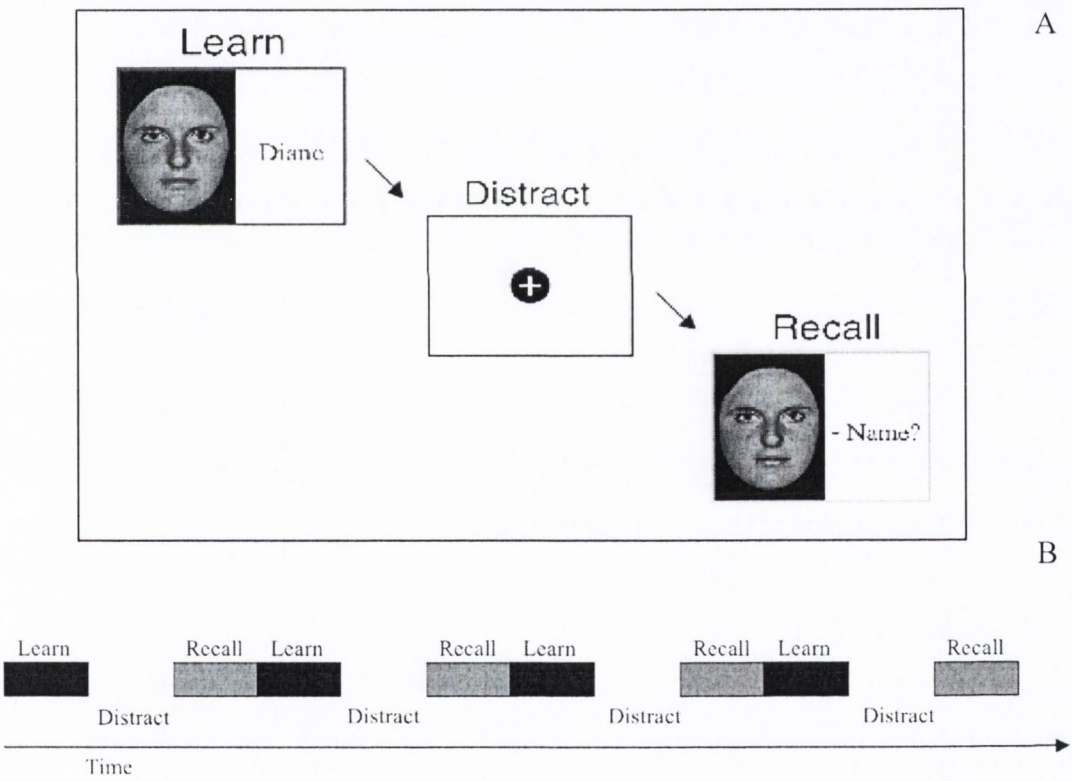


Figure 2.2: Illustration of the face-name pairs task and timeline of the task.

(A) The encoding block consisted of 10 unfamiliar face-name pairs. The subject tried to commit the face-name associations to memory. The recall blocks consisted of a randomised series of the same faces, the subject attempted to verbally recall the correct name. The encoding and recall blocks were separated by a distraction task to prevent rote-rehearsal. **(B)** A timeline showing the running order of the four encoding blocks, interleaved with distraction tasks and four recall blocks.

The recall block consisted of a randomised series of the same faces, where the subject attempted to recall verbally the correct name. The faces appeared on the screen for 3500ms again, with a 50ms interval between faces. Encoding and recall blocks were separated by a distraction task that consisted of a fixation cross on a white screen. A black circle flashed very briefly on screen (for a duration of between 500ms and 3500ms) in place of the cross and the participant was required to press a button on the response pad before it disappeared. This prevented rote rehearsal between encoding and recall blocks. The total duration of each distraction task was 40s. Subjects viewed the same face name combinations four times per task (see timeline in Figure 2.2 B). Four different versions of the task were used, one for each set of cognitive tests.

2.2.5.2. Stroop task

The Stroop word-colour task activates the anterior cingulate cortex. The E Prime suite of applications was used to run this task. The version of the Stroop word-colour task used consisted of the four words: "red", "yellow", "green" and "blue", presented in random order on a white background. The words were presented in one of four font colours (either red, yellow, green or blue). The majority of the stimuli were congruent: for example "blue" presented in a blue font, with random and infrequent incongruent stimuli: for example "green" presented in a yellow font (see Figure 2.3). Subjects were required to inhibit their automatic response to read the word and instead reported the colour the word was presented in. Participants recorded their responses on a response pad with four correspondingly coloured keys.

2.2.5.3. N-back task

The N-back task assesses working memory. The E Prime suite of applications was used to run this task. It was completed only once per testing session (post-acute bout). The 0-back, 1-back and 2-back versions of this task were used. The 0-back task involved a series of numbers (from 1 to 4) presented on a screen, one at a time, in random order. The participant responded to the number stimulus by pressing the numbered button on a response pad corresponding to the number on the screen. The 1-back task involved a similar protocol to the 0-back with one alteration; the number on the screen was no longer the correct response. Instead, the correct response was the number that immediately preceded the number on the screen. Similarly for the 2-back, the correct response for the first two numbers to appear on the screen was no-response. When the

third number appeared, the correct response was the first number that was seen and so on (Figure 2.4).

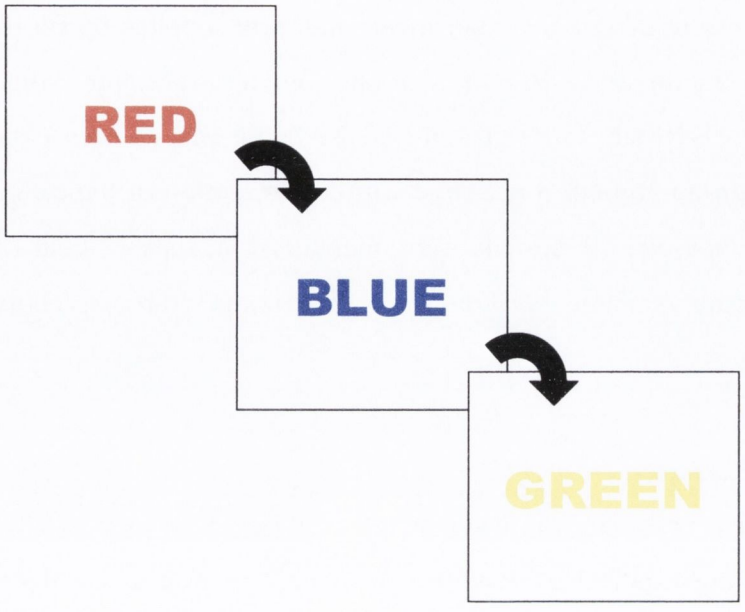


Figure 2.3: Illustration of the Stroop word colour task.

A series of congruent trials: "red" and "blue" above, and an incongruent trial: "green" in yellow font. The subject was required to inhibit the response to read the word and instead pressed the button on the response pad corresponding to the colour of the font.

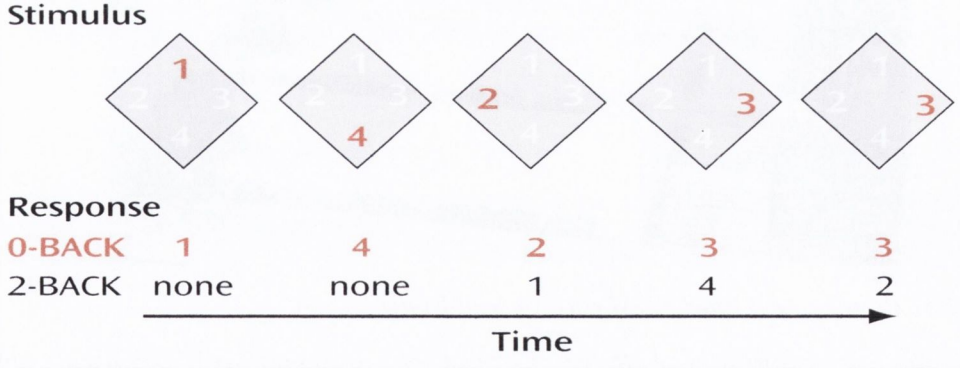


Figure 2.4: Illustration of the N-back 0-back and 2-back task.

One diamond-shaped stimulus was presented every 1.8 seconds. In the 0-back condition, a button corresponding to the number currently displayed was to be pressed. In the 2-back condition, the button corresponding to the number presented two trials before was to be pressed (Meyer-Lindenberg *et al.*, 2001).

2.3. Rodent Studies

2.3.1. Animals

Male Wistar rats were used in all experiments and were supplied by BioResources Unit, Trinity College Dublin, from an inbred strain. Animals were approximately 3 months old and weighed between 250 and 350g at the beginning of each experiment. They were generally group-housed, 3 per cage with the exception of the cannulated animals that were singly housed. All animals were maintained under a 12-hour light-dark cycle with food and water available *ad libitum*. Ambient temperature was controlled between 20°C and 23°C.

2.3.2. Exercise

A forced exercise protocol was used in all exercise experiments. The exercise was performed on motorised rodent treadmills (Figure 2.5). The treadmills were equipped with wire loops at one end of the moving belt, through which a mild electric shock was delivered. These shocks provided motivation for the animals to run constantly, allowing for a controlled duration and intensity of exercise. The animals were monitored constantly to ensure they were running and to assess them for signs of stress.

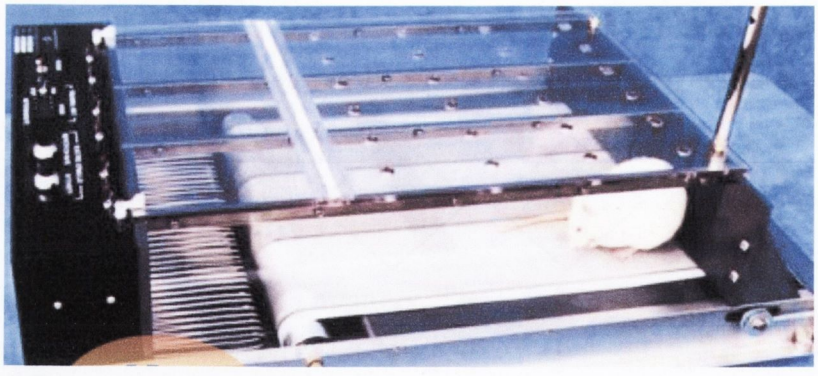


Figure 2.5: Picture of a motorised rodent treadmill

Exer 3/6 treadmill, Columbus instruments, equipped with electrical stimulus system and 3 individual lanes.

2.3.3. Behaviour

Two types of object recognition task were used: object displacement (OD), a spatial variant of the object recognition task, and object substitution (OS), the classic novel-object recognition task. Prior to all tasks, animals were familiarised to the arena (a black circular open field, diameter, 0.9m; height, 0.48m, Figure 2.6). All of the objects used were constructed from Lego blocks and were fixed to the floor of the arena (Figure 2.7). For both OS and OD tasks, the training phase consisted of three 5min trials, with an inter-trial rest period of 5min. The animals were put in a holding cage for the 5min rest period in order to minimise the disturbance to the other animals in the home cage. Objects were cleaned thoroughly between trials to eliminate any olfactory cues. For the testing phase, animals were reintroduced to the arena 6hrs or 24hrs post-training for one 5min trial. The time spent exploring each object was recorded using stopwatches. The rats were considered to be exploring only when they were touching the objects with their paws or noses and were actively investigating, not just sitting on the object. Measurement of the time spent actively exploring each object was recorded and expressed as a percentage of total exploration time in seconds.

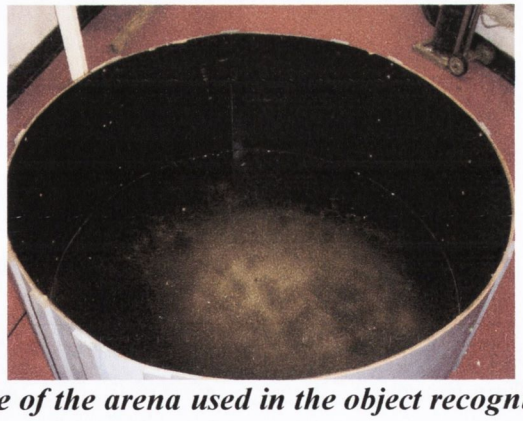


Figure 2.6: Picture of the arena used in the object recognition tasks

The arena consists of a black circular open field (diameter, 0.9m; height, 0.48m), placed in a dimly lit room.

For the object displacement task three objects were positioned in the open field and a spatial cue was fixed to the wall. For the testing phase, one of the three objects was displaced. Stopwatches were used to record the total time spent exploring the three objects and the amount of time spent exploring the displaced object. For the object

substitution task two or three objects were positioned in the arena (Figure 2.7, a) and for the testing phase, one object was substituted for a novel object (Figure 2.7, b). Discrimination between familiar and novel objects is measured by time spent exploring and not on the basis of the rat's first choice. A three-object variant of this task was used to increase the difficulty of the task. Time spent exploring each of the objects was recorded and expressed as a percentage of the total exploration time.

(a) (b)

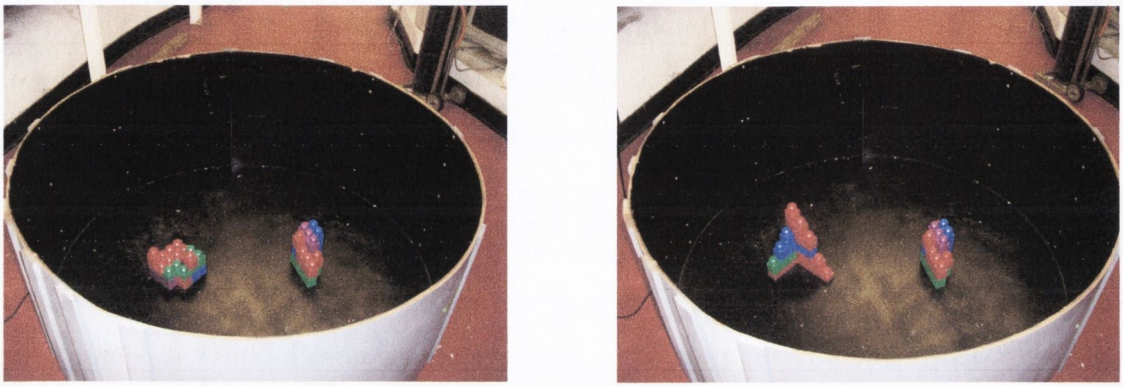


Figure 2.7: Pictures of the arena containing example objects

(a) Objects A and B (constructed from lego) used for training in the object substitution task. (b) Objects A and C used for testing in the object substitution task.

2.3.4. Blood sampling

Animals were anaesthetised by halothane inhalation. Anaesthesia was confirmed by the absence of the pedal reflex. The hind limb was shaved with an electric razor and an elastic band and surgical clamp were used to occlude venous flow. A heparinised, 26-gauge needle was inserted into the saphenous vein, which became prominent upon venous occlusion. Once blood flow was initiated through the needle, samples were collected into microvettes. Both plain and lithium-heparin coated microvettes were used for serum and plasma collection respectively. The plain microvettes were incubated at room temperature for 20min to allow clotting and centrifuged for 20min at 10,000rpm to obtain serum. The supernatant was removed and stored at -80°C for later analysis. The lithium-heparin coated microvettes for plasma analysis were similarly spun down and the supernatant stored at -80°C.

2.3.5. Preparation of tissue

Animals were stunned and sacrificed by decapitation. The brains were quickly removed and placed on ice and the perirhinal cortices, hippocampi and dentate gyri were dissected free. The entire brain dissection took approximately 2min. Tissue from each of the three brain regions dissected was taken as slices (see section 2.3.5.1) or taken for RNA extraction (see section 2.3.5.4*)*. Samples of the muscle and liver were also taken for RNA extraction.

2.3.5.1. Preparation of slices for freezing

The freshly dissected tissue of the dentate gyrus, hippocampus and perirhinal cortex was sliced bi-directionally to a thickness of 350μm using a McIlwain tissue chopper and rinsed in ice-cold 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 then were rinsed twice more with Krebs $CaCl_2$. Finally, slices were rinsed with ice-cold Krebs $CaCl_2$ containing DMSO (final concentration: 10%) and were stored in this solution at -20°C until required for analysis.

2.3.5.2. Preparation of homogenate from slices

Tissue slices from the dentate gyrus, hippocampus and perirhinal cortex were defrosted on ice, washed in ice-cold Krebs $CaCl_2$ and then homogenised (x 15 strokes) in Krebs $CaCl_2$ (500μl) using a 1ml glass homogeniser. The homogenate was aliquotted into microtubes (100μl/aliquot) and stored at -20°C until required.

2.3.5.3. Preparation of samples for gel electrophoresis

Aliquots of dentate gyrus, hippocampus and perirhinal cortex homogenates were thawed on ice and equalised for protein concentration (Bradford, 1976). The samples 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)) and boiled for 5 min in a heating block.

2.3.5.4. Preparation of samples for RNA extraction

Samples of dentate gyrus, hippocampus and perirhinal cortex from each rat were placed in RNase-free tubes containing RNA*later*™ and stored for 4 weeks at 4°C. All samples were then removed from the RNA*later*™ solution, transferred to fresh RNase-free tubes and frozen at -80°C until RNA extraction was performed.

2.3.5.5. Preparation of tissue for PCR

RNA extraction was performed using a Nucleospin® RNA II isolation kit (Technopath). Samples were removed from the -80°C freezer, placed in RA1 buffer (350µl) containing β–mercaptoethanol (3.5µl) and homogenised using a polytron tissue disrupter (Kinetatica). The sample homogenate was added to Nucleospin® filter units, as per the manufacturer's user manual and filtered by centrifugation (13,000rpm, 1min). Ethanol (350µl, 70% v/v) was added to each sample lysate and mixed by pipetting vigorously, approximately 5 times. Each sample mix was placed in Nucleospin® RNA II columns and centrifuged (13,000rpm, 30sec), allowing the RNA to bind to the silica membrane. Following centrifugation, the column was placed in a new collecting tube and membrane desalting buffer (350µl) was added to the column. The column was again centrifuged (13,000rpm, 1min). rDNase (95µl, 1:10 dilution in DNase Reaction Buffer) was pipetted directly into the centre of the silica column, to digest DNA. Samples were incubated with the DNase solution for 15min at room temperature. RA2 buffer (200µl) was then added to the column and centrifuged (13,000rpm, 30sec). The column was placed in a new collecting tube and RA3 buffer (600µl, 1:2 dilution in ethanol) was added to the column and centrifuged (13,000rpm, 30sec). The flow-through was discarded and the column was washed a second time with RA3 buffer (250µl), and then centrifuged again (13,000rpm, 2min). Finally, the column was placed in a fresh RNase–free microtube and RNase–free H_2O (60µl) was added to the column and the RNA was eluted during centrifugation (13,000rpm, 1min). The eluted RNA was stored at -80°C for qualification, quantification and reverse transcription (see below).

In order to check the integrity of the extracted RNA samples the, RNA was separated on a 1% agarose gel. Agarose (1.3g) was dissolved in TBE (130ml; 1.0M Tris, 0.9M Boric acid, 0.01M EDTA) by heating in the microwave. Once the solution had cooled sufficiently, ethidium bromide (1.3µl) was added with due caution and swirled to mix.

The agarose solution was then poured into a sealed agarose gel tray containing a comb and allowed to solidify for approximately 30min. The tray was transferred to a gel tank and flooded with 1X TBE running buffer. Each RNA sample (3µl) was mixed with DEPC H_2O (2µl) and loading dye (1µl) and 4µl of this mixture loaded onto a gel. The RNA was separated with a 90V current, for between 30min and 1.5hrs. The power supply was switched off and disconnected when the yellow dye reached the end of the gel. The gel was then taken to a UV transilluminator and the RNA was visualised. Only extracted RNA that demonstrated visible 28S and 18S ribosomal RNA bands were used for quantification and reverse transcription, as this indicated that the RNA had not been degraded during the extraction process.

The optical density of the extracted RNA was measured using a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific) to determine RNA concentration and purity. The concentration of RNA can be measured due to its ability to absorb light at 260nm. An optical density reading of 1.0 at 260nm is equivalent to an RNA concentration of 40µg/ml. Therefore the RNA concentration of a sample of extracted RNA can be quantified using the following equation:

$$RNA = OD_{260} \text{ X dilution factor X } 40\mu g/ml.$$

The absorbance is also measured by the spectrophotometer at 280nm. This reading can be used to ascertain the purity of the RNA. A ratio of optical $density_{260}$: optical $density_{280}$ of approx. 1.8-2.0 is indicative of pure RNA. RNA concentrations were equalised with RNase-free H_2O in order that equal concentrations of RNA could be used as a template for cDNA transcription.

A high capacity cDNA archive kit (Applied Biosystems) was used to reverse transcribe the equalised RNA samples. Equalised RNA (20µl) was mixed in a PCR mini-tube with an equal volume of 2X master mix (containing: 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 a thermal cycler (PTC-200 Peltier Thermal Cycler, Biosciences, Dublin, Ireland) and incubated at 25°C for 10min, then 37°C for 2hrs. The cDNA was stored frozen at -20°C for later real-time polymerase chain reaction (PCR) analysis.

2.3.6. Protein quantitation using Bradford assay

Protein concentrations were quantified using the Bradford method (1976). Samples were analysed in triplicate (5µl/well) on 96-well plate (microtest plate; Sarstedt, Ireland). A 7-point standard curve was prepared from a stock solution bovine serum albumin (BSA, 1000µg/ml, diluted in deionised water) ranging from 1000µg/ml to 15.625µg/ml. A blank of deionised water was also included. Bio-Rad reagent (195µl, 1:5 dilution in ddH_2O) was added to samples and standards. The absorbance was assessed at 620nm using a 96-well plate reader (Sunrise basic, Tecan, Austria). Regression analysis was used to calculate protein concentrations and values were expressed as mg protein per ml.

2.3.7. Enzyme linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISA) were used to examine the concentration of BDNF, IGF-1, β-NGF, & NT-4/5 protein in brain tissue homogenate. Commercially available ELISA kits were used; human BDNF, mouse IGF-1, rat β-NGF and human NT-4 DuoSet ELISA Development systems kits (R&D Systems Europe, Oxon, United Kingdom). According to the manufacturer, the human BDNF kit, mouse IGF-1 kit and the human NT-4 kit are 100% cross-reactive with rat BDNF, IGF-1 and NT-4 respectively. BDNF and IGF-1 protein concentrations were also assessed in serum, plasma and muscle and liver tissue homogenates

For each ELISA, a 96-well plate was coated with capture antibody (see table 2.1, diluted in PBS, 50µl/well) and incubated overnight at room temperature. The plate was then washed with wash buffer (0.05% Tween®20 in PBS) using an automated plate washer (Columbus Plus, Tecan, Austria) and blocked with a reagent diluent (1% BSA in PBS, 150µl/well), for 1 hour at room temperature. After washing, samples and standards were added (50µl/well) and incubated for 2 hours at room temperature. The plates were washed and incubated in detection antibody (see table 2.1, diluted in reagent diluent, 50µl/well) for 2 hours at room temperature, then washed and reacted with Streptavidin-HRP (1:200 dilution in reagent diluent; 50µl/well) for 20min. The plates were washed and substrate solution was added (50µl/well) and incubated in the dark for a minimum of 20min. H_2SO_4 was used to stop the reaction (50µl/well) once colour development had occurred. The absorbances of samples and standards were read

at 450nm on a plate reader (Sunrise basic, Tecan, Austria). Standard curves were constructed for each plate and concentrations of BDNF, IGF-1, NGF and NT-4/5 in the samples were extrapolated from these curves.

ELISA	Capture Antibody	Standard (top standard conc.)	Detection Antibody
Human BDNF	Mouse anti-human BDNF ($2\mu g.ml^{-1}$)	Recombinant human BDNF ($1500pg.ml^{-1}$)	Biotinylated mouse anti-human BDNF ($25ng.ml^{-1}$)
Mouse IGF-1	Hamster anti-mouse IGF-1 ($4\mu g.ml^{-1}$)	Recombinant mouse IGF-1 ($2000pg.ml^{-1}$)	Biotinylated goat anti-mouse IGF-1 ($80ng.ml^{-1}$)
Rat β-NGF	Goat anti-rat β-NGF ($0.4\mu g.ml^{-1}$)	Recombinant rat β-NGF ($1000pg.ml^{-1}$)	Biotinylated goat anti-rat β-NGF ($100ng.ml^{-1}$)
Human NT-4	Mouse anti-human NT-4 ($1.0\mu g.ml^{-1}$)	Recombinant human NT-4 ($2000pg.ml^{-1}$)	Biotinylated mouse anti-human NT-4 ($100ng.ml^{-1}$)

Table 2.1: ELISA antibodies and concentrations

The human BDNF, mouse IGF-1, Rat β-NGF and human NT-4 Duoset ELISA development kits were manufactured by R&D Systems Europe, Oxon, United Kingdom.

2.3.8. Gel electrophoresis

Acrylamide gels, 7.5% or 10% 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. Samples prepared in sample buffer (10µl), or pre-stained molecular weight markers (5µl; BioRad) were loaded into the wells and run at 30mA for approximately 40mins.

2.3.9. Western Immunoblotting

The gel was rinsed 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 grade), were cut to the size of the gel and soaked in transfer buffer for 2-3min. A sandwich was made of

these with the nitrocellulose placed on top of one sheet of filter paper, followed by the gel and finally the second sheet of filter paper. The sandwich was placed on the anode of the semi-dry blotter (Apollo Instruments, Alpha Technologies, Dublin, Ireland) that had been moistened with transfer buffer and any air bubbles were removed. The lid, containing the cathode, was placed firmly on top and the transfer was carried out at 225mA for 80min.

Once the transfer was complete, the blots were blocked for non-specific binding overnight at 4°C or for 2hrs at room temperature with a solution of TBS-T (10ml) containing BSA (5% (w/v)) and probed with an antibody raised against the protein of interest. 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 film (Hyperfilm, Amersham, UK) and developed using a Fuji Processor.

2.3.10. Densitometric analysis

All protein bands were quantitated by densitometric analysis using the Gel Doc It Imaging System (UVP, Medical Supply Company, Ireland) in conjunction with LabWorks (Lablogics Inc, Mission Viejo, California, USA).

2.3.11. Polymerase chain reaction (PCR)

Gene expression of targets (see Table 2.2) was assessed using Taqman gene expression assays containing specific target primers, and FAM-labelled MGB target probes. β–actin gene expression was used to normalise gene expression between samples, and was quantified using a β–actin endogenous control gene expression assay containing specific primers, and a VIC-labelled MGB probe for rat β–actin.

For multi-target (multiplex) Q-PCR, cDNA (see section 2.3.5.5) was diluted 1:4 with RNase-free water 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). Electronic pipettes (EDP3 20-200µl, 2-20µl, and 10-100µl) were used to ensure pipetting accuracy.

Gene Name	Assay Number	NCBI Gene Reference*
BDNF	Rn00560868_m1	-------
NT4/5	Rn01645205_m1	NM_013184.2
β-NGF	Rn01533872_m1	-------
Trk B	Rn00820626_m1	NM_012731.1
IGF-1	Rn00710306_m1	NM_178866.4 NM_001082478.1 NM_001082479.1 NM_001082477.2
Ki67	Rn01451466_m1	XM_225460.4
VEGF	Rn00582935_m1	NM_0031836.2 NM_00111033.1 NM_001110334.1

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

Samples were placed in a real time PCR thermocycler (Applied Biosystems 7300) using the following successive steps; 95°C for 10 minutes, 95°C for 15 seconds followed by 1 minute at 60°C. The second step was repeated 40 times, and fluorescence read during the annealing and extension phase (60°C) for the duration of the programme.

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, which 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 specificity 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).

The ΔΔCT method of RT_PCR analysis (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 an average of normal/control samples 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.

Chapter 3
Human Studies

Chapter 3: Human Studies

3.1. Introduction

The benefits of regular physical activity to the cardiovascular and respiratory systems are well known. In addition, physically active older people have a decreased risk of Alzheimer's disease (AD) and other forms of dementia relative to their sedentary counterparts (Larson *et al.*, 2006). Moreover, exercise training has been shown to improve both physical fitness and cognitive function in people diagnosed with dementia and related cognitive impairments (Heyn *et al.*, 2004). Unlike the physical benefits of exercise, these associated cognitive enhancements are not well understood. In a review of the literature on the effect of acute exercise on cognition in humans, 60 minutes or less of sub-maximal exercise was reported to facilitate aspects of information processing (Tomporowski, 2003). In agreement with this, improvements in cognition reportedly occurred immediately post-exercise, in trained subjects who exercise for more than 20 minutes. Conversely, fatigue symptoms, coinciding with impairment in information processing speed were reported with exercise bouts lasting more than an hour (Grego *et al.*, 2004).

The assessment of physical activity as a non-pharmacological cognitive enhancer is particularly relevant considering current demographic trends towards an increasingly elderly population. The result of these demographic changes is that the problem of age-related cognitive decline is becoming increasingly prevalent in our society (Mount & Downton, 2006). Physical exercise may prove to be a simple means of ameliorating this problem. A role for the neurotrophins in the link between exercise and cognitive function has been proposed. Neurotrophins are secreted peptides that promote the growth and differentiation of developing neurons and support phenotypic maintenance and survival of adult mature neurons. It has been suggested that alterations in neurotrophin concentrations contribute to the characteristic neurodegeneration seen in AD and Parkinson's disease among others (Siegel & Chauhan, 2000). Diminished regulated secretion of BDNF reportedly results in impaired episodic memory in humans (Hariri *et al.*, 2003). Furthermore, evidence suggests that acute moderate exercise can induce transient increases in serum BDNF concentrations in humans (Gold *et al.*,

2003), although the effect of exercise on concentrations of neurotrophins in the brain of humans is as yet unknown.

The aim of this study was to investigate the effect of physical exercise on cognitive function and to assess the role of neurotrophins in this system. The effects of both acute and chronic exercise on serum BDNF and IGF-1 concentrations were investigated and the impact of these exercise protocols on cognitive function was assessed in young sedentary men.

3.2. Methods

An undergraduate student from the Physiology Department, Trinity College Dublin, Carole Foley assisted with participant recruitment and data collection, in particular for the 5-week chronic exercise group, while completing her final year project in our laboratory.

3.2.1. Participants

The Ethical Committee for Research Involving Human Participants, Faculty of Health Sciences, Trinity College Dublin approved the experimental protocol for this study. Participants were recruited through poster advertisements, mainly from the student population of Trinity College Dublin. 47 healthy male students volunteered to participate (age, height, weight: 22 ± 2.01yrs, 180 ± 1.23cm, 82 ± 2.03kg respectively, mean ± SEM). All were sedentary, that is, not involved in any regular physical training prior to commencement of the study. Exercising participants completed a medical questionnaire (see appendix), received a routine medical examination and gave written informed consent (see appendix) in accordance with the Declaration of Helsinki (see appendix). Exclusion criteria included any contraindications to intense exercise discovered at the medical, intake of prescription medication, history of neurological or medical problems, pre-existing injuries, smoking and intake of recreational drugs. All subjects were required to refrain from consumption of stimulants (i.e. caffeine and nicotine) for 12 hours prior to attendance at the lab and to fast for 2 hours prior to testing.

3.2.2. Experimental Protocol

Initially, participants were allocated to either an exercise group (EX, n=32), or a control group (CON, n=15). For testing session 1 (see Figure 3.1), all participants performed a set of cognitive tests, including the face-name matching task and the Stroop task. Following this, the EX group completed a VO_2 max test, which served as the acute-exercise bout, while the acute bout for CON participants was a 30min rest. A second set of cognitive tests, which included another version of the face-name matching task, the Stroop task and the N-back task was performed post-acute bout. Blood samples were taken from the EX group throughout this testing session, to assess the effect of acute exercise on concentrations of circulating BDNF and IGF-1 (see section 3.2.3).

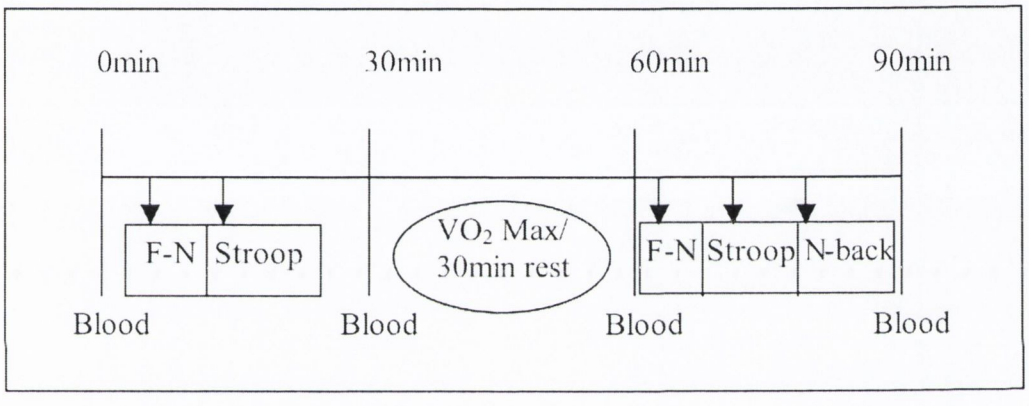


Figure 3.1: Timeline of testing sessions 1 & 2.

A baseline blood sample was taken at 0min, followed by the first set of cognitive tests. Another blood sample was taken at 30min before the VO_2 max test was completed (30min rest period for CON). A third, post-acute exercise, blood sample was taken at 60min, the second set of cognitive tests was completed and a final blood sample was drawn at 90min.

After testing session 1, in order to analyse the effect of chronic exercise on cognition and circulating concentrations of BDNF and IGF-1, the EX group was further divided into subgroups. Two training protocols were used, which were identical in all aspects except for duration. The effects of both 3 and 5 weeks of chronic exercise were assessed by repeating the testing session after the aerobic training interventions (see Figure 3.2). The EX group was divided into 2 chronic exercise groups; C-EX3 (n=9) and C-EX5 (n=9), and 2 acute exercise control groups; A-EX3 (n=5) and A-EX5 (n=9). C-EX3 completed 3 weeks of aerobic training and C-EX5 completed 5 weeks of

aerobic training (see section 3.2.6), prior to completion of testing session 2. A-EX3 and A-EX5 completed testing session 2 after 3-week and 5-week intervals respectively, but remained sedentary during the chronic phase. The CON group remained completely sedentary during the chronic phase, and completed testing session 2 after either 3 weeks (n=7) or 5 weeks (n=8) interval.

3.2.3. Blood sampling

Blood samples were taken through an in-dwelling catheter placed in the anticubital vein as described previously (see section 2.3.4). Four samples were taken per session, at time 0, 30, 60 and 90min (see Figure 3.1). The blood samples were allow to clot prior to centrifugation and the supernatant was removed and stored at -80°C for later analysis of the serum concentration of IGF-1 and BDNF.

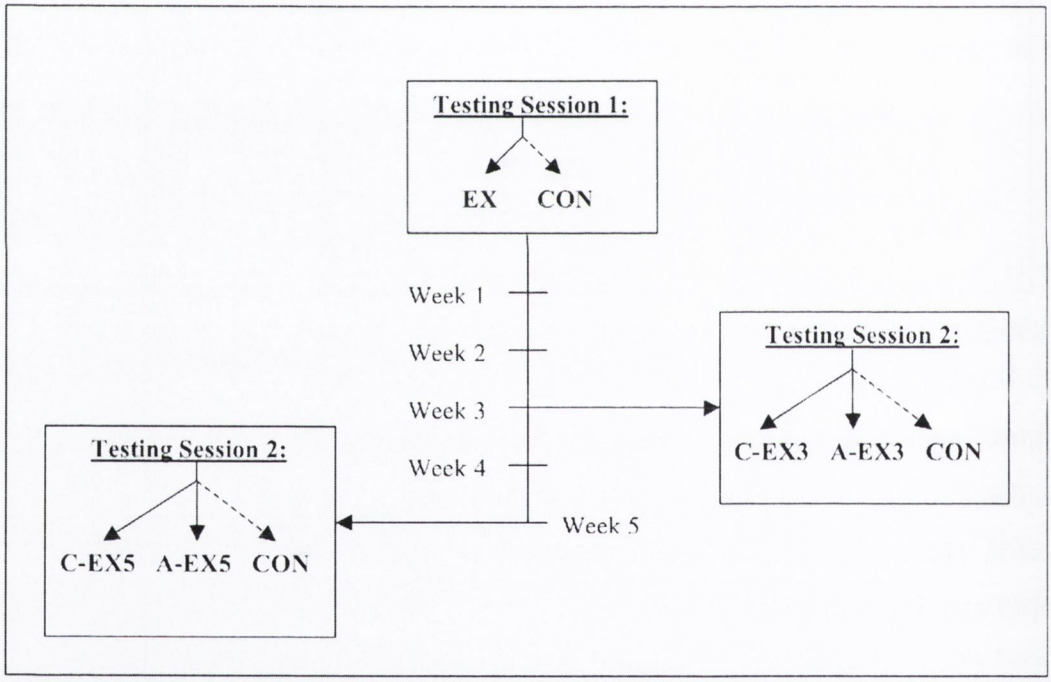


Figure 3.2: Overview of experimental protocol and groups.

The effect of acute exercise was assessed during testing session 1. EX completed an acute exercise bout, while CON rested for a corresponding 30min period. The effect of both 3 and 5 weeks chronic exercise was assessed by subdividing the EX group. The subgroups; C-EX3 and C-EX5 completed 3 and 5 weeks of chronic-exercise respectively, prior to testing session 2. The acute exercise control groups; A-EX3 and A-EX5 remained sedentary for the corresponding 3-week or 5-week chronic phase. The CON group completed testing session 2 after a 3 or 5-week chronic phase and remained sedentary throughout.

3.2.4. Cognitive testing

The face-name task is a cognitive function test involving encoding and retrieval processes that recruit the hippocampus and associated cortical areas of the medial temporal lobe (see section 2.2.5.1). The encoding block consisted of a series of ten unfamiliar faces, which were paired with names. The participant attempted to commit the face-name associations to memory. The recall block consisted of a randomised series of the same faces, where the subject attempted to recall verbally the correct name. Subjects viewed the same face name combinations four times per task (giving a maximum possible score of 40). Four different versions of the task were used, one for each set of cognitive tests.

The Stroop word-colour task activates the anterior cingulate cortex (see section 2.2.5.2) and was tested in this study in order to determine if the effect of exercise on cognitive function was global or specific to particular brain regions (i.e the medial temporal lobe). Participants were required to inhibit their automatic response to read the word stimuli presented, and instead reported the colour of the font the word was presented in. Results are presented as percentage response accuracy.

The N-back task assesses working memory. It was completed only once per testing session (see Figure 3.1). The 0-back, 1-back and 2-back versions of this task were used (see section 2.2.5.3). The participants responded to the number stimuli by pressing a numbered button on a response pad corresponding to the number on the screen or the number previously on the screen, depending on the trial. Results are presented as percentage response accuracy.

3.2.5. Acute exercise

The acute exercise consisted of an incremental exercise test to volitional exhaustion, performed on a stationary cycle ergometer (see section 2.2.4), to establish maximal oxygen consumption rate (VO_2 max). This acute exercise bout was completed by the EX group during testing session 1 and by the EX subgroups, A-EX3, A-EX5, C-EX3 & C-EX5 during testing session 2. The CON group rested for a corresponding 30min period, during testing session 1 and 2.

3.2.6. Aerobic Training

The chronic exercise groups; C-EX3 and C-EX5 attended the lab three times per week for 3 weeks and 5 weeks respectively. Aerobic training was performed on stationary cycle ergometers (Monark) for between 30min and 60min per session. The workload and duration of the exercise were increased gradually to maintain the workload at approximately 60% of V_{O_2} max, over the 3 or 5 weeks. Heart rate was monitored at each session in order to ensure the training was sub-maximal and progressive.

3.2.7. Analysis of BDNF & IGF-1 in Serum

Blood samples were not taken from the CON group due to the limited availability of medical support and the reluctance of some participants, hence no data is included for this group in the serum analysis. An enzyme-linked immunosorbent assay (ELISA) was used to examine the concentration of BDNF and IGF-1 in the serum using commercially available kits. For the BDNF analysis, BDNF $E_{max}^{®}$ Immunoassay system (Promega Corporation, Madison, WI, USA) was used. For the IGF-1 analysis, Human IGF-1 DuoSet ELISA Development kit (R&D Systems Europe, Oxon, United Kingdom) was used.

For the BDNF ELISA, 96-well plates were coated with anti-BDNF monoclonal antibody (1:1000 dilution in carbonate coating buffer; 50µl/well) and incubated overnight at 4°C. The plates were washed with TBST wash buffer using an automated plate washer (Columbus Plus, Tecan, Austria) and blocked with block and sample buffer (1:5 dilution, 5X solution provided with the kit; 100µl/well), for an hour at room temperature. The plates were washed again with TBST and samples and standards were added (50µl/well) and incubated for 2 hours at room temperature on an automated plate shaker. After a further five washes with TBST, anti-Human BDNF polyclonal antibody was added (1:500 dilution in 1X block buffer; 50µl/well) and incubated for 2 hours at room temperature. The plate was rinsed five times with TBST; subsequently anti-IgY HRP conjugate (1:200 dilution in 1X block buffer; 50µl/well) was added to the plates and incubated for 1 hour at room temperature on a plate shaker. After a final wash with TBST, TMB One solution (50ul/well) was added and incubated for 30mins on a plate shaker. The reaction was stopped with 1N HCL (50µl/well) and the absorbances of samples and standards were read on a plate reader at 450nm.

Similarly for the IGF-1 ELISA, 96-well plates were coated with capture antibody (mouse anti-human IGF-1, 1:180 dilution in PBS; 80μl/well) and incubated overnight at room temperature. The plates were then washed and blocked with a block buffer, (5% Tween 20, 5% Sucrose in PBS). Samples and standards were added (50μl/well) and incubated for 2 hours at room temperature. The plates were washed and incubated in detection antibody (biotinylated goat anti-human IGF-1, 1:180 dilution in reagent diluent; 80μl/well) for 2 hours at room temperature, then reacted with Streptavidin-HRP (1:200 dilution in reagent diluent; 80μl/well) for 20min. H_2SO_4 was used to stop the reaction (50μl/well). The absorbances of samples and standards were read at 450nm on a plate reader (Sunrise basic, Tecan, Austria). Standard curves were constructed for each plate and concentrations of BDNF and IGF-1 in the samples were extrapolated from these curves.

3.2.8. Statistical Analysis

All statistical analyses were performed using Graphpad Prism 5 for Mac OSX. Data are expressed as mean ± standard error of the mean (SEM). All data were examined for outliers, which were excluded if they were at least two standard deviations outside the mean. For the analysis of the face-name task and the Stroop task, two-way repeated measures analysis of variance (ANOVA) were used, to assess both the effect of trial (the repeated measure) and the effect of group. Where a significant difference occurred, Bonferroni *post hoc* analyses were performed.

The N-back task was completed by participants only once per testing session. Hence, unpaired Student's *t* tests and one-way ANOVA with *post hoc* Newman-Keuls were used to compare results from the 2 groups in the acute analysis and 3 groups in the chronic analysis, respectively.

The CON group completed testing session 2 after a 3-week (n=7) or 5-week (n=8) interval. Results for the 3-week and 5-week subsets of this group were compared for all cognitive function tasks and found to be not different in all cases. That is, re-testing in all three cognitive function tasks was not affected by a difference in the duration of the sedentary chronic phase between testing sessions. Therefore the CON group (n=15) was not subdivided for the chronic analysis (section 3.3.2).

For the serum analysis, no blood samples were taken from the CON group, hence one-way repeated measures ANOVA with *post hoc* Newman-Keuls were used to analyse serum BDNF changes over time for testing session 1. Two-way repeated measures ANOVA with *post hoc* Bonferroni were used to analyse BDNF concentrations for testing session 2, to assess both the effect of time (the repeated measure) and group.

3.3. Results

3.3.1. Acute exercise

3.3.1.1.Cognitive function

Acute exercise induced an enhancement in face-name task performance (Figure 3.3). There was a significant effect of trial ($P<0.0001$, $F_{(1,41)}=19.42$) indicating that the number of face-name pairs recalled post-acute bout was greater that pre-acute bout. There was also a significant effect of group ($P=0.0219$, $F_{(1,41)}=5.681$) indicating a difference in performance between the groups. *Post hoc* analysis revealed that while CON scores increased, (pre-acute bout: 12.15 ± 0.87 pairs recalled, post-acute bout: 17.08 ± 1.90 pairs recalled, $P<0.05$), EX improved in the performance of the task to a greater extent, (pre-acute exercise: 16.03 ± 1.05 pairs recalled, post-acute exercise: 21.20 ± 1.28 pairs recalled, $P<0.001$). This suggests that although familiarization with the task may have resulted in an improved score, the acute exercise intervention resulted in an enhancement in the performance of this task. Results are expressed as means ± SEM of the total number of pairs recalled across the 4 recall blocks, hence 40 was the maximum score possible. The statistical analyses used were two-way repeated measures ANOVA and *post hoc* Bonferroni (CON n=13, EX n=30). There was a significant effect of matching ($P=0.0080$).

Acute exercise did not alter performance of the Stroop word-colour task, in either congruent trials (Figure 3.4, A) or incongruent trials (Figure 3.4, B). There was an overall effect of trial ($P=0.0133$, $F_{(1,40)}=6.714$) for the congruent Stroop, however, *post hoc* analysis revealed no within-group differences. There was also an overall effect of trial for the incongruent Stroop ($P=0.0124$, $F_{(1,40)}=6.813$), suggesting that familiarization with the task may enhance response accuracy, and a significant

interaction (P=0.0462, $F_{(1,40)}$=4.233) indicating a difference in the response between groups. *Post hoc* analysis revealed a significant increase in CON, (pre-acute bout: 97.25 ± 0.65, post-acute bout: 98.61 ± 0.31, P<0.05), indicating that familiarization with the Stroop task resulted in enhanced performance in the incongruent trials. Results are expressed as percentage response accuracy, mean ± SEM. The statistical analyses used were two-way repeated measures ANOVA and *post hoc* Bonferroni (CON n=14, EX n=28). There was a significant effect of matching for the incongruent Stroop (P<0.0001).

Acute exercise did not alter performance of the N-back; 0-back (Figure 3.5, A), 1-back (Figure 3.5, B) or 2-back (Figure 3.5, C) tasks. Results are expressed as percentage response accuracy, mean ± SEM. The statistical analysis used were unpaired Student's *t* tests, CON n=15, EX n=32.

3.3.1.2.Analysis of BDNF & IGF-1 in Serum

Acute exercise induced a significant increase in serum BDNF concentration (Figure 3.6). There was a significant effect suggesting the mean BDNF concentrations changed over time (P=0.0126, $F_{(3,93)}$=3.813). *Post hoc* analysis revealed BDNF concentrations increased immediately post-acute exercise (60min: 1294.0 ± 145.0pg.ml^{-1}), relative to baseline (0min: 974.7 ± 131.1pg.ml^{-1}, P<0.05) and relative to the 30min timepoint, (30min: 1004.0 ± 122.7pg.ml^{-1}, P<0.05). At 30min post-acute exercise serum BDNF was not significantly different to baseline, 30min or to 60min (90min: 1254.0 ± 154.0pg.ml^{-1}). Results are expressed as pg BDNF/ ml serum, mean ± SEM. Data were analysed by a one-way repeated measures ANOVA with *post hoc* Newman-Keuls multiple comparison tests (n=32). There was significant matching (P<0.0001).

Acute exercise did not alter serum IGF-1 concentrations (Figure 3.7). Results are expressed as pg IGF-1/ ml serum (0min: 1087.0 ± 302.3pg.ml^{-1}, 30min: 1020.0 ± 291.6pg.ml^{-1}, 60min: 1041.0 ± 291.9pg.ml^{-1}, 90min: 1084.0 ± 296.4pg.ml^{-1}; mean ± SEM). Data were analysed by a one-way repeated measures ANOVA (n=32). There was significant matching (P<0.0001).

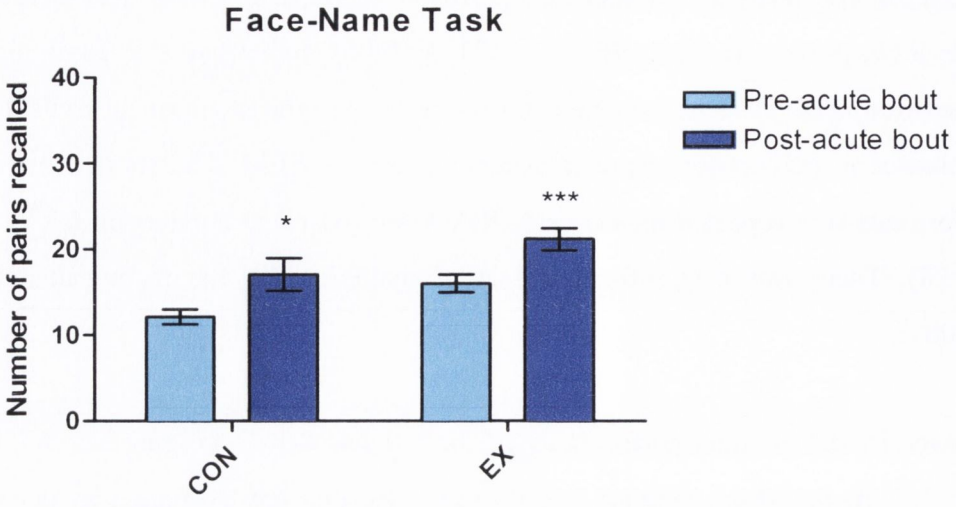


Figure 3.3: Effect of acute exercise on the face-name task

Acute exercise enhanced performance of the face-name task. There was a significant effect of trial ($P<0.0001$, $F_{(1,41)}=19.42$) indicating that the number of pairs recalled post-acute bout was greater that pre-acute bout. There was also a significant effect of group ($P=0.0219$, $F_{(1,41)}=5.681$) indicating a difference in performance between the groups. *Post hoc* analysis revealed that while CON scores increased post-acute bout (* indicates significant difference within the group, $P<0.05$), EX improved post-acute exercise to a greater extent (*** indicates significant difference within the group, $P<0.001$). Results are expressed as number of face-name pairs recalled, mean ± SEM. Statistical analyses: two-way repeated measures ANOVA and *post hoc* Bonferroni. CON n=13, EX n=30.

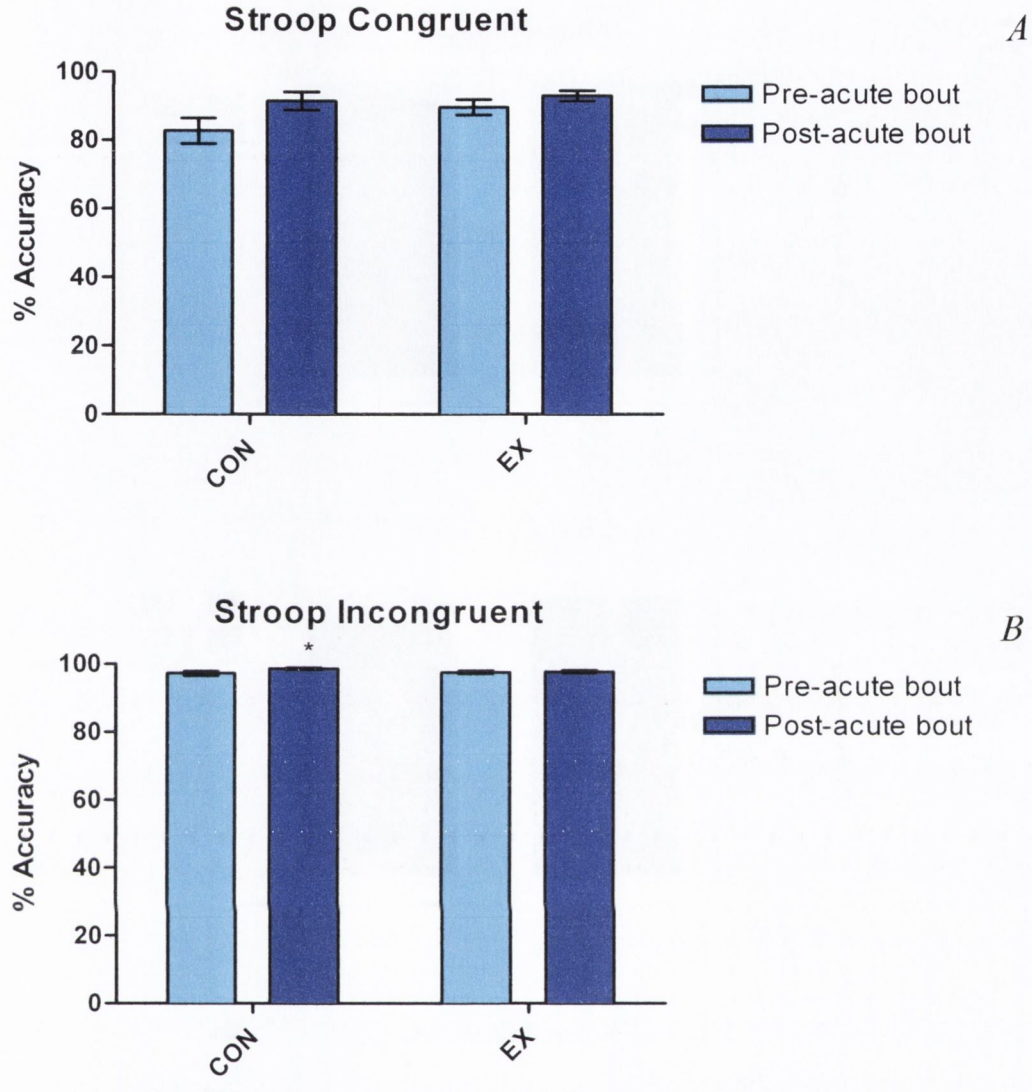


Figure 3.4: Effect of acute exercise on the Stroop word-colour task

Acute exercise did not alter performance of the Stroop word-colour task, in either congruent trials (A) or incongruent trials (B). There was an overall effect of trial ($P=0.0133$, $F_{(1,40)}=6.714$) for the congruent Stroop, but Bonferroni *post hoc* analysis revealed no within-group differences. There was also an overall effect of trial for the incongruent Stroop ($P=0.0124$, $F_{(1,40)}=6.813$) and a significant interaction ($P=0.0462$, $F_{(1,40)}=4.233$). Bonferroni *post hoc* analysis revealed a significant increase in CON (* represents $P<0.05$). Results are expressed as percentage response accuracy, mean ± SEM. Statistical analyses: two-way repeated measures ANOVA and *post hoc* Bonferroni, CON n=14, EX n=28.

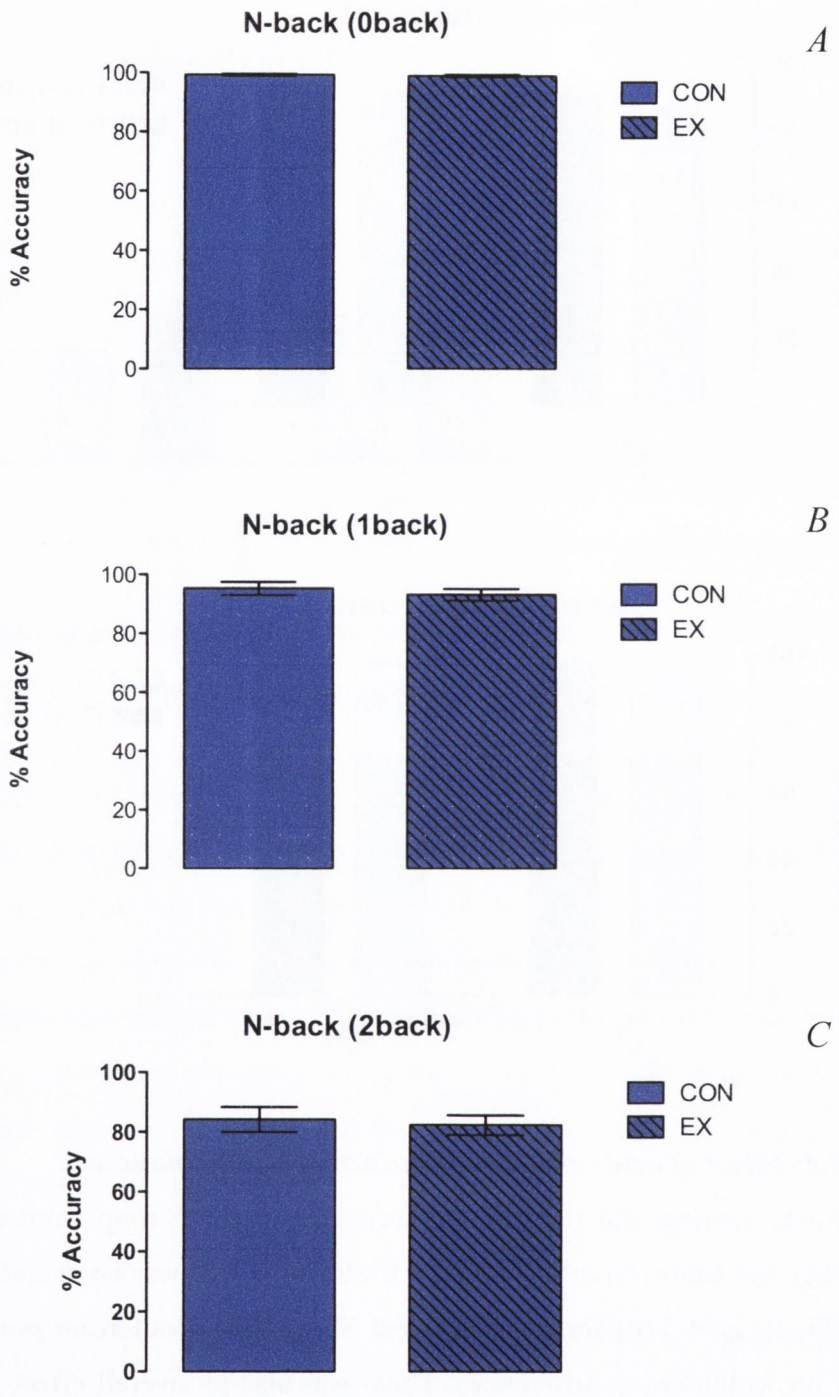


Figure 3.5: Effect of acute exercise on the N-back task

Acute exercise did not alter performance of the N-back (A) 0-back, (B) 1-back or (C) 2-back tasks. Results are expressed as percentage response accuracy, mean ± SEM. Statistical analysis used were an unpaired Student's *t* tests, CON n=15, EX n=32.

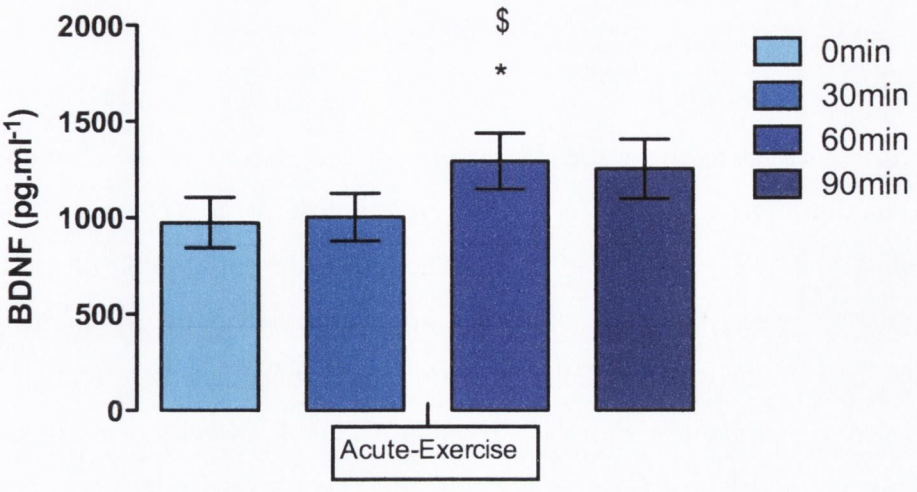


Figure 3.6: Effect of acute exercise on circulating BDNF concentrations

Acute exercise increased serum BDNF concentration (P=0.0126, $F_{(3,93)}$=3.813). Newman-Keuls Multiple comparison tests revealed the increase occured immediately post acute-exercise (60min) (* represents P<0.05 relative to 0min, $ represents P<0.05 relative to 30min). Results are expressed as pg BDNF/ ml serum, means ± SEM. Statistical analyses: one-way repeated measures ANOVA and *post hoc* Newman-Keuls, n=32.

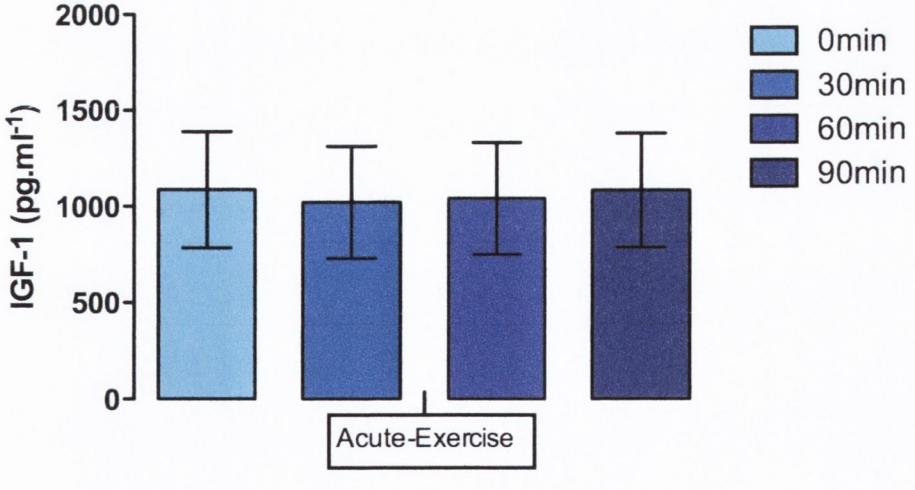


Figure 3.7: Effect of acute exercise on circulating IGF-1 concentrations

Acute exercise did not alter serum IGF-1 concentrations. Results are expressed as pg IGF-1/ ml serum, mean ± SEM. Statistical analysis: one-way repeated measures ANOVA, n=32.

3.3.2. Chronic exercise

3.3.2.1. Training

Aerobic fitness, as assessed by the VO_2 max test, was altered by the chronic exercise training paradigms. There was an overall effect of trial on VO_2 max (Figure 3.8, $P=0.0190$, $F_{(1,24)}=6.321$), indicating a difference between pre-chronic phase and post-chronic phase values. Although there was no overall effect of group, there was a significant interaction ($P=0.0335$, $F_{(2,24)}=3.924$), indicating that the effect of trial was not the same for all groups. *Post hoc* analysis revealed 5 weeks of chronic exercise significantly increased post-chronic exercise VO_2 max scores relative to pre-chronic exercise values in the C-EX5 group (pre-chronic exercise: $39.70 \pm 2.6 ml.min.kg^{-1}$, post-chronic exercise: $49.26 \pm 1.9 ml.min.kg^{-1}$, $P<0.05$). However 3 weeks of aerobic training had no effect on VO_2 max scores in the C-EX3 group (pre-chronic exercise: $44.69 \pm 3.5 ml.min.kg^{-1}$, post-chronic exercise: $47.65 \pm 2.7 ml.min.kg^{-1}$). Also, there was no difference between VO_2 max scores for A-EX group (A-EX3 and A-EX5 pooled together), across a 3 to 5 week sedentary period (pre-chronic phase: 50.88 ± 2.8 $ml.min.kg^{-1}$, post-chronic phase: $49.88 \pm 2.9 ml.min.kg^{-1}$). Results are expressed as ml oxygen consumed per min per kg body mass, means ± SEM. The statistical analyses used was a two-way repeated measures ANOVA and *post hoc* Bonferroni (A-EX: n=11, C-EX3: n=9, C-EX5: n=7). There was significant subject matching ($P=0.0007$).

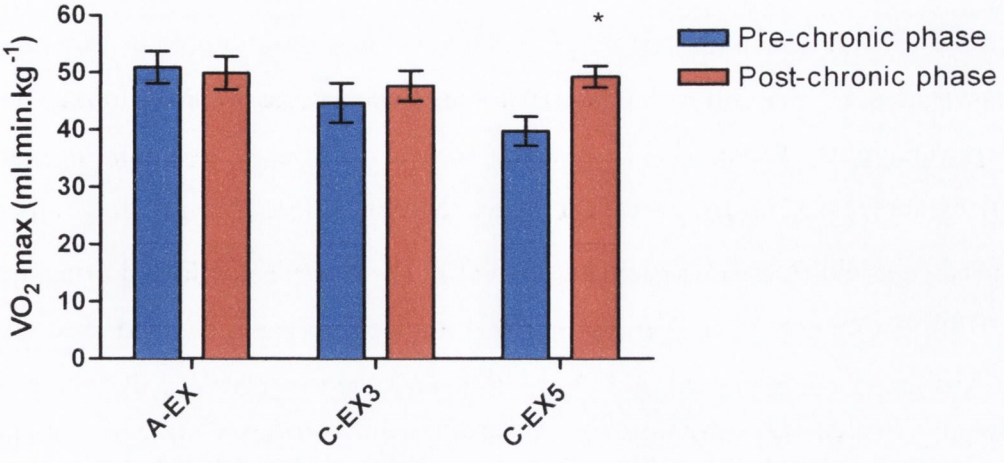


Figure 3.8: Effect of chronic exercise on VO_2 max scores

Chronic exercise had an overall effect on VO_2 max scores. There was a significant effect of trial ($P=0.0190$, $F_{(1,24)}=6.321$), indicating a difference between pre-chronic phase and post-chronic phase values. There was also a significant interaction ($P=0.0335$, $F_{(2,24)}=3.924$), indicating that the effect of trial was not the same for all groups. *Post hoc* analysis revealed no difference between VO_2 max scores for the A-EX group, across a 3 to 5 week sedentary period. Also, 3 weeks of chronic exercise did not affect VO_2 max scores. However, 5 weeks of chronic-exercise increased post-chronic exercise VO_2 max scores relative to pre-chronic exercise values, * indicates $P<0.05$. Results are expressed as ml oxygen consumed per min per kg body mass, means ± SEM. Statistical analysis; two-way repeated measures ANOVA and *post hoc* Bonferroni, A-EX: n=11, C-EX3: n=9, C-EX5: n=7.

3.3.2.2. Cognitive function

Face-name task performance was not altered by 3 weeks of chronic exercise (Figure 3.9) in the CON (pre-chronic phase: 14.200 ± 1.59pairs, post-chronic phase: 16.333 ± 2.00pairs), A-EX3 (pre-chronic phase: 19.800 ± 4.48pairs, post-chronic phase: 19.800 ± 4.40pairs) or C-EX3 (pre-chronic exercise: 16.556 ± 2.62pairs, post-chronic exercise: 19.222 ± 2.31pairs) groups. However, 5 weeks of chronic-exercise enhanced performance in the face-name task (Figure 3.10). There was a significant effect of trial ($P=0.0172$, $F_{(1,28)}=6.414$) indicating that the number of pairs recalled post-chronic phase was greater than pre-chronic phase. *Post hoc* analysis revealed that there was a significant increase in face-name performance in the C-EX5 group post-chronic exercise (pre-chronic exercise: 13.625 ± 1.55pairs, post-chronic exercise: 21.125 ± 2.18pairs, $P<0.05$), while the CON and A-EX5 (pre-chronic phase: 15.375 ± 1.57pairs, post-chronic phase: 17.375 ± 2.34pairs) groups remained unchanged. Results are expressed as total number of face-name pairs recalled across the 4 recall blocks (maximum 40 pairs), mean ± SEM. Data were analysed by a two-way repeated measures ANOVAs with Bonferoni *post hoc* tests, CON n=15, A-EX3 n=5, C-EX3 n=9, A-EX5 n=8, C-EX5 n=8.

Stroop word-colour task performance was not altered by 3 weeks of chronic exercise in congruent trials (Figure 3.11, A) or incongruent trials (Figure 3.11, B). There was an overall effect of trial ($P=0.0371$, $F_{(1,25)}=4.852$) for the congruent Stroop, but *post hoc* analysis revealed no significant differences within the groups; CON (pre-chronic phase: 82.433 ± 3.69%, post-chronic phase: 91.7 ± 6.56%), A-EX3 (pre-chronic phase: 92.75 ± 7.25%, post-chronic phase: 98.50 ± 0.89%) and C-EX3 (pre-chronic exercise: 78.67 ± 9.40%, post-chronic exercise: 98.33 ± 0.49%). However, 5 weeks of chronic exercise enhanced performance in the Stroop word-colour task in congruent trials (Figure 3.12, A). There was an overall effect of trial ($P=0.0154$, $F_{(1,28)}=6.66$) and *post hoc* analysis revealed a significant increase in response accuracy in the C-EX5 group (pre-chronic exercise: 78.69 ± 6.50%, post-chronic exercise: 96.69 ± 0.63%, $P<0.05$). Conversely, 5 weeks of chronic exercise decreased response accuracy in incongruent trials (Figure 3.12, B). There was an overall effect of trial ($P=0.0005$, $F_{(1,27)}=15.71$) and *post hoc* analysis revealed a significant decrease in the C-EX5 group (pre-chronic exercise: 96.63 ± 0.81%, post-chronic exercise: 82.13 ± 5.41%, $P<0.05$). Results are expressed as

percentage response accuracy, mean ± SEM. Data were analysed by two-way repeated measures ANOVA and *post hoc* Bonferroni, CON n=15, A-EX3 n=4, C-EX3 n=9, A-EX5 n=9, C-EX5 n=9.

Performance in the N-back task was not altered by 3 weeks of chronic exercise in the 0back (Figure 3.13, A), 1back (Figure 3.13, B), or 2back (Figure 3.13, C) tasks. Similarly, 5 weeks of chronic exercise had no effect on the N-back 0back (Figure 3.14, A), 1back (Figure 3.14, B), or 2back (Figure 3.14, C) tasks. Results are expressed as percentage response accuracy, mean ± SEM. Data were analysed by one-way ANOVA, CON n=15, A-EX3 n=5, C-EX3 n=9, A-EX5 n= 9, C-EX5 n=9.

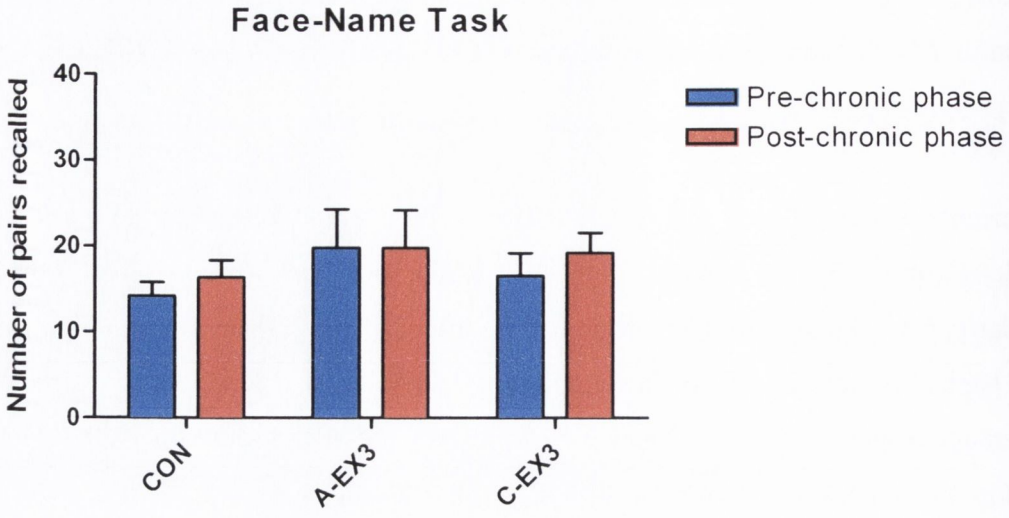


Figure 3.9: Effect of 3 weeks chronic exercise on the face-name task

3 weeks of chronic exercise did not alter face-name task performance. Results are expressed as total number of face-name pairs recalled, mean ± SEM. Statistical analysis: two-way repeated measures ANOVA and *post hoc* Bonferroni, CON n=15, A-EX3 n=5, C-EX3 n=9.

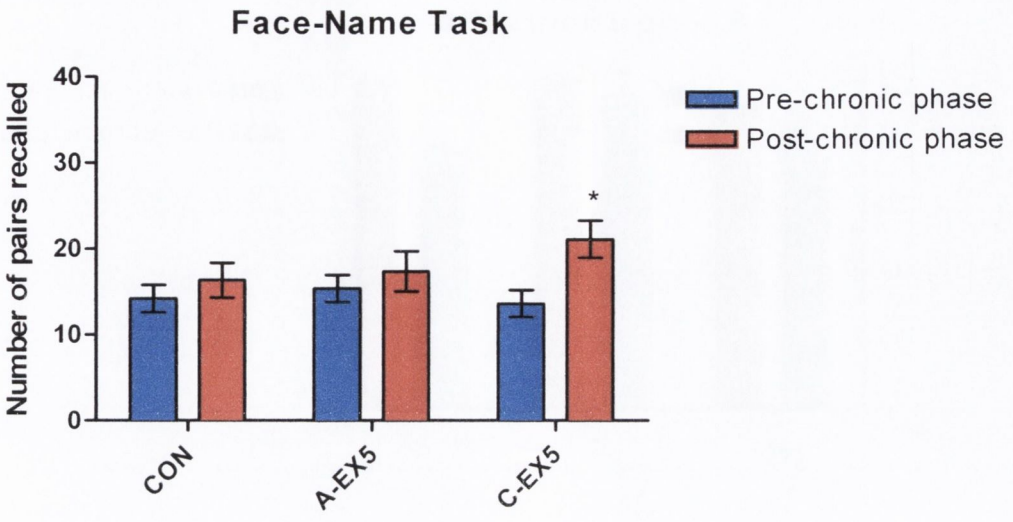
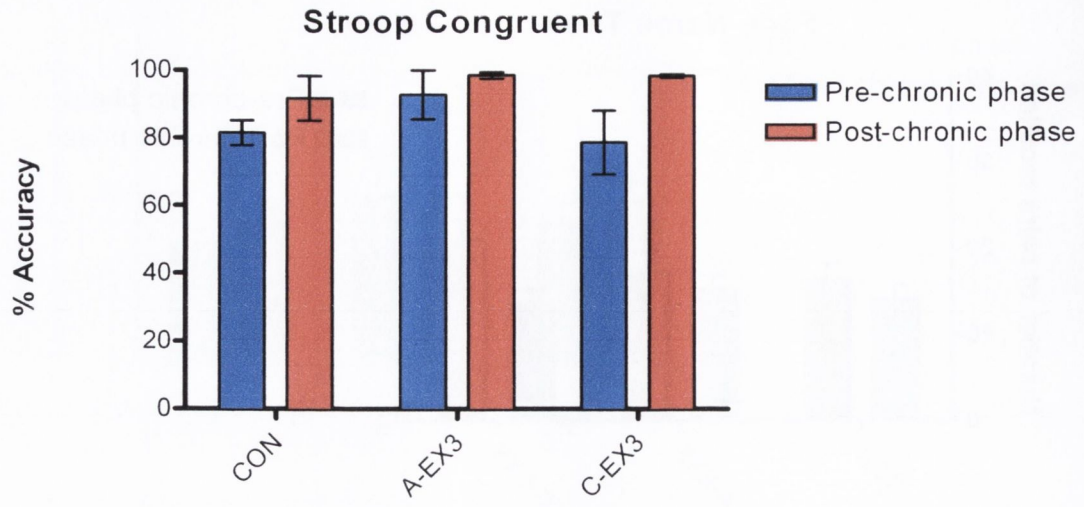


Figure 3.10: Effect of 5 weeks chronic exercise on the face-name task

5 weeks of chronic exercise enhanced performance of the face-name task. There was a significant effect of trial ($P=0.0172$, $F_{(1,28)}=6.414$) indicating that the number of pairs recalled post-chronic phase was greater than pre-chronic phase. *Post hoc* analysis revealed that there was a significant increase in face-name performance in the C-EX5 group (* represents $P<0.05$). Results are expressed as total number of face-name pairs recalled, mean ± SEM. Statistical analysis: 2-way repeated measures ANOVA, CON n=15, A-EX5 n=8, C-EX5 n=8.

A



B

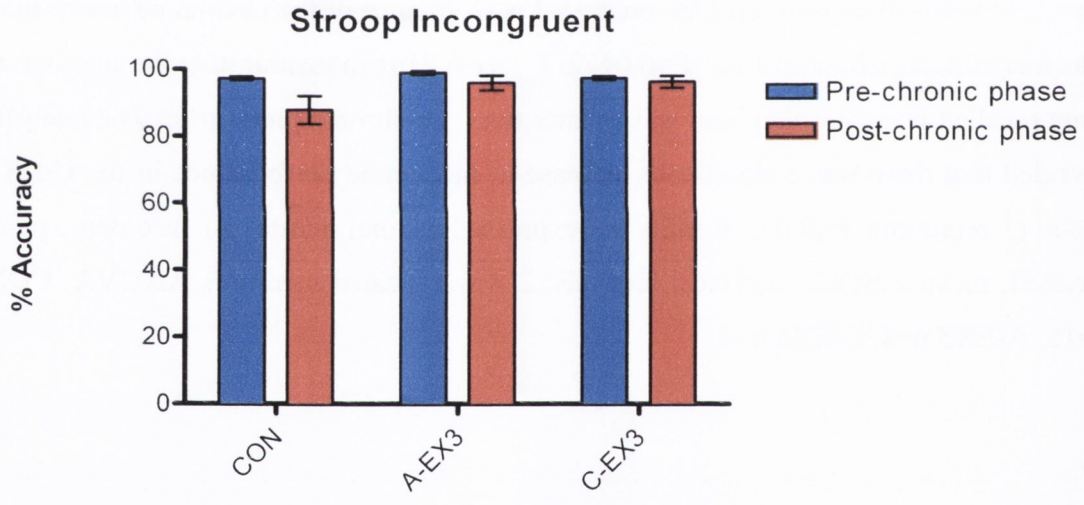


Figure 3.11: Effect of 3 weeks chronic exercise on the Stroop word-colour task.

3 weeks of chronic exercise did not alter Stroop word-colour task performance in either congruent trials (A) or incongruent trials (B). Results are expressed as percentage response accuracy, mean ± SEM. Statistical analysis: two-way repeated measures ANOVA and *post hoc* Bonferroni, CON n=15, A-EX3 n=4, C-EX3 n=9.

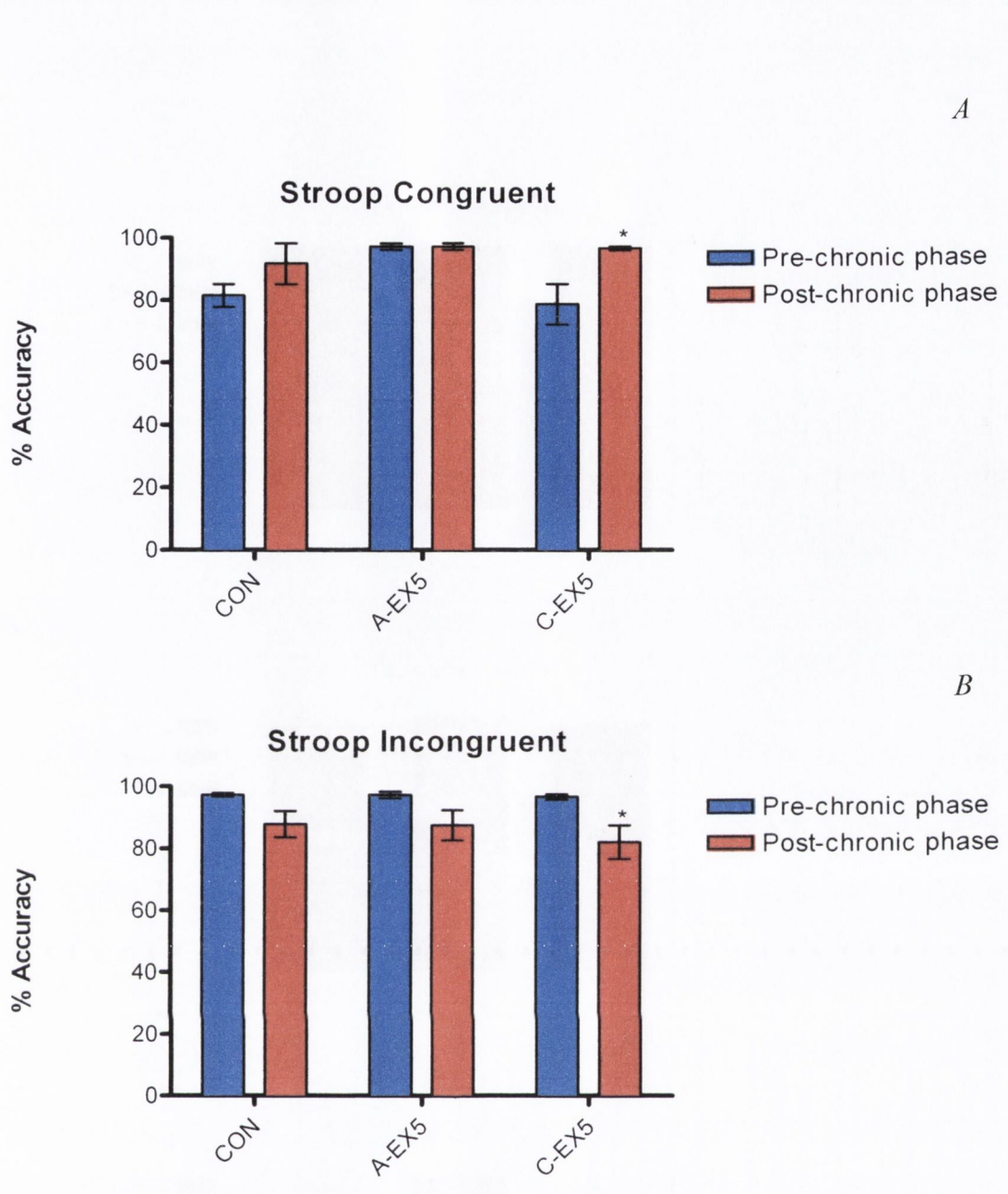


Figure 3.12: Effect of 5 weeks chronic exercise on the Stroop word-colour task.

(A) 5 weeks of chronic exercise enhanced performance in the Stroop word-colour task in congruent trials. There was an overall effect of trial ($P=0.0154$, $F_{(1,28)}=6.66$), *post hoc* analysis revealed a significant increase in response accuracy in the C-EX5 group (* represents $P<0.05$). (B) 5 weeks of chronic exercise decreased response accuracy in incongruent trials. There was an overall effect of trial ($P=0.0005$, $F_{(1,27)}=15.71$), *post hoc* analysis revealed a significant decrease in the C-EX5 group ($P<0.05$). Results are expressed as percentage response accuracy, mean ± SEM. Statistical analysis: two-way repeated measures ANOVA and *post hoc* Bonferroni, CON n=15, A-EX5 n=9, C-EX5 n=9.

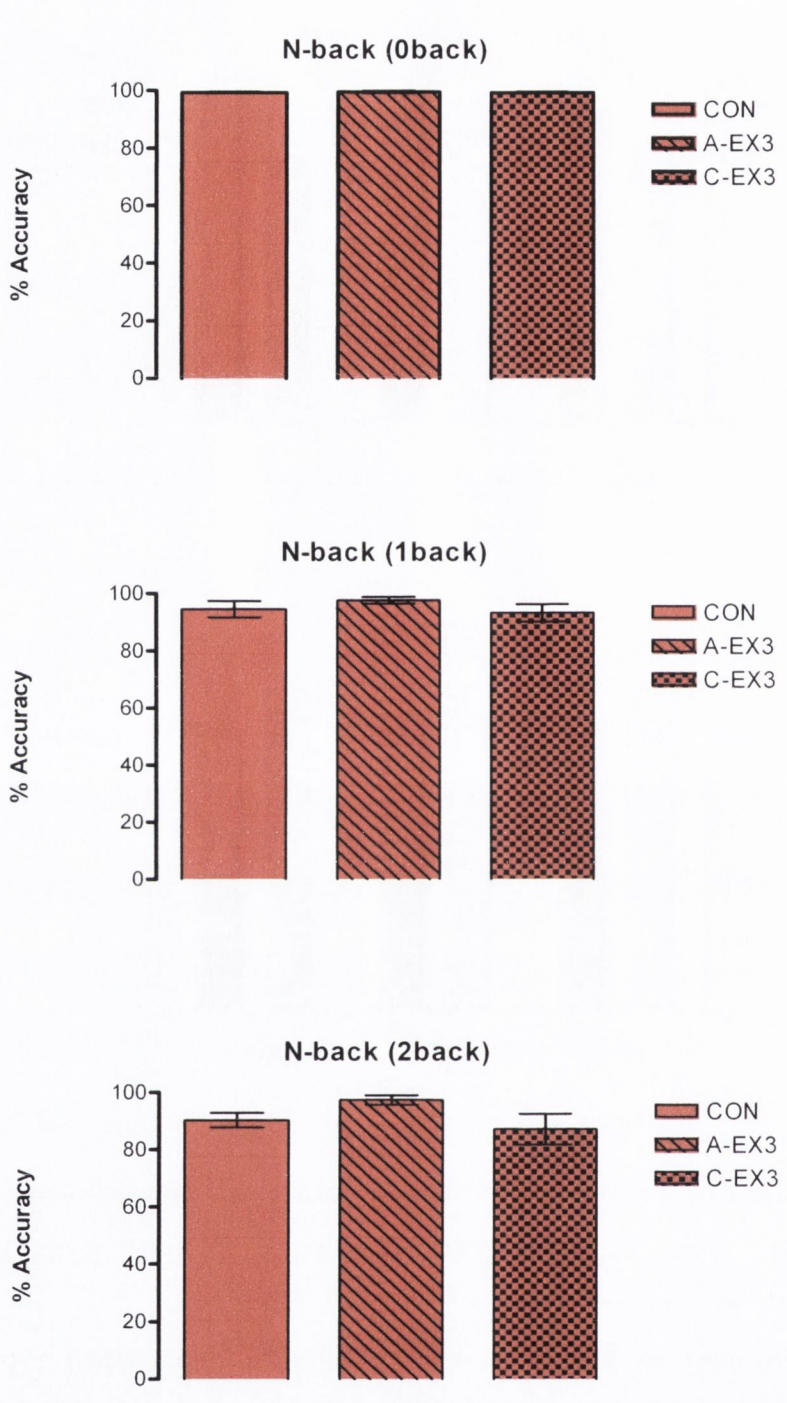


Figure 3.13: Effect of 3 weeks chronic exercise on the N-back task.

3 weeks of chronic exercise has no effect on the N-back 0back (A), 1back (B) or 2back (C) tasks. Results are expressed as percentage response accuracy, mean ± SEM. Statistical analysis: one-way ANOVA, CON n=15, A-EX3 n=5, C-EX3 n=9.

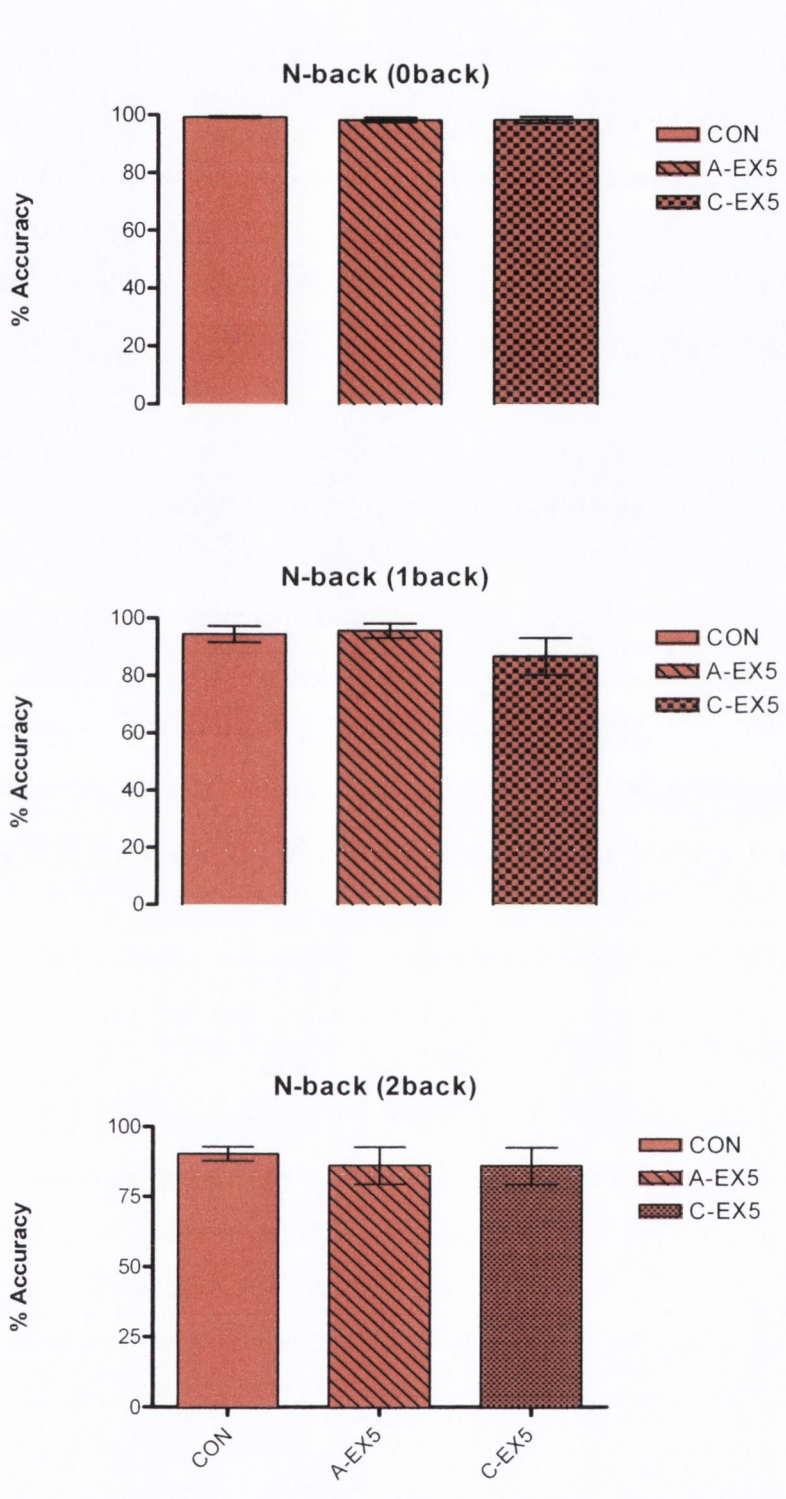


Figure 3.14: Effect of 5 weeks chronic exercise on the N-back task.

5 weeks of chronic exercise had no effect on the N-back 0back (A), 1back (B), or 2back (C) tasks. Results are expressed as percentage response accuracy, mean ± SEM. Statistical analysis: one-way ANOVA, CON n=15, A-EX5 n= 9, C-EX5 n=9.

3.3.2.3. Analysis of BDNF & IGF-1 in serum

Previously (Figure 3.6) an acute exercise bout was shown to induce an increase in serum BDNF concentration, immediately post-exercise. Similarly, in the A-EX3 (Figure 3.15) and A-EX5 groups (Figure 3.16), acute exercise induced an increase in BDNF immediately post-exercise; this increase was sustained at 30min post-exercise. Both 3 weeks and 5 weeks of chronic exercise altered the profile of this BDNF induction by acute exercise.

For the 3 week chronic phase (Figure 3.15), there was an overall effect of time ($P=0.007$, $F_{(3,33)}=4.725$) and an overall interaction ($P=0.0109$, $F_{(3,33)}=4.349$), indicating that BDNF concentration changed over time but the effect was not the same for both groups. *Post hoc* analysis revealed that a significant increase occured in the A-EX3 group immediately post-exercise (60min: 1225.50 ± 478.81pg.ml^{-1}; 0min: 339.04 ± 194.49pg.ml^{-1}, $P<0.01$; 30min: 531.13 ± 292.48pg.ml^{-1}, $P<0.05$) which was sustained at 30min post-exercise (90min: 1058.67 ± 516.00pg.ml^{-1}, $P<0.01$). However, serum BDNF concentration did not change from baseline in the C-EX3 group (0min: 898.323 ± 265.45pg.ml^{-1}, 30min: 882.363 ± 273.98pg.ml^{-1}, 60min: 878.863 ± 166.86pg.ml^{-1}, 90min: 955.732 ± 186.91pg.ml^{-1}). The absence of the BDNF response to acute-exercise in the C-EX3 group indicates that 3 weeks of chronic exercise had some impact on this system.

For the 5 week chronic exercise protocol (Figure 3.16) there was an overall effect of time ($P=0.0001$, $F_{(3,42)}=8.973$) indicating that BDNF concentration changed over time. *Post hoc* analysis revealed that a significant increase occured in the A-EX5 group immediately post-exercise (60min: 1484.960 ± 392.56 pg.ml^{-1} relative to 0min: 608.436 ± 190.53pg.ml^{-1}, $P<0.001$; 60min relative to 30min: 741.869 ± 181.14pg.ml^{-1}, $P<0.01$) which was sustained 30min post-exercise (90min: 1326.456 ± 456pg.ml^{-1}, $P<0.01$ relative to 0min; $P<0.05$ relative to 30min). There was also a significant increase in the C-EX5 group, which did not occur until 30min post-exercise (90min: 1345.044 ± 230.11pg.ml^{-1} relative to 0min: 778.411 ± 162.00pg.ml^{-1}, $P<0.05$; 90min relative to 30min: 602.764 ± 120.37pg.ml^{-1}, $P<0.01$; 90min relative to 60min: 851.730 ± 216.25pg.ml^{-1}). Indicating that 5 weeks of chronic exercise has altered the temporal profile of the BDNF response to acute exercise. Results are expressed as pg BDNF per

ml serum, mean ± SEM. The statistical analyses used were two-way repeated measures ANOVA with *post hoc* Bonferroni (A-EX3 n=5, C-EX3 n=9; A-EX5 n=6, C-EX5 n=8). There was significant subject matching for both chronic exercise protocol analyses (3 week: $P<0.0001$, 5week: $P<0.0001$).

Previously, (Figure 3.7) it was shown that an acute exercise bout had no effect on serum IGF-1 concentration. Similarly, in the A-EX3 (Figure 3.17) and A-EX5 acute exercise subgroups (Figure 3.18), acute exercise had no effect on serum IGF-1 concentration. Although the range of IGF-1 concentrations recorded for the 5-week chronic exercise group are considerably lower than the 3-week chronic exercise group, both 3 weeks and 5 weeks of chronic exercise induced a decrease in serum IGF-1 concentration immediately post-exercise.

There was no overall effect of 3 weeks chronic exercise on serum IGF-1 concentration (Figure 3.17). Although, there was no overall effect of time, *post hoc* analysis revealed a slight decrease in IGF-1 concentration in the C-EX3 group immediately post-acute exercise (60min: 1177.714 ± 526.30pg.ml^{-1}) this decrease was not different to the 0min (1446.545 ± 549.64pg.ml^{-1}) and 30min (1491.675 ± 577.31pg.ml^{-1}) time points, only to the 90min (1716.351 ± 715.66pg.ml^{-1}, $P<0.01$) time point. Similarly, 5 weeks of chronic exercise resulted decreased serum IGF-1 concentration, immediately post-acute exercise (Figure 3.18). There was an overall effect of time ($P=0.002$, $F_{(3,39)}=5.898$), indicating that IGF-1 concentration changed over time. *Post hoc* analysis revealed a decrease in IGF-1 concentration in the C-EX5 group immediately post-acute exercise (60min: 461.815 ± 181.30pg.ml^{-1}) relative to 0min (527.928 ± 158.85pg.ml^{-1}, $P<0.05$), 30min (524.143 ± 172.21pg.ml^{-1}, $P<0.05$) and relative to 90min (547.704 ± 167.72pg.ml^{-1}, $P<0.01$). Results are expressed as pg IGF-1 per ml serum, mean ± SEM. The statistical analyses used were two-way repeated measures ANOVA with *post hoc* Bonferroni (A-EX3 n=4, C-EX3 n=7; A-EX5 n=6, C-EX5 n=9). There was significant matching ($P<0.0001$).

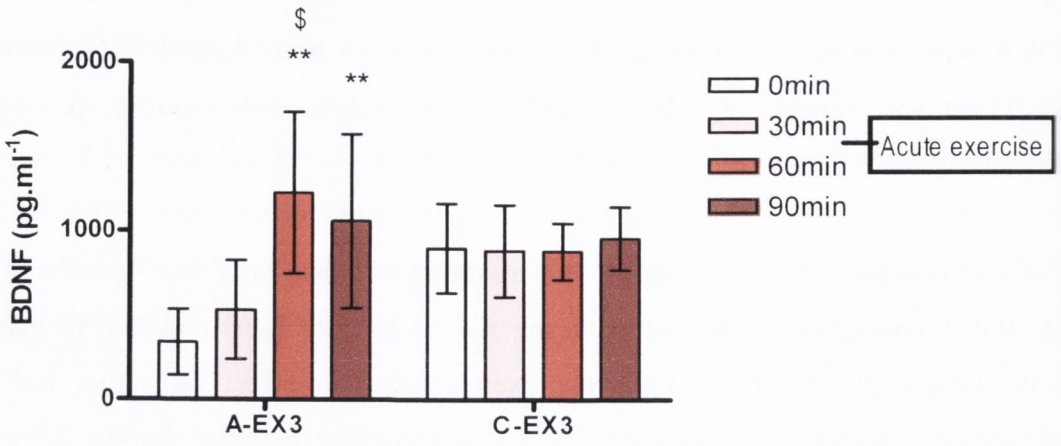


Figure 3.15: Effect of 3 weeks chronic exercise on serum BDNF concentration.

3 weeks of chronic exercise altered the profile of BDNF induction by acute exercise. There was an overall effect of time ($P=0.007$, $F_{(3,33)}=4.725$) and an overall interaction ($P=0.0109$, $F_{(3,33)}=4.349$), indicating that BDNF concentration changed over time but the effect was not the same for both groups. *Post hoc* analysis revealed that a significant increase occured in the A-EX3 group immediately post-acute exercise (60min; ** represents $P<0.01$ relative to 0min, $ represents $P<0.05$ relative to 30min) which was sustained at 30min post-acute exercise (90min; $P<0.01$). Serum BDNF concentration did not change from baseline in the C-EX3 group. Results are expressed as pg BDNF/ ml serum, mean ± SEM. Statistical analyses: two-way repeated measures ANOVA with *post hoc* Bonferroni, A-EX3 n=4, C-EX3 n=9.

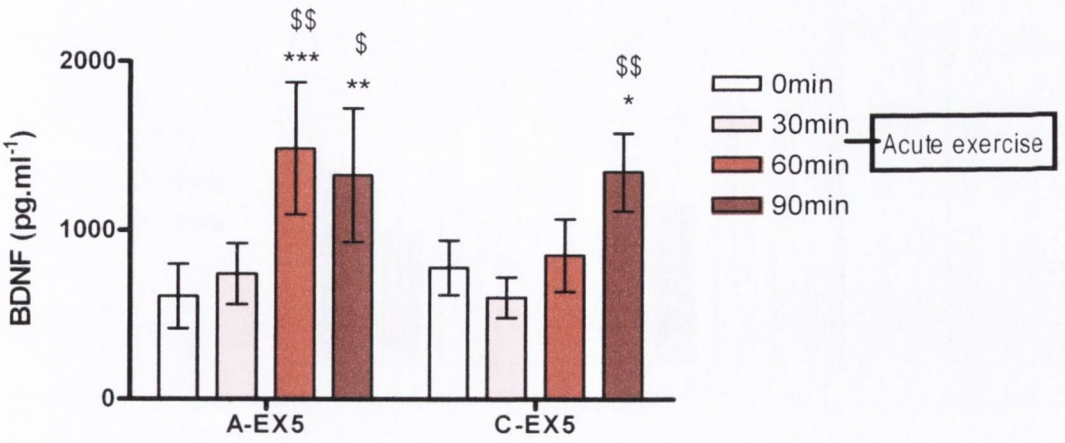


Figure 3.16: Effect of 5 weeks chronic exercise on serum BDNF concentration.

5 weeks of chronic exercise altered the profile of serum BDNF induction by acute exercise. There was an overall effect of time ($P=0.0001$, $F_{(3,42)}=8.973$) indicating that BDNF concentration changed over time. *Post hoc* analysis revealed that a significant increase occured in the A-EX5 group immediately post-acute exercise (60min; *** represents $P<0.001$ relative to 0min, $$ represents $P<0.01$ relative to 30min) which was sustained 30min post-acute exercise (90min; ** represents $P<0.01$ relative to 0min, $ represents $P<0.05$ relative to 30min). There was also a significant increase in the C-EX5 group, which did not occur until 30min post-acute exercise (90min; * represents $P<0.05$ relative to 0min, $$ represents $P<0.01$ relative to 30min). Results are expressed as pg BDNF/ ml serum, mean ± SEM. Statistical analyses: two-way repeated measures ANOVA with *post hoc* Bonferroni, A-EX5 n=6, C-EX5 n=8.

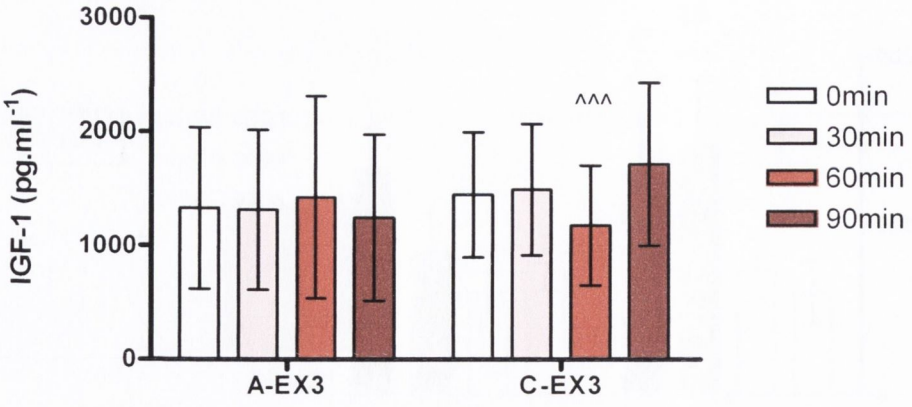


Figure 3.17: Effect of 3 weeks chronic exercise on serum IGF-1 concentration.

3 weeks of chronic exercise had no overall effect on serum IGF-1 concentration. However, *post hoc* analysis revealed a slight decrease in IGF-1 concentration in the C-EX3 group immediately post-acute exercise (60min, ^^^ represents $P<0.01$ relative to 90min). Results are expressed as pg IGF-1/ ml serum, mean ± SEM. Statistical analysis: two-way repeated measures ANOVA with *post hoc* Bonferroni. A-EX3 n=4, C-EX3 n=7.

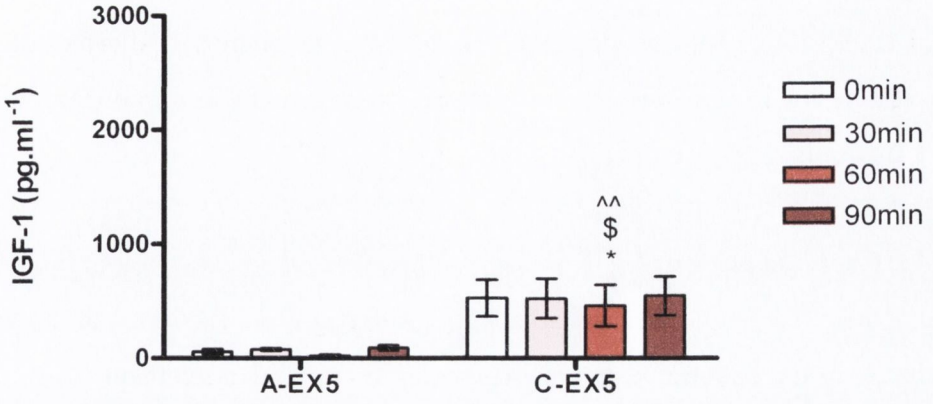


Figure 3.18: Effect of 5 weeks chronic exercise on serum IGF-1 concentration.

5 weeks of chronic exercise resulted in a decrease in serum IGF-1 concentration immediately post-acute exercise. IGF-1 concentration changed significantly over time ($P=0.002$, $F_{(3,39)}=5.898$). *Post hoc* analysis revealed a decrease in IGF-1 concentration in the C-EX5 group immediately post-acute exercise (60min, *, $ represent $P<0.05$ relative to 0min and 30min respectively; ^^ represents $P<0.01$ relative to 90min). Results are expressed as pg IGF-1/ ml serum, mean ± SEM. Statistical analysis: two-way repeated measures ANOVA with *post hoc* Bonferroni. A-EX5 n=6, C-EX5 n=9.

3.4. Discussion

This study was completed in an effort to examine the link between physical activity and improved cognition in human subjects. The aim was to examine the effect of both an acute exercise bout and chronic exercise on the serum growth factor concentrations and cognitive function.

3.4.1. Acute exercise

Maximal acute exercise induced an enhancement in cognitive function, as shown by the improvement in face-name task performance. This is in agreement with previous studies which suggest that intense acute exercise enhances learning and memory as assessed by a language-learning model (Winter *et al.*, 2007), and the Stroop task (Ferris *et al.*, 2007). In contrast, maximal acute exercise had no effect on the Stroop word-colour task, which recruits the anterior cingulate cortex (Leung *et al.*, 2000). Although some improvement in the incongruent trials was seen in the control group, it is likely that this is an effect of practice, as there was less to distract the control participants during the 30min interval between tests. Similarly, maximal acute exercise had no effect on the N-back task, which primarily recruits the dorsolateral prefrontal cortex (Callicott *et al.*, 1999).

Previously, face recognition was shown to recruit the right medial-temporal lobe (MTL), as evidenced by an inability of patients with right amygdalo-hippocampectomy to recognise previously viewed faces (Crane & Milner, 2002). It has also been repeatedly demonstrated, using high-resolution functional magnetic resonance imaging (fMRI) acquisition and analysis methods, that the face-name association task used in the present study, engages the hippocampus (Zeineh *et al.*, 2003) and nearby MTL cortical areas, including the amygdala, parahippocampal cortex, perirhinal cortex and entorhinal cortex (Kirwan & Stark, 2004). Hence, in the present study the acute exercise-induced cognitive enhancement appears to be selectively MTL-dependent. To my knowledge, this is the first evidence for a maximal acute exercise-induced enhancement in cognitive function in humans, which is selectively MTL-dependent.

In the case of Winter *et al.* (2007), language-learning was reportedly accelerated by 20% following intense exercise, and the authors suggest that the improvement is mediated through BDNF. However, the single nucleotide polymorphism in the BDNF

gene, which affects activity-dependent BDNF secretion, reportedly has no effect on semantic memory, working memory/executive function or recall of word-lists, all of which contain a large pre-frontal component (Egan *et al.*, 2003). Hence, the enhancement in language learning is unlikely to be mediated through BDNF, based on our knowledge of the functions of BDNF in hippocampal-dependent learning. Furthermore, the post-exercise improvements in the performance of the Stroop word and colour tests reported by Ferris *et al* (2007) are somewhat questionable in light of the experimental design used. The possibility that these increases were as a result of a practise effect has not been accounted for as there were no control groups included in the study. Alterations in the performance of the Stroop colour-word test (except in the case of an intense, endurance cycle) were not seen and the author admits "the Stroop scores, even where significant increases occurred, were not uniform" (Ferris *et al.*, 2007).

In agreement with the literature, the serum analysis revealed an acute exercise-induced increase in serum BDNF in sedentary young men. Previously, serum BDNF was reported to increase in response to incremental exercise to exhaustion but not low-intensity exercise, in recreational athletes (Rojas Vega *et al.*, 2006). Moreover, BDNF reportedly increased by 30% following a graded exercise test in physically active men and women (Ferris *et al.*, 2007). Moderate acute exercise, in the form of a 30min sub-maximal endurance test on a bicycle ergometer, has also been shown to induce a transient increase in serum BDNF in humans which returns to baseline at 30min post-exercise (Gold *et al.*, 2003). The transient nature of the BDNF increase was common to all three of these studies. In contrast, in the present study, maximal exercise induced an increase in BDNF that had not quite returned to baseline at 30min post-exercise. The disparity in these findings may be a result of differences in exercise intensity. Gold *et al*'s study used sub-maximal exercise at 60% of the VO_2 max, while our participants cycled until they reached their maximum oxygen consumption rate. Alternatively, the fitness level of the participants might be a factor; the participants in Rojas Vega *et al*'s study achieved a VO_2 max of 56.6 ± 8.6ml.min.kg^{-1} (mean ± SEM) at an average heart rate of 189.3 ± 10.3bpm (mean ± SEM), indicating that they were relatively fit, in comparison to our sedentary participants. It remains to be elucidated if the BDNF response to exercise differs between athletes and sedentary individuals (Rojas Vega *et*

al., 2006) but this may account for the discrepancy in the results of these two studies, as the protocols were very similar.

The acute exercise bout did not alter serum IGF-1 concentrations. This result is in agreement with the literature. It has been reported that 1 hour of treadmill running had no effect on serum IGF-1 concentrations in the rat, although increased uptake of serum IGF-1 into the brain was reported (Carro *et al.*, 2000). Acute, high-intensity exercise causes a significant elevation of cortisol in men and women (Borst *et al.*, 2002) and corticosteroids exert inhibitory effects on the synthesis of IGF-1 by the liver (Trejo *et al.*, 2001). Hence, it is possible that an acute exercise-induced increase in cortisol prevented an increase in the concentration of circulating IGF-1. Alternatively, serum IGF-1 levels may have remained unchanged because an increase in circulating IGF-1 that was not detected due to increased uptake into target organs such as muscle and brain.

3.4.2. Chronic exercise

An increase in VO_2 max score indicates an improvement in aerobic fitness. A comparison of the two incremental exercise tests to volutional exhaustion revealed that while 3 weeks of chronic exercise was insufficient to affect VO_2 max scores, 5 weeks of aerobic training increased maximal oxygen consumption rates, relative to pre-chronic phase values. This is particularly interesting when we consider the face-name task results. While 3-weeks of chronic exercise had no effect on MTL-dependent learning and memory, 5 weeks of chronic exercise enhanced face-name task performance. The 3-week chronic phase protocol also had no effect on the Stroop word-colour task or the N-back task. The Stroop task measures the ability of a participant to inhibit the automatic reading response and instead report the colour of the font the word is presented in (Stroop, 1935). The incongruent trials provided the cognitive interference associated with the Stroop effect. Therefore, although there was some improvement in the performance of Stroop congruent trials in the 5-week chronic exercise group, indicating perhaps increased attention, there was a decrease in response accuracy for this group in the incongruent Stroop, indicating an exercise-induced inhibition in the performance of this task. The 5-week chronic exercise protocol had no effect on the N-back task. Hence, 5 weeks of aerobic training resulted in increased

aerobic fitness and an enhancement in cognitive function that was selectively MTL-dependent.

Taken together these results indicate that both acute exercise and 5 weeks of chronic exercise enhance MTL-dependent learning. However, the question remains: what is the mechanism by which physical activity enhances MTL-dependent cognition? An increase in serum BDNF concentration with acute exercise has been demonstrated and was associated with a concomitant enhancement in the performance of the face-name task. Furthermore, this acute exercise-induced BDNF increase was shown to be reproducible in testing session 2. The serum BDNF concentration increased immediately post-exercise in the acute-exercise control groups (A-EX) and was sustained at 30min post-exercise. However, in order for this exercise-induced serum BDNF increase to be functionally relevant, BDNF must be able to gain access to the brain.

It is generally accepted that BDNF can cross the blood-brain-barrier, although the mechanism by which this occurs is somewhat controversial. There is evidence to suggest the presence of a saturable transport system of the blood-brain-barrier for BDNF, which is unidirectional from blood to brain. Conversely, the movement of BDNF from brain to blood is said to occur via bulk flow associated with the reabsorption of the cerebrospinal fluid (Pan *et al.*, 1998). Alternatively, it has been suggested that exercise transiently increases the permeability of the blood-brain-barrier as demonstrated by an increase in the extravasation of Evans blue albumin into the brain following 30min of forced swim exercise (Sharma *et al.*, 1991). Regardless of its method of transit across the blood-brain-barrier, there is strong evidence from animal studies that increased BDNF concentration may have functional consequences for cognition. The binding of BDNF to its receptor Trk B mediates plasticity changes involved in recognition memory in sheep (Broad *et al.*, 2002). Furthermore, exercise was shown to enhance object recognition learning in association with an increased concentration BDNF in the dentate gyrus of young rats (O'Callaghan *et al.*, 2007).

Evidence from the literature suggests that BDNF can facilitate neurotransmitter release and enhance synaptic transmission (Jovanovic *et al.*, 2000; Xu *et al.*, 2000), leading to the hypothesis that the acute exercise-induced enhancement in hippocampal function

may be mediated by the actions of BDNF on synaptic transmission. However, neither 3 weeks nor 5 weeks of chronic exercise had an effect on basal BDNF levels. Furthermore, following 3 weeks of chronic exercise, the effect of acute exercise on serum BDNF was absent. Similarly, 5 weeks of chronic exercise altered the profile of the BDNF response to acute exercise, in that the BDNF increase was delayed until 30min post-exercise. These results indicate that aerobic training is having some effect on BDNF induction by acute exercise, altering the temporal profile of the acute-exercise effect. It is unclear whether this is a result of increased uptake by target tissues or an alteration in the mechanism of BDNF release.

It is possible that the effect of chronic exercise on cognition may be mediated by an alternative mechanism involving BDNF. BDNF infusion has been shown to induce neurogenesis in rats (Scharfman *et al.*, 2005). It has been demonstrated that hippocampal neurogenesis also occurs in the dentate gyrus of adult humans (Eriksson *et al.*, 1998). Furthermore, any potential functional consequences of adult neurogenesis must occur as long-term adaptations, rather than acute benefits, due to the length of time it takes neurons to mature and become integrated into a network (Kempermann *et al.*, 2004). Evidence suggests that newly generated granule cells are functionally integrated into the circuitry by 4 weeks (van Praag *et al.*, 2002). The timeline of the 5-week chronic exercise study would allow for an exercise-induced enhancement in neurogenesis to result in neurons with functional properties similar to those of mature dentate granule cells in the adult hippocampus (van Praag *et al.*, 2002), while any new neurons generated during the 3-week chronic phase training protocol may not yet be functionally integrated.

The results from the IGF-1 serum analysis showed an acute-exercise related transient decrease in circulating IGF-1 in both 3 weeks and 5 weeks chronic exercise groups. There was a slight decrease in IGF-1 concentration in the 3-week chronic exercise group immediately post-acute exercise and a more robust decrease in the 5-week chronic exercise group at the same time point. Exercise has previously been shown to increase brain IGF-1 concentrations via increased uptake of serum IGF-1, through the blood-CSF pathway in rodents (Carro *et al.*, 2000). Furthermore, using a subcutaneous infusion of a blocking IGF-1 antiserum, it was demonstrated that uptake of serum IGF-1 into the brain is necessary for exercise-induced enhancements in neurogenesis (Trejo

et al., 2001). It is possible that the decrease in serum IGF-1 in the present study is a result of increased uptake of IGF-1 into target tissues, although Carro and colleagues reported no alterations in serum IGF-1 concentration following 1 hour of treadmill exercise. Perhaps aerobic training enhances the efficacy of IGF-1 uptake into target tissues, which may have implications for the rate of neurogenesis.

3.4.3. Summary

These results provide evidence for a link between acute-exercise and cognitive function. Acute-exercise has been shown to increase serum BDNF and a concomitant improvement in selective, MTL-dependent memory has been demonstrated. Hence, BDNF is proposed as a mediator of the cognitive enhancements described, possibly through its role in enhanced neurotransmitter release and synaptic plasticity. Furthermore, it has been shown that while the 3-week chronic exercise programme was insufficient to improve aerobic fitness or augment memory test performance, the 5-week chronic exercise programme resulted in enhanced fitness scores and improvements in selective MTL-dependent cognition. The possibility that neurogenesis may be a factor in the chronic-exercise induced enhancements in cognition is proposed. Moreover, a role for peripheral growth factors in the alterations to the central nervous system was suggested, presuming that BDNF and IGF-1 are crossing the blood-brain-barrier. In the next chapter a rodent model was used in order to study the role of neurotrophins in exercise-induced cognitive enhancement, more mechanistically.

Chapter 4
Rodent Studies

Chapter 4: Rodent Studies

4.1. Introduction

Through the use of a rodent model, it is possible not only to study the relationship between physical activity and cognitive function, but also to investigate the underlying mechanisms at the cellular and molecular levels. The neurotrophins are proposed as candidate mediators of exercise-induced cognitive enhancement. In particular, BDNF is known to be a major regulator of activity-dependent plasticity in the hippocampus, and exercise, along with several other factors including learning (Silhol *et al.*, 2007) and environmental enrichment (Nilsson *et al.*, 1999; Ickes *et al.*, 2000), has been shown to increase BDNF expression in the rat hippocampus (Neeper *et al.*, 1996; Adlard *et al.*, 2004). Evidence suggests a causal link between BDNF induction and neuronal plasticity, such as hippocampal dependent learning (Anderson *et al.*, 2000) and adult neurogenesis (van Praag *et al.*, 1999; van Praag *et al.*, 2005). Moreover, evidence from our laboratory shows that an exercise-induced increase in the concentration of BDNF in the dentate gyrus is associated with a concomitant enhancement in novel object recognition, but not spatial memory (O'Callaghan *et al.*, 2007), indicating that exercise may selectively enhance cognitive function. Spatial memory is widely accepted to be a hippocampal-dependent form of learning, while the dentate gyrus (Kelly *et al.*, 2003) and perirhinal cortex (Wan *et al.*, 1999) are associated with the novel object recognition.

There is evidence to suggest that exercise can modulate expression of the BDNF receptor, Trk B (Wu *et al.*, 2007; Wu *et al.*, 2008) and alter expression or activation of downstream signalling molecules of Trk B activation, ERK activation, synapsin 1 expression and P-CaMKii expression in the hippocampus (Shen *et al.*, 2001; Ding *et al.*, 2006). Furthermore, exercise is also known to affect BDNF expression in the periphery, including increased concentrations in the muscle and serum (Gomez-Pinilla *et al.*, 2002; Ferris *et al.*, 2007). Neurotrophins can cross the blood brain barrier (Poduslo & Curran, 1996) and BDNF influx has been demonstrated through a rapid saturable transport system that is unidirectional from blood to brain, while BDNF efflux is associated with CSF reabsorption (Pan *et al.*, 1998). It has been proposed that circulating BDNF concentrations reflect BDNF concentrations in the brain during

development and aging (Karege *et al.*, 2002), with a possible correlation between serum BDNF concentrations and cortical plasticity (Lang *et al.*, 2007). However, the impact of alterations in circulating BDNF on brain BDNF concentration and vice versa has not yet been defined.

The objective of this study was to further elucidate the role of neurotrophins in the link between exercise and improved cognition, using a rodent model. The aim was firstly to establish the effect of forced exercise on both spatial and non-spatial memory in the male Wistar rat. It is proposed that exercise enhances cognitive function through a BDNF mediated mechanism. Therefore, the concentration of BDNF protein and mRNA expression in specific brain regions was analysed. Moreover, considering the evidence that BDNF crosses the blood-brain-barrier in both directions (Pan *et al.*, 1998), BDNF protein concentrations in the serum, plasma, muscle and liver were also measured. A potential role for the other neurotrophic factors was also considered. Hence, analysis of the concentration of NT-4/5 and NGF protein and mRNA in the brain was completed. An assessment of the effect of the 7-day forced exercise protocol on neurogenesis was made, using both BrdU labelling and the expression of Ki67 mRNA as markers. Finally exogenous BDNF and anti-BDNF infusion studies were completed in an effort to confirm the emerging role of BDNF in exercise-induced enhancement in cognitive function.

4.2. Methods

Amy Birch and Ranya Bechara assisted in collecting the behavioural data in the overtraining study and the forced exercise, 3-object substitution task respectively.

4.2.1. Animals

Male Wistar rats, supplied by the BioResources Unit in Trinity College Dublin from an inbred strain, were used in all experiments. The animals were approximately 3 months old and weighed between 250 and 350g at the beginning of the experiments. They were group-housed, 3 per cage, with the exception of the rats in the cannulation and infusion studies that were singly housed. In all cases animals were maintained under a 12-hour light-dark cycle with food and water available *ad libitum*. Ambient temperature was controlled between 20°C and 23°C. Rats were tail marked and split at random into either an exercise (EX) or a control (CON) group.

4.2.2. Forced exercise

The forced exercise protocol was performed on motorized rodent treadmills (Exer 3/6 treadmill, Columbus instruments) fitted with wire loops (see Figure 2.2), which were activated at a low intensity, averaging 3 on a scale of 1 to 10 (10 being the most intense shock) and were adjusted as necessary. All animals were familiarized to the treadmill for 3 days prior to the commencement of training. The familiarization protocol involved the animals being placed on a rodent treadmill, 3 animals per treadmill, separated into lanes by transparent partitions. The animals were left for 10min to adjust to their surroundings then the treadmill was switched on at a speed of 9m/min, with a low shock-intensity, for 10min.

Following familiarization, the 7-day forced exercise protocol began (Figure 4.1). This consisted of 1hr of running per day for the exercise group, at a rate of 10 to 15m/min, which can be considered low intensity, being below the lactate threshold in the rat. The lactate threshold is the point at which lactate levels begin rising significantly from baseline, is approximately 20m/min for male Wistar rats (Soya *et al.*, 2007). Animals were monitored constantly to ensure they were running and to assess them for signs of stress. Control animals were exposed to stationary treadmills for 1hr each day for the duration of the forced exercise protocol. Training in an object recognition task occurred on the final day of the exercise protocol, post-exercise. Object recognition testing was

completed either 6hrs later or 24hrs later (on day 8). In all cases, animals were sacrificed immediately post object recognition testing and tissue was collected and prepared as described earlier (section 2.3.5).

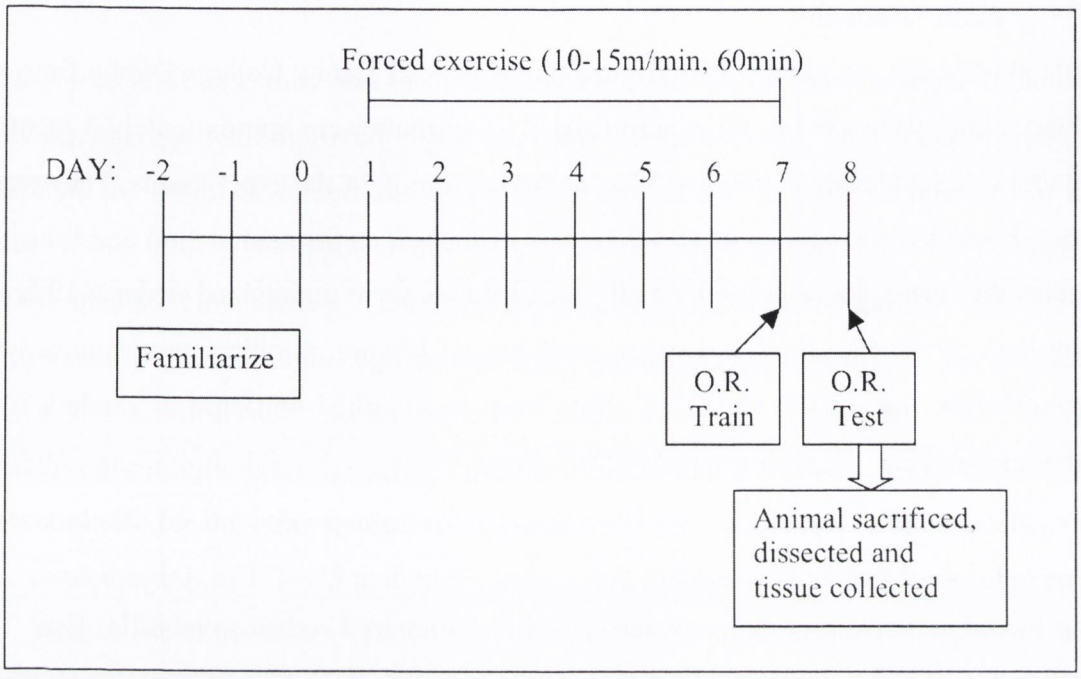


Figure 4.1: Timeline for 7-day forced exercise experimental protocol

Animals were familiarized to the treadmills for 3 days (days; -2, -1, 0). The forced exercise protocol involved animals running at a rate of 10 to 15m/min, 60min per day for 7 days (day 1-7). Animals were trained in an object recognition task on day 7, post-exercise and tested on day 8. Immediately post-testing animals were sacrificed and the tissue collected for later analysis.

4.2.3. Object recognition tasks

Rats were familiarized to the apparatus, which consisted of a black, circular open field (diameter 0.9m, height 48cm) placed in a dimly lit room, for two days prior to training in the object recognition tasks. During familiarization, animals were introduced to the open field in pairs on the first day, for a period of 10min. On the second day of familiarization the animals explored the arena alone for 5min. The object displacement task was used to assess spatial recognition memory and the object substitution task was used to assess non-spatial recognition memory.

4.2.3.1. Object Displacement

For the training phase of the object displacement task, objects (A, B & C), which were constructed of Lego bricks, were positioned in the open field and a spatial cue was fixed to the wall. Each animal was allowed to explore the arena for three 5min trials with an inter-trial rest period of 5min. The time spent in active exploration of object A and the total exploration time were recorded using stopwatches. For the testing phase, object A was displaced. Animals were reintroduced to the arena 6hrs post-training, for one 5min trial. Stopwatches were again used to record the total time spent exploring the three objects and the amount of time spent exploring object A, the displaced object. Time spent exploring object A was expressed as a percentage of the total exploration time.

4.2.3.2. Object substitution (2 object)

For the training phase of the object substitution task (2-object variant), objects A and B were positioned in the arena. Each animal was allowed to explore the arena for three 5min trials as before. The time spent exploring objects A & B was recorded using stopwatches. For the testing phase, object B was replaced with the novel object C. Animals were reintroduced to the arena 6hrs later, for one 5min trial. Stopwatches were used to record the time spent exploring the familiar object A and the novel object C, which were expressed as a percentage of the total exploration time.

4.2.3.3. Object substitution (3object)

For the training phase of the 3-object variant of the object substitution task, objects A, B and C were positioned in the arena. Each animal was allowed to explore the arena for three 5min trials as before. The time spent exploring objects A, B and C was recorded using stopwatches. For the testing phase, object C was replaced with the novel object D. Animals were reintroduced to the arena 24hrs later, for one 5min trial. Stopwatches were used to record the time spent exploring the familiar objects A and B and the novel object D, which were then expressed as a percentage of the total exploration time.

4.2.3.4. Overtraining

An overtraining experiment was also completed on the 3-object variant of the object substitution task. The objective of the overtraining was to verify that control animals could learn the 3-object substitution task, given enough time to explore the objects.

Hence, this task represents a more difficult version of the standard 2-object substitution task. For the overtraining phase of this task, objects A, B and C were positioned in the arena. Each animal was allowed to explore the arena for three 5min trials as before. The animal was reintroduced into the arena for another three 5min training trials following a 90min interval and again following another 90min interval, giving a total of nine 5min training trials. The time spent exploring objects A, B and C was recorded using stopwatches. For the testing phase, object C was replaced with the novel object D. Animals were reintroduced to the arena 24hrs following the first training trial, for one 5min trial. Stopwatches were used to record the time spent exploring the familiar objects A and B and the novel object D, which were expressed as a percentage of the total exploration time.

4.2.4. Blood sampling

Blood samples were taken through the saphenous vein under halothane anaesthesia (see section 2.3.4). Both plain and anti-coagulant coated microvettes were used for serum and plasma collection respectively. The plain microvettes were allowed to clot prior to centrifugation. The supernatant was removed from all samples and stored at -80°C for later analysis.

4.2.5. Analysis of protein expression by ELISA

The concentration of BDNF, IGF-1, β-NGF, & NT4/5 protein in brain tissue homogenate was analysed using commercially available ELISA kits. These were human BDNF, mouse IGF-1, rat β-NGF and human NT4/5 DuoSet ELISA Development systems kits (R&D Systems Europe, Oxon, United Kingdom). According to the manufacturer, the human BDNF kit, mouse IGF-1 kit and the human NT4/5 kit are 100% cross-reactive with rat BDNF, IGF-1 and NT4/5 respectively. BDNF and IGF-1 protein concentrations were also assessed in serum, plasma, muscle and liver tissue homogenates. The tissue homogenates was prepared as described in section 2.3.5 and protein content of the samples was assessed as described in section 2.3.6. The ELISAs were performed as described in section 2.3.7.

4.2.6. Real Time PCR

Analysis of BDNF, Trk B, IGF-1, β-NGF, NT 4/5, Ki67 and VEGF messenger RNA was performed using multiplex RT-PCR with ß-actin serving as endogenous control for each of the targets. The procedure is described in section 2.3.11.

4.2.7. Analysis of protein expression by Western immunoblotting

Trk B expression, ERK activity, P-Synapsin 1 and P-CaMKII expression were assessed using gel electrophoresis and Western immunoblotting (as described in sections 2.3.8 and 2.3.9). Briefly, nitrocellulose membranes were blocked against non-specific binding in TBS-T containing bovine serum albumin (BSA, 5% w/v), with the exception of probing for Trk B which required blocking in PBS containing non-fat dried milk powder (3% w/v). The membranes were incubated with primary antibody (see table 4.1) overnight at 4°C or at room temperature for 2hrs with constant agitation. The membranes were then washed 3x 10min with TBS-T (or twice with distilled water if probing for Trk B) and incubated with a secondary antibody (see table 4.1) for 1hr at room temperature with constant agitation. Following this, membranes were washed 3x 10min with TBS-T (2 washes with distilled water, 3x 10min with PBS-T and then 4x rinse with distilled water, if probing for Trk B). Supersignal® reagent was then added for 5min. The membrane was exposed to photographic film in the dark and the film was developed using a Fuji X-ray processor. The membrane was then stripped using Reblot super strength (1:10 dilution in dH_2O, 10ml) for 10min and reprobed for β-actin (for P-ERK analysis the membrane was reprobed for T-ERK expression). All protein bands were quantitated by densitometric analysis using the Gel Doc It Imaging System (UVP, Medical Supply Company, Ireland) in conjunction with LabWorks (Lablogics Inc, Mission Viejo, California, USA).

Primary	Dilution	Incubation	Secondary	Dilution	Incubation
Mouse Anti-ß-Actin (Sigma)	1:1000 TBS-T with BSA (2% w/v)	2hr, Room temp.	Goat anti-mouse IgG HRP (Sigma)	1:2000 in TBS-T with BSA (2% w/v)	1hr at Room temp.
Rabbit Anti-Trk B antibody (Upstate)	1:1000 PBS with non-fat Milk (2% w/v)	Overnight, 4°C	Goat anti-rabbit IgG HRP (Sigma)	1:5000 in PBS with non-fat Milk (2% w/v)	90min at Room temp.
Mouse P-ERK antibody (Santa Cruz)	1:2000 TBS-T with BSA (2% w/v)	2hr, Room temp.	Goat anti-mouse IgG HRP (Sigma)	1:6000 TBS-T with BSA (2% w/v)	1hr at Room temp.
Mouse ERK2 IgG (Santa Cruz)	1:1000 TBS-T with BSA (2% w/v)	2hr, Room temp.	Goat anti-mouse IgG HRP (Sigma)	1:1000 in TBS-T with BSA (2% w/v)	1hr at Room temp.
Rabbit Anti-P-Synapsin 1 antibody (Chemicon)	1:1000 TBS-T with BSA (2% w/v)	2hr, Room temp.	Goat anti-Rabbit IgG HRP (Sigma)	1:2000 in TBS-T with BSA (2% w/v)	1hr at Room temp.
Rabbit Anti-P- CaMKii antibody (Chemicon)	1:1000 TBS-T with non-fat milk (3% w/v)	2hr, Room temp.	Goat anti-Rabbit IgG HRP (Sigma)	1:5000 in TBS-T with non-fat milk (3% w/v)	1hr at Room temp.

Table 4.1: Dilutions, incubations and antibodies used for Western immunoblotting

4.2.8. BrdU administration and detection by immunohistochemistry

Animals completed the 7-day forced exercise protocol as previously described (section 4.2.2). The animals also received daily intra-peritoneal injections of bromodeoxyuridine (BrdU, 50mg.kg^{-1}) during the 7-day exercise protocol (Figure 4.2). Following the forced exercise and injection protocol the animals were sacrificed with an overdose of Urethane (30% w/v) via intra-peritoneal injection and perfused transcardially with a paraformaldehyde solution (4% w/v) to fix the tissue.

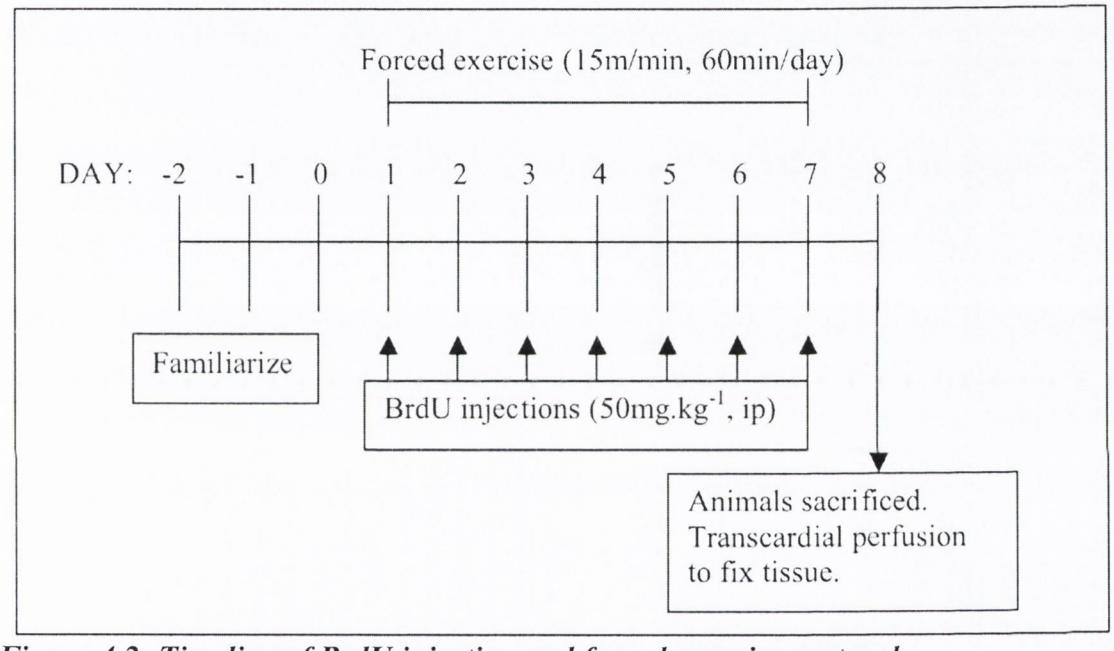


Figure 4.2: Timeline of BrdU injection and forced exercise protocol

Animals were familiarized with the treadmills for 3 days (days; -2, -1, 0). The forced exercise protocol involved animals running at a rate of 15m/min, 60min per day for 7 days (day 1-7). Animals were sacrificed on day 8 and perfused transcardially with paraformaldehyde to fix the tissue. The brains were dissected out for later analysis of BrdU expression.

4.2.8.1. Transcardial perfusion

Absence of the pedal reflex was confirmed prior to dissection. While the heart was still beating, the chest cavity was opened, the diaphragm cut, the ribs removed and the heart exposed. With a scissors the apex of the heart was cut and a small incision was made in the right atrium. A gavage was inserted into the left ventricle and clamped in place. Using a syringe (50ml) attached to the gavage, the rat was perfused transcardially with a 0.089% saline solution (100ml), which was heparinised (25units/ml). The colour of the liver was observed to change from dark red to tan in colour, as the blood was being flushed out, which confirmed that the saline was perfusing through the systemic circulation. The animal was then perfused with a paraformaldehyde solution (200ml, 4% w/v). The brains were dissected out and post-fixed overnight in 4% paraformaldehyde. Following this the tissue was cryoprotected overnight in a sucrose solution (20% sucrose in PBS), covered in a supporting medium, (OCT) and then flash frozen in liquid nitrogen. The tissue was stored at -80°C until ready to use, at which time it was equilibrated to 20°C in a cryostat for 1hr, then cut into 10μm coronal sections, placed on gelatin-coated slides and frozen at 20°C until ready to stain.

4.2.8.2. BrdU immunohistochemistry

The slides were defrosted thoroughly prior to staining, and pre-treated in a bath of acetone for 15min. The sections were then dehydrated in a succession of alcohol baths (90%, 70% alcohol) and washed in PBS. An antigen retrieval step was performed where the slides were immersed in a citrate buffer (0.1M) and heated in a microwave (800W) at 100%, 80% and 60% of the maximum power output, for 2.5min each. Slides were allowed to cool in a water bath for 30min and washed in PBS (2x 5min) prior to permeabilization in a Triton X solution (0.25% Triton X-100 in PBS, 10min). The sections were blocked by submersion in endogen peroxidase (1:100 dilution of H_2O_2 in distilled H_2O) prior to blocking against non-specific binding using normal horse serum (100μl, 1:5 dilution in PBS containing BSA (1% w/v)). Sections were incubated in primary antibody (mouse anti bromodeoxyuridine monoclonal antibody, Chemicon, 50μl, 1:100 dilution in PBS containing BSA (1% w/v)) overnight at 4°C. The sections were washed in PBS (3x 5min) and then incubated in secondary antibody (biotinylated horse anti-mouse IgG, Vector, 75μl, 1:300 dilution in PBS containing BSA (1% w/v), 30min room temperature). The ABC complex was prepared 30min prior to use (2drops

solution A, 2 drops solution B in 5ml PBS, Vector) and was applied to the slides after washing in PBS (3x 5min). Following the incubation in ABC complex (2 drops per section, 30min room temperature) and a wash in PBS (3x 5min), DAB chromogen (100µl, 1tablet in 10ml PBS, Dako Cytomation) was applied to the sections until colour development was visualized under the microscope (15min incubation). The reaction was stopped in PBS and the slides washed in distilled H_2O. The slides were immersed in a hematoxylin counterstain (Sigma) to stain the nuclei and cytosol. Following a thorough wash in H_2O, the sections were dehydrated in a succession of alcohol baths (70%, 90%, 100%), fixed in xylene and mounted in DPX.

4.2.9. Cannulation

Animals were anaesthetised (2µl/g body weight; ketamine 37.5% (w/v), xylazine 25% (w/v)). Once anaesthesia was confirmed by absence of the pedal reflex, animals were placed in a stereotaxic frame. An incision was made in the scalp and bregma was visualized. A guide cannula was fixed to the movable arm of the stereotaxic frame and placed above bregma. A hole was drilled through the skull at the coordinates: 0.9mm ventral, 1.6mm lateral to bregma. The menenges were perforated gently with a sterile needle and the guide cannula was implanted into left ventricle at a depth of 2.6mm. Three more sites were drilled part-way through the skull, ventral, dorsal and lateral to the cannula site and screws were fixed in place. The guide cannula was fixed in place by attaching it to the screws using dental cement (Dentalon plus, Heraeus Kulzer Ltd., Belgium) and the incision was closed using surgical staples. The guide cannula was sealed with a dummy cannula which screwed in place.

Animals were allowed to recover from surgery for at least 2 weeks prior to completion of any infusion protocols. For infusions into the left ventrical, the dummy cannula was replaced with an internal cannula which had a 1mm protrusion, hence the depth of the infusion was 3.6mm in total, from the surface of the skull. Infusions were made with a 10µl syringe which was fixed to the internal cannula using sterile rubber tubing.

4.2.10. Exogenous BDNF infusion protocol

One bolus infusion of recombinant Human BDNF (10ng), was administered intracerebroventricularly (icv) into the left ventricle. The dosage was calculated based on the exercise-induced increase in hippocampal-BDNF protein measured following the forced exercise protocol (48.22pg.mg protein^{-1}) and the average weight of the hippocampus for rats of a similar age and size (160mg). The calculated dose (7.72ng) was rounded up to allow for some loss during infusion. Control animals received an infusion of cytochrome C (10ng), icv to control for the effect of infusing a large molecule directly into the brain. All infusion solutions were diluted in an artificial cerebrospinal fluid (NaCL 150mM, KCl 3mM, $CaCl_2$ 0.19mM, $MgCl_2$ 0.8mM, Na_2HPO_4 0.8mM, NaH_2PO_4 0.2mM). 30min post-infusion, all animals were trained in the 3-object variant of the object substitution task (section 4.2.3.3). Animals were tested 24 hours later. Exploration of the objects was recorded and expressed as a percentage of the total exploration time.

4.2.11. Anti-BDNF & forced exercise

Animals were familiarized to the motorized rodent treadmills for 2 days prior to the commencement of training, as previously described (section 4.2.2). Animals were then trained in the 3-object variant of the object substitution task (section 4.2.3.3) and tested 24hrs later. Exploration of the objects was recorded and expressed as a percentage of the total exploration time. Following familiarization and testing in the object substitution task, the 7-day forced exercise protocol began. This consisted of 1hr of running per day, at a rate of 15m/min. The animals were monitored constantly to ensure they were running and to assess them for signs of stress. On day 2, 4 and 6 of the 7-day exercise protocol animal were given an icv infusion of anti-BDNF (5µl), with control rats receiving sheep serum (5µl, icv). On day 7 of the forced exercise protocol, animals were again trained in the 3-object variant of the object substitution task and tested 24 hours later (see Figure 4.3). Exploration of the objects was recorded and expressed as a percentage of the total exploration time.

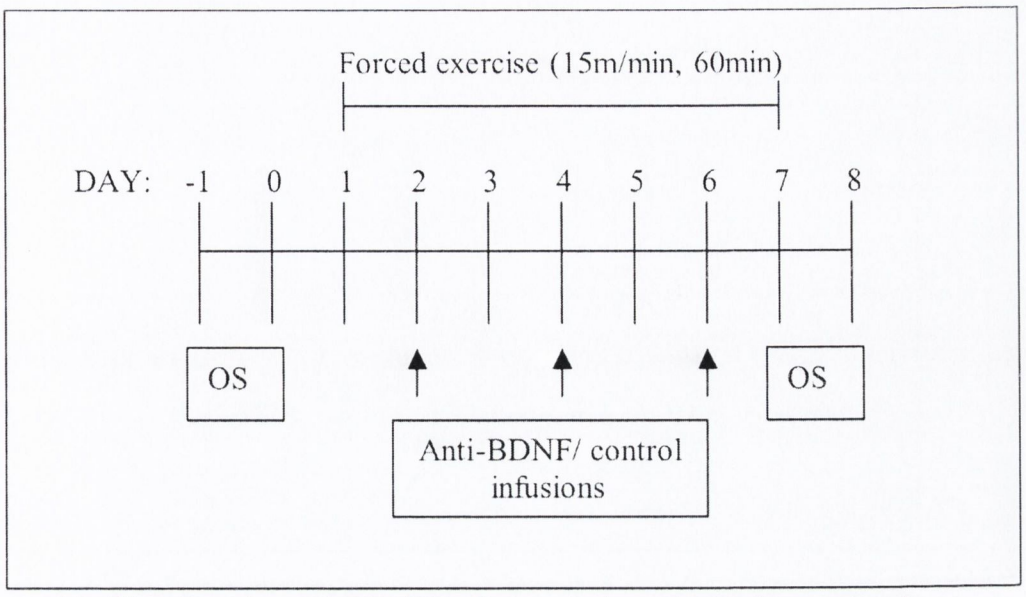


Figure 4.3: Timeline for anti-BDNF infusion & forced exercise protocol

On day -1, all animals completed training in the 3-object substitution task (OS) and were tested on day 0. The 7-day forced exercise protocol commenced on day 1, all animals ran at 15m/min for 60min per day. On days 2, 4 and 6, the treatment group of animals received anti-BDNF (5μl) while control animals received sheep serum (5μl). on day 7, following exercise, animals were trained in the 3-object substitution task and were tested 24hrs later (day 8).

4.2.12. Statistical analysis

All statistical analyses were performed using Graphpad Prism 5 for Mac OSX. Data are expressed as mean ± standard error of the mean (SEM). All data were examined for outliers, which were excluded if they were at least two standard deviations outside the mean. For the behavioural testing, two-way ANOVA was used to compare the exploration of objects and the effect of group. Where a significant difference occurred, Bonferroni *post hoc* analysis was performed. The exception was the overtraining behavioural experiment, where one-way ANOVA with *post hoc* Newman-Keuls multiple comparison tests were used to analysed the difference in exploration of the 3 objects.

For the analysis of serum and plasma BDNF in the moderate exercise protocol, two-way ANOVA and *post hoc* Bonferroni were used to compare the effect of day and the effect of group. Serum BDNF for the mild forced exercise study was also analysed using two-way ANOVA and *post hoc* Bonferroni, this time to compare the effect of

exercise and the effect of learning. Unpaired Student's *t* tests were used to compare directly between control and exercise groups for the rest of the analyses.

4.3. Results

4.3.1. Recognition memory and exercise

7 days of forced exercise had no effect spatial recognition memory, as assessed by the object displacement task (Figure 4.4). During training, both groups of rats spent approximately one third of the total exploration time exploring the object that was later displaced (CON: 33.98 ± 1.40%, EX: 30.89 ± 3.18%), indicating there was no preference for any one of the three objects. There was a significant effect of trial ($P<0.0001$, $F_{(1,44)}=45.08$) and *post hoc* analysis revealed that both groups spent significantly more time exploring the displaced object during the testing trial than during the training trials (CON: 59.28 ± 4.94%, EX: 49.50 ± 2.51%, $P<0.001$). This indicates that the animals learned the arrangement of the 3 objects during the training phase and could recognise the displaced object during the testing phase. However, there was no difference between groups, indicating that 7 days of forced exercise had no effect on exploration of the displaced object. Data are presented as a percentage of the total exploration time, mean ± SEM. The statistical analysis used was a two-way ANOVA with *post hoc* Bonferroni, n=12 per group.

7 days of forced exercise did enhance novel object recognition memory, as assessed by the object substitution task (2-object variant). During the training phase both groups spent approximately equal amounts of time exploring the two objects A and B, indicating there was no preference for either one of the objects (Figure 4.5, A; CON object A: 46.44 ± 4.13%, CON object B: 53.57 ± 4.13%, EX object A: 43.55 ± 4.856%, EX object B: 56.45 ± 4.856%). For the testing phase, object B was substituted for the novel object C and the animals were re-exposed to the arena (Figure 4.5, B). There was an overall effect of object ($P=0.0004$, $F_{(1,26)}=16.27$) indicating a preference for the novel object. *Post hoc* analysis revealed that the EX group spent significantly more time exploring the novel object C (object A: 26.31 ± 9.197%, object C: 73.69 ± 9.197%, $P<0.01$). This indicates that the exercised animals remembered the familiar object A and could identify the novel object C. Although there appears to be trend for CON animals to favour the novel object, this did not reach statistical significance

(object A: 37.41 ± 8.61%, object C: 62.59 ± 8.61%, P>0.05). Time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM. The statistical analyses were two-way ANOVA with *post hoc* Bonferroni. CON n=7, EX n=8, 1 animal was excluded from the analysis as it did not explore object A during the training trials.

7 days of forced exercise enhanced novel object recognition memory as assessed by the 3-object variant of the object substitution task. Both groups spent equal amounts of time exploring objects A, B and C during the training phase (Figure 4.6, A). However, during the testing trials (Figure 4.6, B) there was an overall effect of object (P=0.044, $F_{(2,39)}$=6.245) and a significant interaction (P=0.0002, $F_{(2,39)}$=10.41), indicating that a preference occurred for one of the objects but that the two groups responded differently. *Post hoc* analysis revealed a significant preference for the novel object D in the EX group only (object A: 23.34 ± 5.115%; object B: 19.613 ± 4.922%; object D: 57.047 ± 7.919%, P<0.001). Data are presented as a percentage of the total exploration time, mean ± SEM. The statistical analyses used were two-way ANOVA and post hoc Bonferroni, CON n=7, EX n=8.

Overtraining in the 3-object variant of the object substitution task resulted in learning in a control population of animals. The animals spent equal amounts of time exploring the 3 objects during the training phase (Figure 4.7, A). There was an overall significant effect of the means (P=0.0005, $F_{(2,21)}$=10.99) in the testing phase (Figure 4.7, B), indicating a preference for the novel object D. *Post hoc* analysis revealed a significant preference for the novel object D (object A: 30.17 ± 9.177%, P<0.05; object B: 10.23 ± 4.028%, P<0.001; object D: 59.61 ± 8.245%). Data are presented as a percentage of the total exploration time, mean ± SEM. The statistical analyses used were one-way ANOVA and *post hoc* Newman-Keuls, n=8.

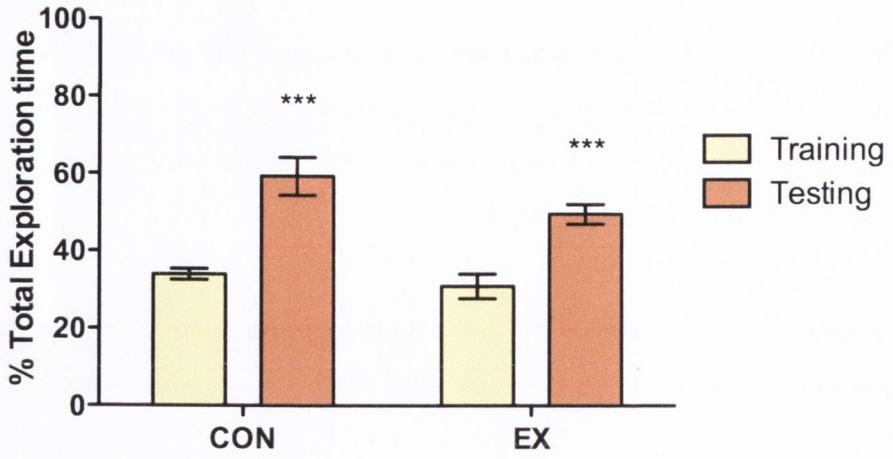


Figure 4.4: Effect of 7 days of forced exercise on spatial recognition memory

7 days of forced exercise had no effect on the object displacement task. There was a significant effect of trial ($P<0.0001$, $F_{(1,44)}=45.08$), indicating that animals spent a significantly greater amount of time exploring the displaced object during the testing trial. *Post hoc* analysis revealed that both groups spent significantly more time exploring the displaced object during the testing trial than during the training trials (*** represents $P<0.001$). Data are presented as a percentage of the total exploration time, mean ± SEM, Statistical analysis: two-way ANOVA with *post hoc* Bonferroni, n=12 per group.

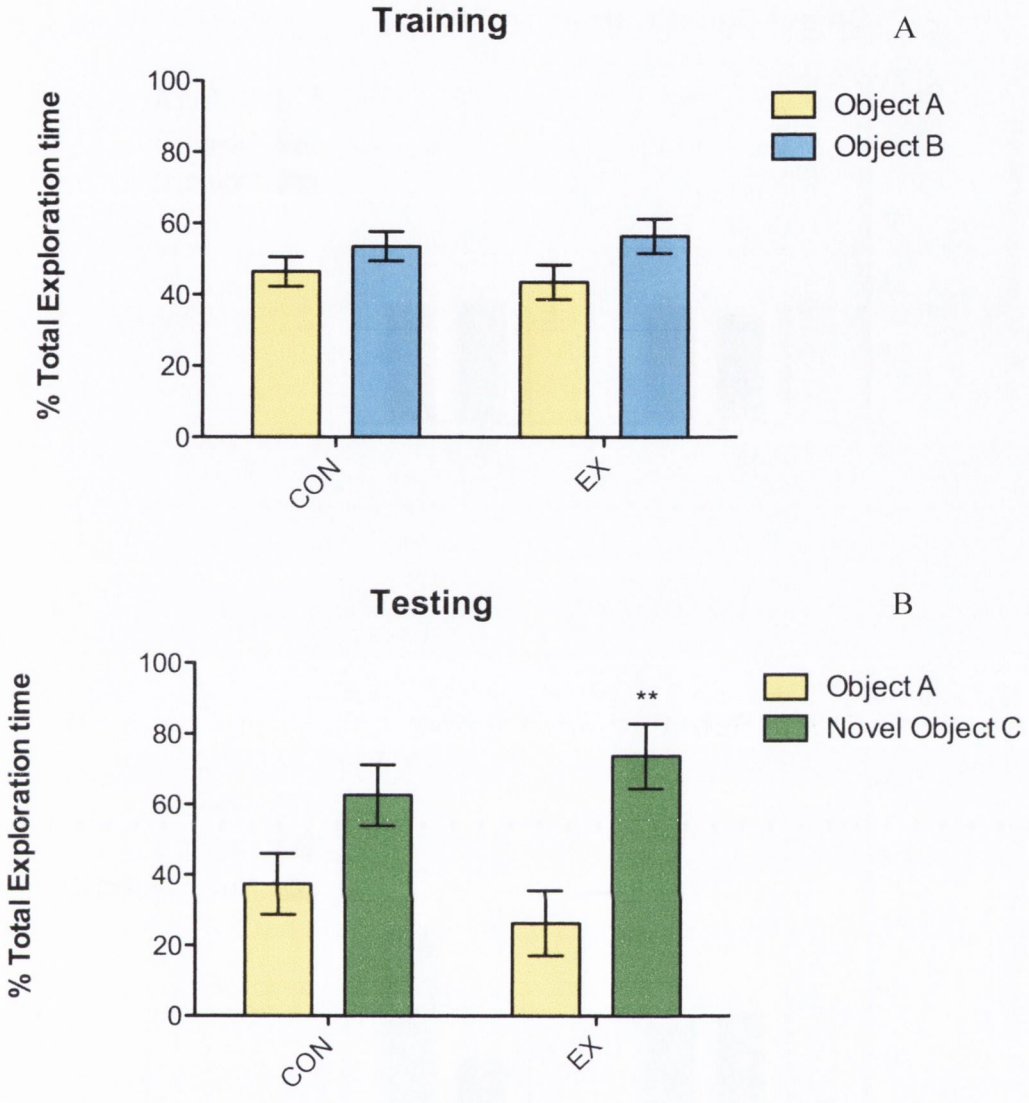
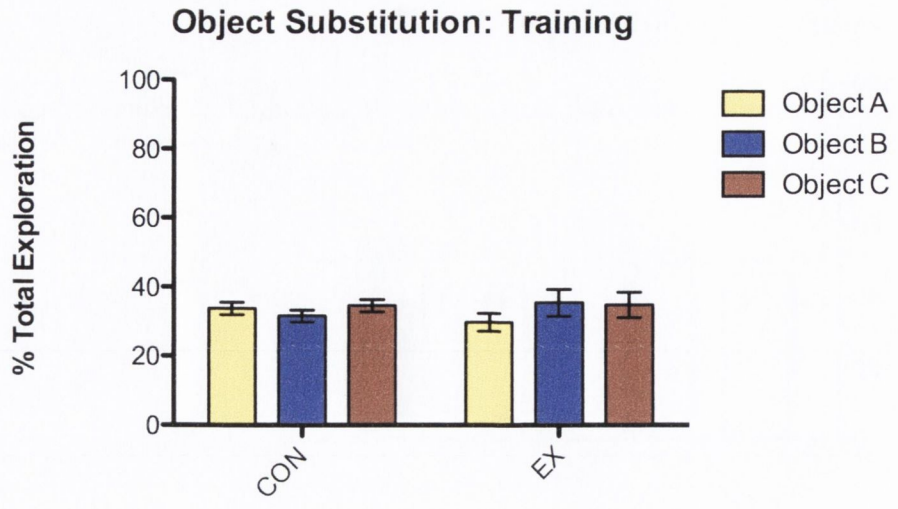


Figure 4.5: Effect of 7 days of forced exercise on the object substitution task

7 days of forced exercise enhanced performance in the object substitution task (2-object variant). **(A)** Both groups spent equal amounts of time exploring objects A and B during the training trials. **(B)** During the testing trials there was an overall effect of object ($P=0.0004$, $F_{(1,26)}=16.27$) indicating a preference for the novel object C. *Post hoc* analysis revealed that the EX group spent significantly more time exploring the novel object C relative to their exploration of the familiar object A (** represents $P<0.001$). Data are presented as a percentage of the total exploration time, mean ± SEM. Statistical analysis: two-way ANOVA and *post hoc* Bonferroni, CON n=7, EX n=8.

A



B

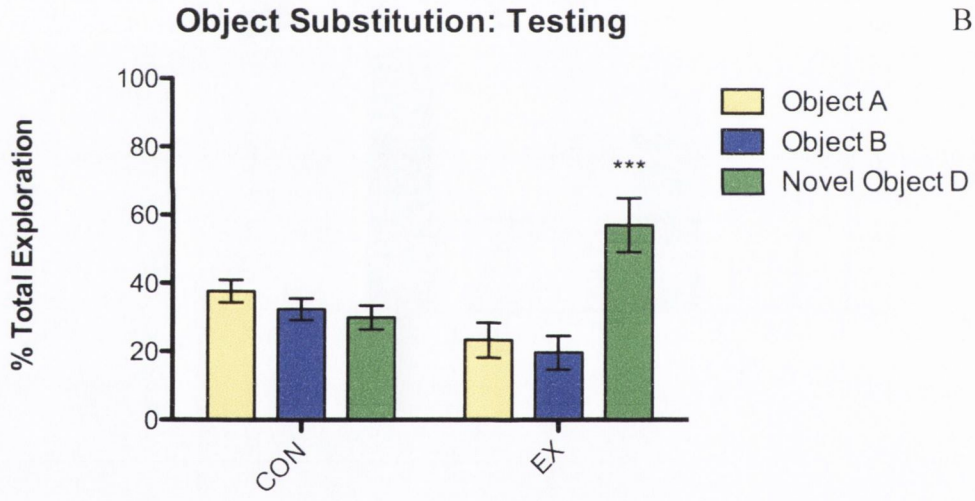


Figure 4.6: Effect of 7 days of forced exercise on the 3-object variant of the object substitution task

7 days of forced exercise enhanced performance in the 3-object variant of the object substitution task. **(A)** Both groups spent equal amounts of time exploring the 3 objects during the training phase. **(B)** During the testing trials there was an overall effect of object ($P=0.044$, $F_{(2,39)}=6.245$) and a significant interaction ($P=0.0002$, $F_{(2,39)}=10.41$). *Post hoc* analysis revealed a significant preference for the novel object D in the EX group only (*** represents $P<0.001$). Data are presented as a percentage of the total exploration time, mean ± SEM. Statistical analysis: two-way ANOVA and *post hoc* Bonferroni, CON n=7, EX n=8.

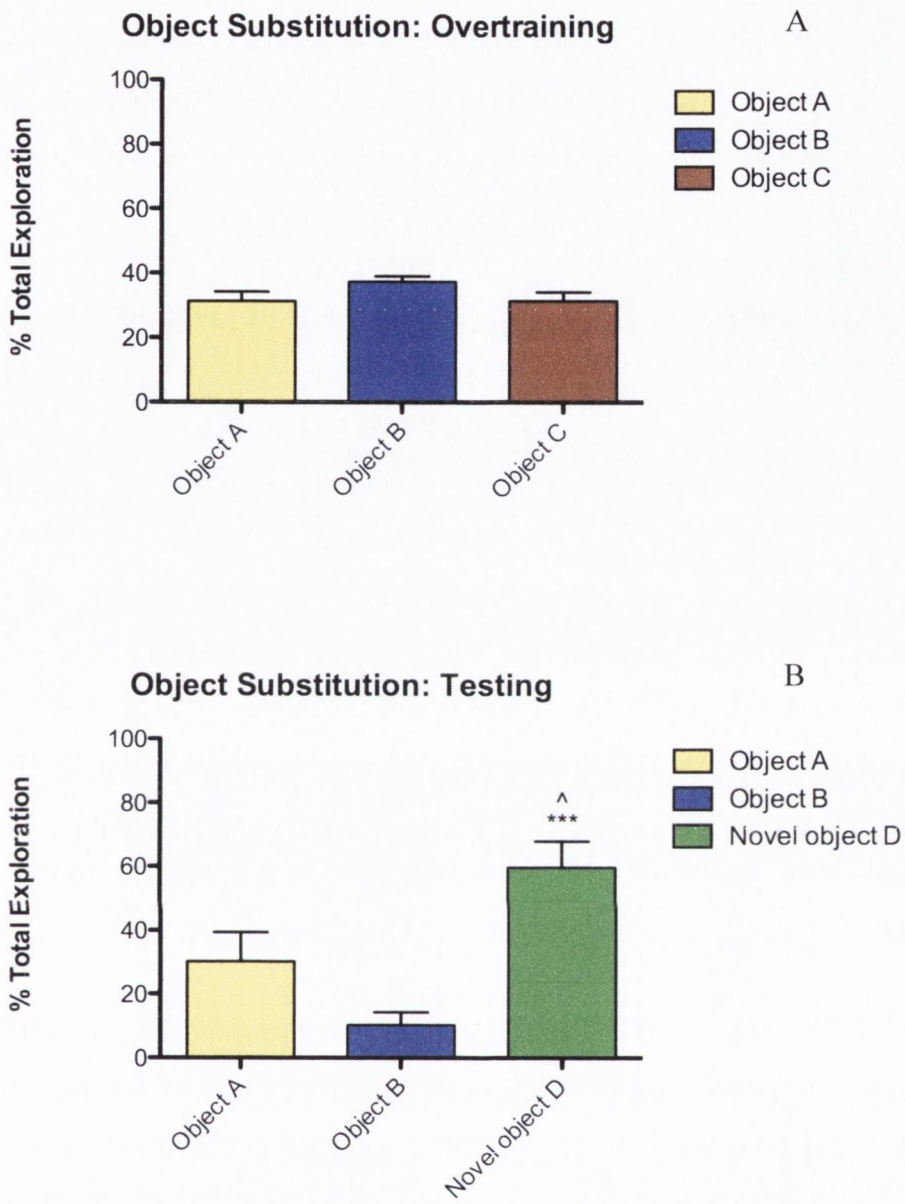


Figure 4.7: Effect of overtraining on the 3-object variant of the object substitution task

Overtraining in the 3-object variant of the object substitution task resulted in learning in sedentary rats. **(A)** The animals spent equal amounts of time exploring the 3 objects during the training phase. **(B)** During the testing trial there was a significant overall effect of the mean ($P=0.0005$, $F_{(2,21)}=10.99$), indicating a preference for the one of the objects. *Post hoc* analysis revealed a significant preference for the novel object D (^ represents $P<0.05$ relative to object A, ***represents $P<0.001$ relative to object B). Data are presented as a percentage of the total exploration time, mean ± SEM. Statistical analyses: one-way ANOVA and *post hoc* Newman-Keuls, n=8.

4.3.2. BDNF concentrations and exercise

Serum BDNF concentration increased across the 7-day forced exercise protocol (Figure 4.8, A). There was a significant overall effect of day (P=0.0001, $F_{(2,41)}$=11.50), indicating that BDNF concentration increased across the 7 days. However, there was no significant effect of group, that is, no difference between exercised and sedentary rats. *Post hoc* analysis revealed no differences between the groups for the 3 days sampled (Day 1: CON: 25.694 ± 17.225pg.ml^{-1}, EX: 36.417 ± 14.152pg.ml^{-1}; Day 5: CON: 70.486 ± 16.431pg.ml^{-1}, EX: 124.236 ± 48.098pg.ml^{-1}; Day 8: CON: 240.313 ± 46.081pg.ml^{-1}, EX: 157.197 ± 22.378 pg.ml^{-1}). Samples of serum and plasma were collected through the saphenous vein on day 1 and day 5. Trunk blood was taken on day 8, post-behavioural testing, for serum analysis only. Hence, plasma BDNF concentrations were only measured for 5 days. Plasma BDNF was not affected by 5 days of forced exercise (Figure 4.8, B). There was no effect of day or group (Day 1; CON 30.313 ± 6.504pg.ml^{-1}, EX 36.493 ± 10.233pg.ml^{-1}. Day 5; CON: 38.792 ± 11.781pg.ml^{-1}, EX 37.188 ± 17.146pg.ml^{-1}). Circulating BDNF data is represented as pg BDNF per ml serum or plasma, mean ± SEM. Data were analysed by two-way ANOVA and *post hoc* Bonferroni, Day1 & Day 5: CON n=6, EX n=6, Day 8: CON n=12, EX n=11.

The effect of 7 days of forced exercise with and without learning, on serum BDNF was also analysed. Treadmill exercise with learning induced an increase in the concentration of serum BDNF (Figure 4.9). There was an overall effect of group (P=0.0093, $F_{(1,24)}$=7.998) indicating that there was significantly more BDNF in serum of EX animals relative to CON. *Post hoc* analysis revealed a significant increase in BDNF occurred in the EX with learning group relative to CON with learning (CON: 176.020 ± 56.465pg.ml^{-1}; EX: 383.571 ± 77.785pg.ml^{-1}, P<0.05), indicating that forced exercise with learning (in the object substitution task) caused the biggest increase in serum BDNF. Data are presented as pg BDNF per ml serum, mean ± SEM. The statistical analysis used was a two-way ANOVA and *post hoc* Bonferroni. CON with learning n=7, CON no learning n=6, EX with learning n=8, EX no learning n=7.

BDNF concentrations were also analysed in an exercising muscle of the hind limb and in the liver. 7 days of forced exercise increased BDNF protein concentration in the soleus muscle (Figure 4.10 A; EX: 6.266 ± 1.023pg.mg protein^{-1}, CON: 9.888 ±

1.289pg.mg protein^{-1}, P=0.0376, $t_{(21)}$=2.219). However, there was no effect on BDNF protein in the liver (Figure 4.10 B; EX: 100.6 ± 5.195pg.mg protein^{-1}, CON: 111.3 ± 5.320pg.mg protein^{-1}). Data are represented as pg BDNF per mg protein, mean ± SEM. Data were analysed by unpaired Student's *t* tests. Muscle; CON n=12, EX n=11, Liver; CON n=11, EX n=11.

Concentrations of BDNF protein and mRNA were analysed in the dentate gyrus, the perirhinal cortex and the hippocampus. 7 days of moderate forced exercise increased BDNF protein concentrations. There was a significant increase in BDNF protein in the dentate gyrus (Figure 4.11, A (a); CON: 163.4 ± 19.46pg.mg protein^{-1}, EX: 416.7 ± 71.22pg.mg protein^{-1}, P=0.0039, $t_{(19)}$=3.285), the perirhinal cortex (Figure 4.11, B (a); CON: 43.86 ± 7.483pg.mg protein^{-1}, EX 112.9 ± 22.03pg.mg protein^{-1}, P=0.0071, $t_{(22)}$=2.968) and the hippocampus (Figure 4.11, C (a); CON: 55.78 ± 5.797pg.mg protein^{-1}, EX 104.0 ± 17.08pg.mg protein^{-1}, P=0.0177, $t_{(21)}$=2.574). BDNF protein concentration is reported in pg BDNF per mg protein, mean ± SEM. The statistical analyses used were unpaired Student's *t* tests. Dentate gyrus: CON n=10, EX n=11. Perirhinal cortex: CON n=12, EX n=12. Hippocampus: CON n=11, EX n=12.

Interestingly, 7 days of forced exercise had no effect on BDNF mRNA in the dentate gyrus (Figure 4.11, A (b); CON: 1.124 ± 0.1238, EX: 0.9162 ± 0.1292), the perirhinal cortex (Figure 4.11, B (b); CON: 0.6023 ± 0.0736, EX: 0.6590 ± 0.1266), or the hippocampus (Figure 4.11, C (b); CON: 2.308 ± 0.3030, EX: 2.807 ± 0.3321). Data are represented as relative quotient (RQ), mean ± SEM. The statistical analyses used were unpaired Student's *t* tests. Dentate gyrus: CON n=12, EX n=11. Perirhinal cortex: CON n=12, EX n=11. Hippocampus: CON n=10, EX n=10.

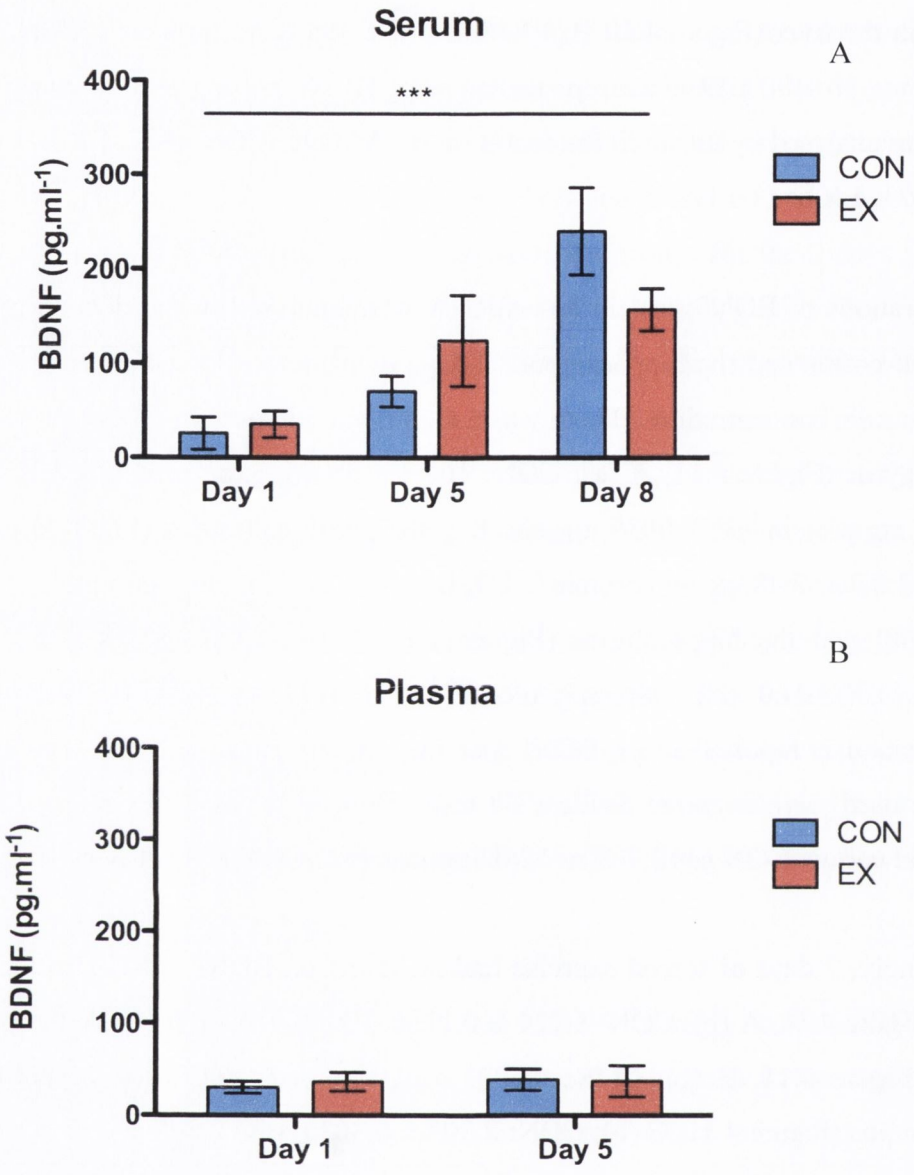


Figure 4.8: Effect of 7 days of forced exercise on circulating BDNF concentrations

(A) Serum BDNF concentration increased across the 7-day forced exercise protocol There was a significant overall effect of day ($P=0.0001$, $F_{(2,41)}=11.50$) but no effect of group. *Post hoc* analysis revealed no between-group differences on any of the 3 days sampled. **(B)** Plasma BDNF concentration was not affected by 1 or 5 days of forced exercise. Data presented as pg BDNF per ml serum or plasma, mean ± SEM. Statistical analyses: two-way ANOVA and *post hoc* Bonferroni. Day1 & Day 5: CON n=6, EX n=6, Day 8: CON n=12, EX n=11.

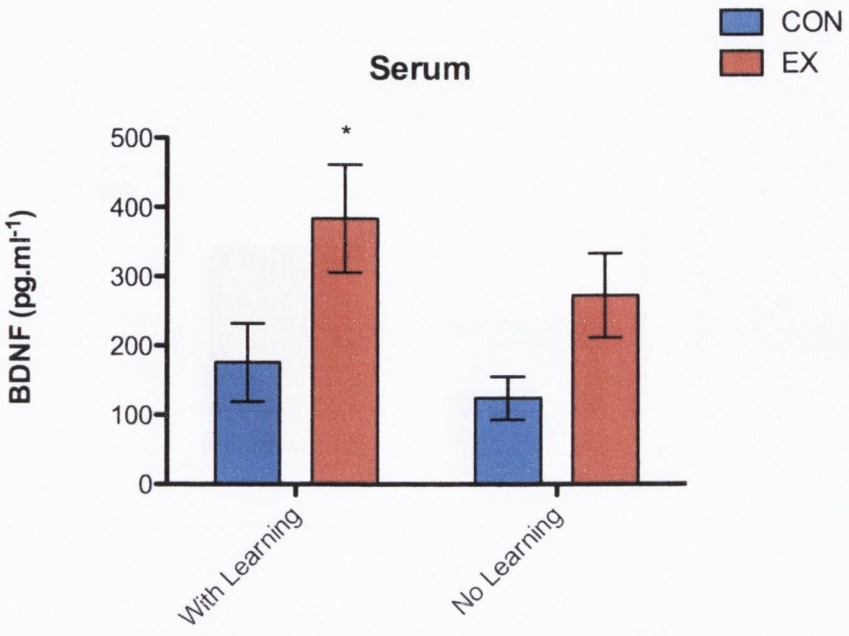


Figure 4.9: Effect of 7 days of forced exercise, with and without learning, on serum BDNF concentrations

Serum BDNF concentration increased with 7 days forced exercise and learning in the object substitution task. There was an overall effect of group ($P=0.0093$, $F_{(1,24)}=7.998$). *Post hoc* analysis revealed the BDNF increase occurred in the EX with learning group (*indicates $P<0.05$). Data are presented as pg BDNF per ml serum, mean ± SEM. Statistical analysis: two-way ANOVA and *post hoc* Bonferroni. CON with learning n=7, CON no learning n=6, EX with learning n=8, EX no learning n=7.

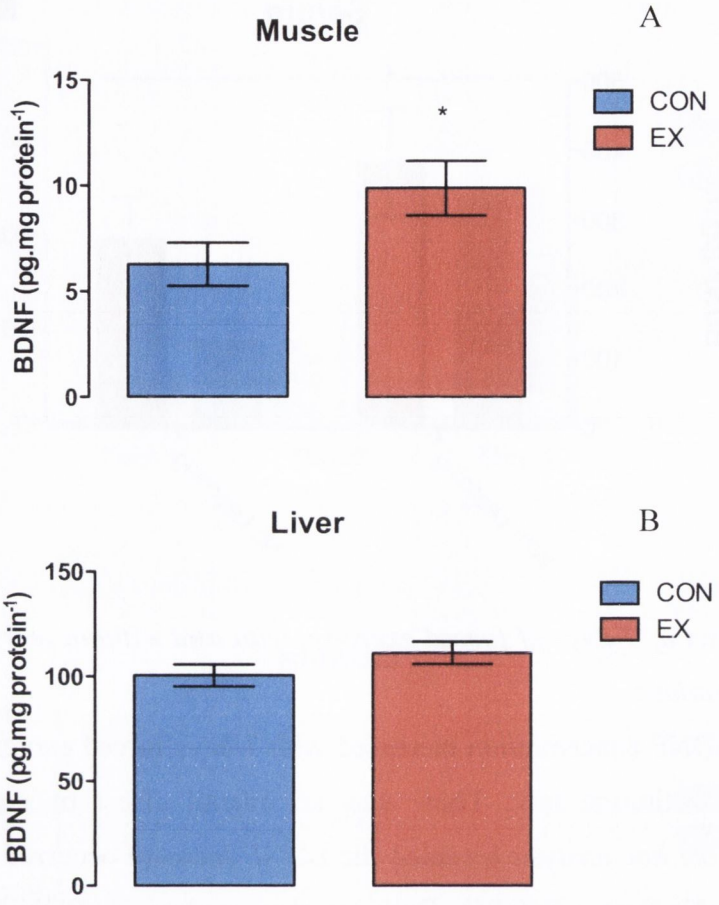


Figure 4.10: Effect of 7 days of forced exercise on BDNF concentrations in muscle and liver

(A) 7 days of forced exercise induced an increase in BDNF protein concentration in the soleus muscle (* represents $P<0.05$, $t_{(21)}=2.219$), (CON n=12, EX n=11). **(B)** There was no effect on BDNF protein concentration in the liver (CON n=11, EX n=11). Data presented as pg BDNF per mg protein, mean ± SEM. Statistical analyses: unpaired Student's *t* tests.

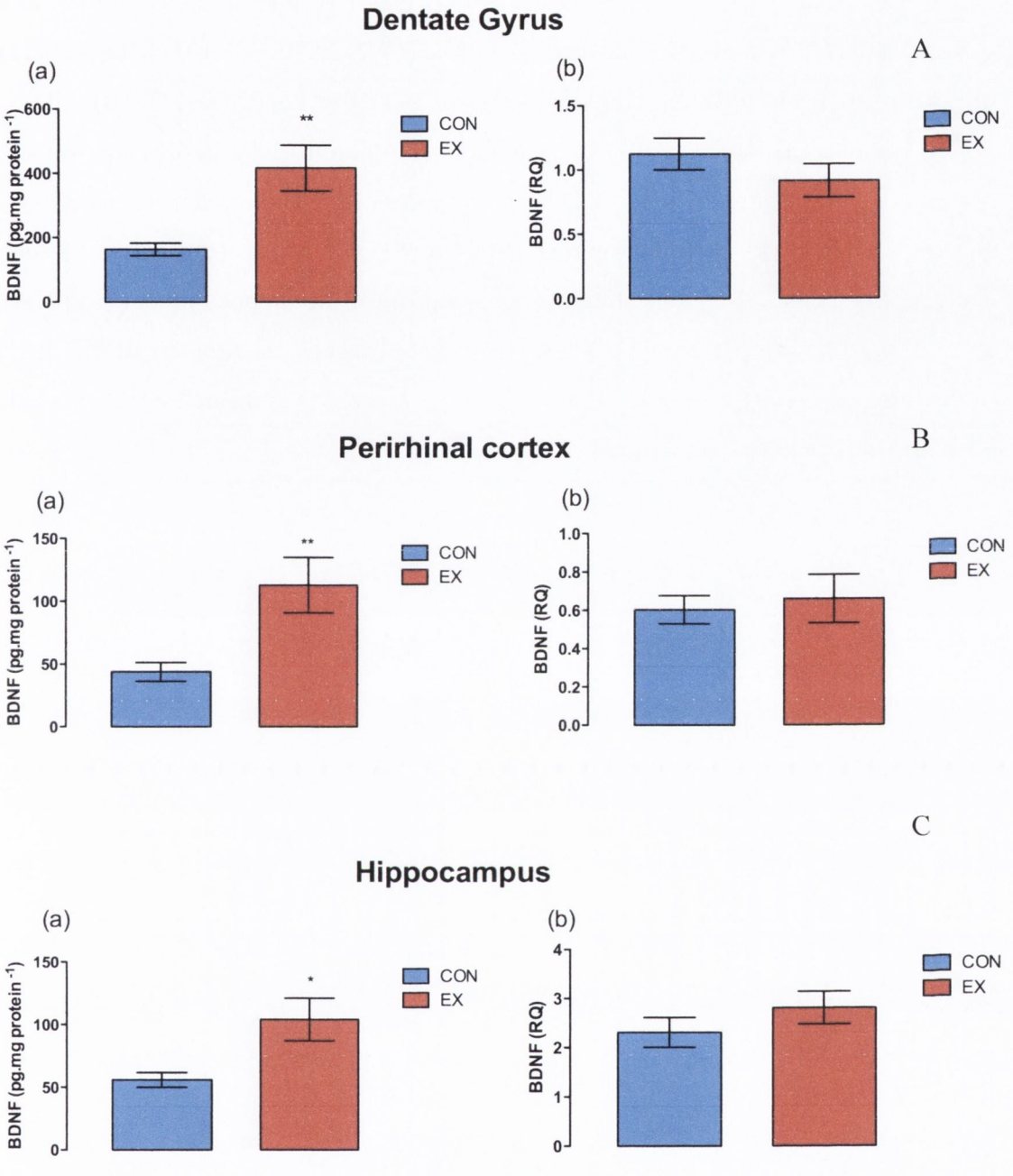


Figure 4.11: Effect of 7 days of forced exercise on BDNF concentration in the dentate gyrus, perirhinal cortex and hippocampus

(A) 7 days of forced exercise (a) increased BDNF protein concentration in the dentate gyrus (** represents $P<0.01$, $t_{(19)}=3.285$), (CON n=10, EX n=11). There was no effect on (b) BDNF mRNA in the dentate gyrus (CON n=12, EX n=11). **(B)** 7 days of forced exercise (a) increased BDNF protein concentration in the perirhinal cortex (** represents $P<0.01$, $t_{(22)}=2.968$), (CON n=12, EX n=12). There was no effect on (b) BDNF mRNA in the perirhinal cortex (CON n=12, EX n=11). **(C)** 7 days of forced exercise (a) increased BDNF protein concentration in the hippocampus (* represents $P<0.05$, $t_{(21)}=2.574$), (CON n=11, EX n=12). There was no effect on (b) BDNF mRNA in the hippocampus (CON n=10, EX n=10). Data presented as mean ± SEM. Statistical analyses: unpaired Student's *t* tests.

4.3.3. Analysis of NT4/5 concentrations

7 days of forced exercise had no effect on NT-4/5 concentrations in the dentate gyrus, perirhinal cortex or hippocampus. NT-4/5 protein was analysed by ELISA and NT-4/5 mRNA was analysed by RT-PCR. The concentrations in the dentate gyrus, of NT-4/5 protein (Figure 4.12, A (a): CON: 325.2 ± 78.28pg.mg protein^{-1}, n=8; EX: 304.6 ± 56.65pg.mg protein^{-1}, n=9) and NT-4/5 mRNA (Figure 4.12, A (b) CON: 0.886 ± 0.083, n=11; EX: 1.295 ± 0.2184, n=12) were not altered by the forced exercise protocol. Similarly, there was no difference between groups in the concentrations of NT-4/5 protein (Figure 4.12, B (a): CON: 1204 ± 292.2pg.mg protein^{-1}, n=12; EX: 1246 ± 271.0pg.mg protein^{-1}, n=8) or NT-4/5 mRNA (Figure 4.12, B (b) CON: 1.079 ± 0.125, n=12; EX: 1.369 ± 0.227, n=11) in the perirhinal cortex. Also, there was no change in NT-4/5 protein concentration in the hippocampus (Figure 4.12, C: CON: 1430 ± 184.1pg.mg protein^{-1}, n=12; EX: 1077 ± 148.9pg.mg protein^{-1}, n=11). Protein data are presented as pg NT-4/5 per mg protein, mean ± SEM, mRNA data are presented as RQ values with β-actin as an endogenous control, mean ± SEM. The statistical analyses used were unpaired Student's *t* tests.

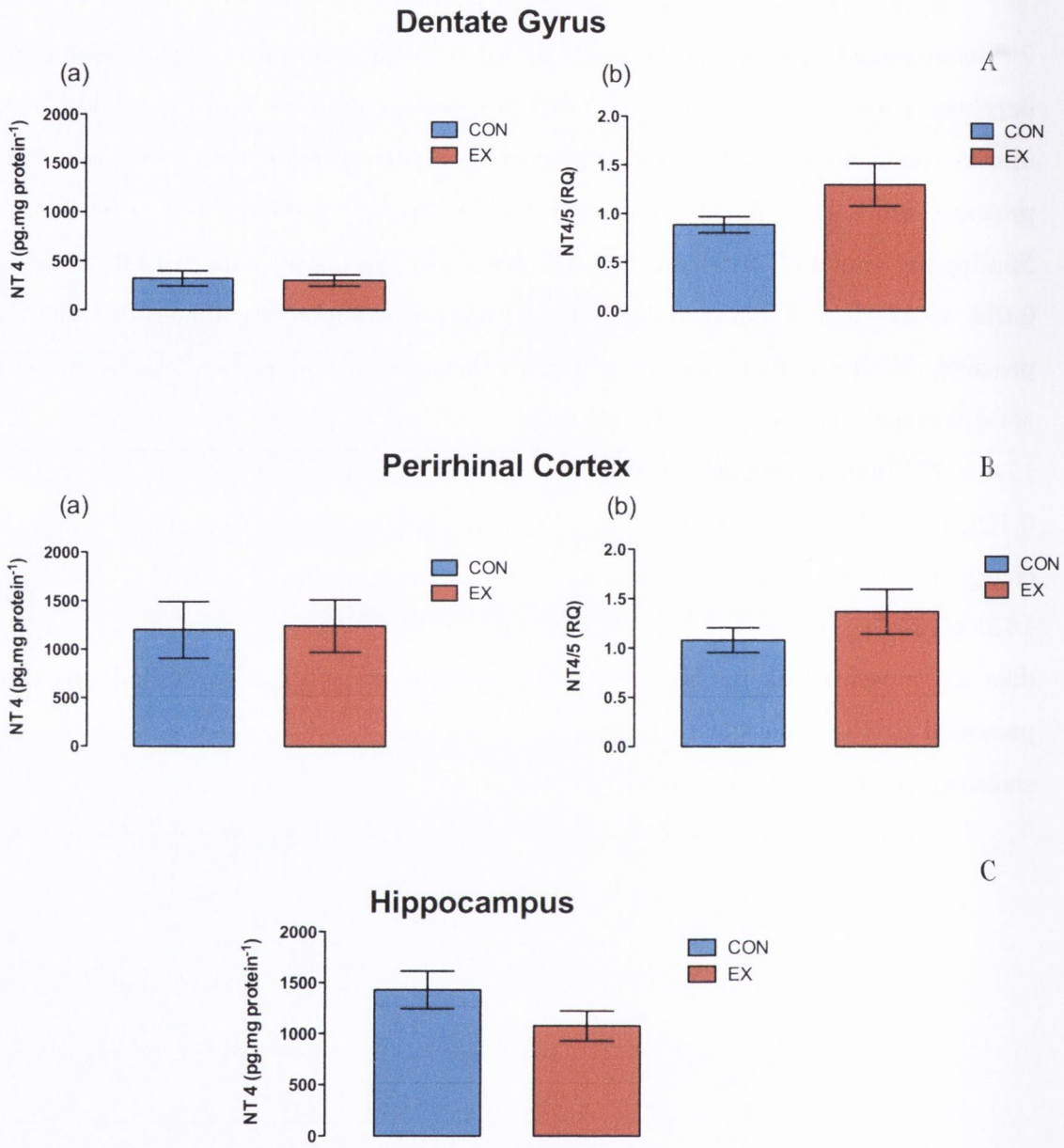


Figure 4.12: Effect of 7 days of forced exercise on NT-4/5 concentrations in the dentate gyrus, perirhinal cortex and hippocampus

(A) 7 days of moderate forced exercise had no effect on (a) NT-4/5 protein concentration (CON n=8, EX n=9) or (b) NT4/5 mRNA concentration in the dentate gyrus (CON n=11, EX n=12). **(B)** 7 days of forced exercise had no effect on (a) NT-4/5 protein concentration (CON n=12 EX n=8) or (b) NT-4/5 mRNA concentration in the perirhinal cortex (CON n=12, EX n=11). ***(C)*** 7 days of forced exercise had no effect on NT-4/5 protein concentration in the hippocampus (CON n=12 EX n=11). Protein data presented as pg NT-4/5 per mg protein, mean ± SEM. NT-4/5 mRNA presented as (RQ) with β-actin as an endogenous control. Statistical analysis: unpaired Student's *t* test.

4.3.4. Analysis of NGF concentrations

7 days of forced exercise had no effect on NGF protein concentration in the dentate gyrus (Figure 4.13, A (a): CON: 134.7 ± 28.62pg.mg protein^{-1}, n=12; EX: 89.63 ± 21.37pg.mg protein^{-1}, n=10). However, the forced exercise protocol resulted in a significant decrease in NGF protein concentration in the perirhinal cortex (Figure 4.13, B (a): CON: 49.65 ± 4.523pg.mg protein^{-1}, n=11; EX: 28.90 ± 7.069pg.mg protein^{-1}, n=8; P=0.019, $t_{(17)}$=2.591) but had no effect on NGF protein in the hippocampus (Figure 4.13, C (a): CON: 54.20 ± 6.041pg.mg protein^{-1}, n=11; EX: 56.71 ± 6.137pg.mg protein^{-1}, n=12). Data are presented as pg NGF per mg protein, mean ± SEM. The statistical analyses used were unpaired Student's *t* tests.

Equally, 7 days of forced exercise had no effect on β-NGF mRNA in the brain. β-NGF mRNA expression was measured by multiplex RT-PCR using ß-actin as an endogenous control. The forced exercise protocol had no affect on β-NGF mRNA in the dentate gyrus (Figure 4.13, A (b): CON: 1.031 ± 0.078, EX: 1.021 ± 0.075), the perirhinal cortex (Figure 4.13, B (b): CON: 1.018 ± 0.064, EX: 0.864 ± 0.071) or the hippocampus (Figure 4.13, C (b): CON: 1.038 ± 0.087, EX: 1.025 ± 0.069). Data are presented as RQ values, mean ± SEM, n=12 per group, with the exception of CON in the perirhinal cortex, in which n=11. The statistical analyses used were unpaired Student's *t* tests.

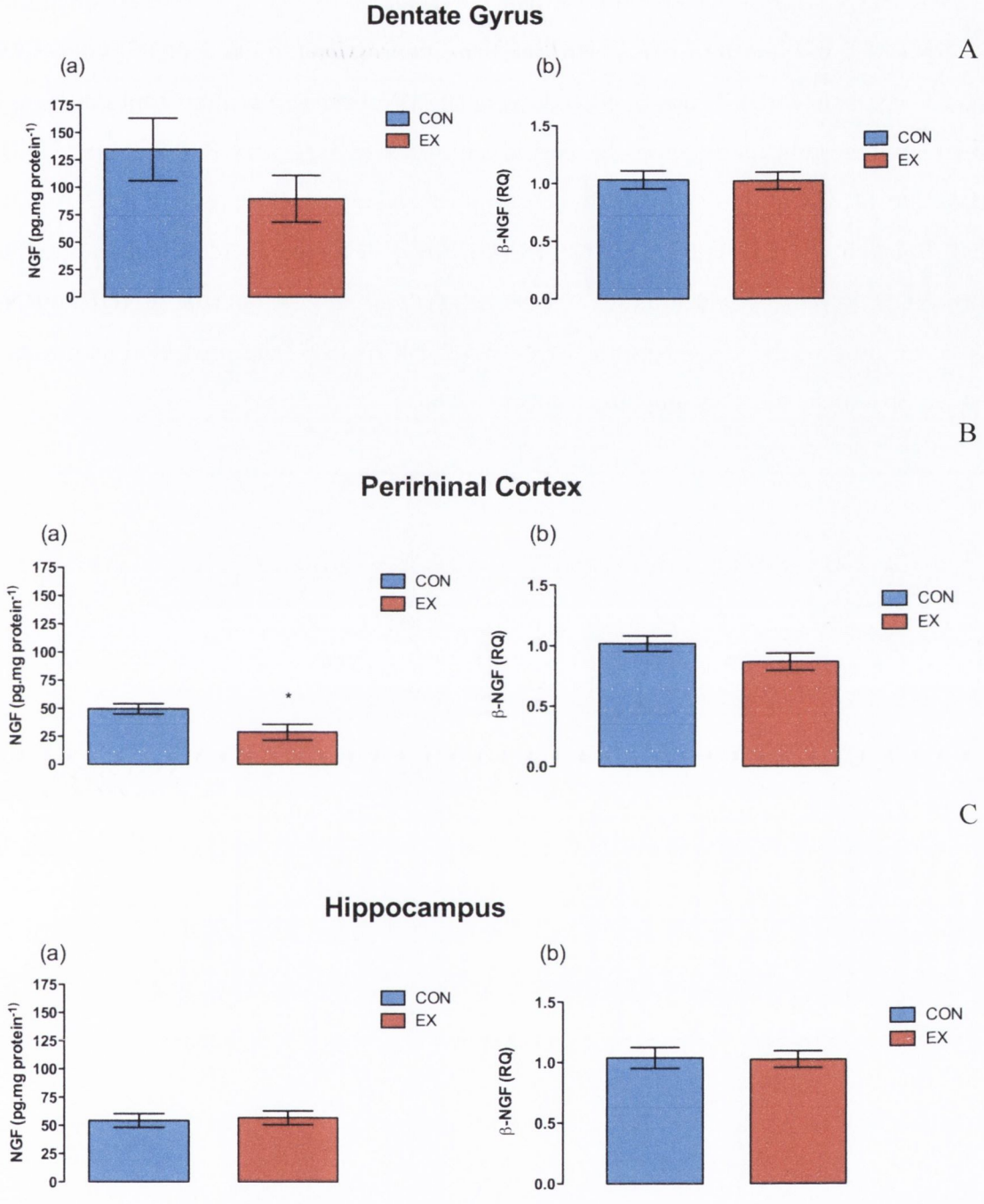


Figure 4.13: Effect of 7 days of forced exercise on NGF concentrations in the dentate gyrus, perirhinal cortex and hippocampus

(A) 7 days of forced exercise had no effect on (a) NGF protein concentration (CON n=12, EX n=10) or (b) β-NGF mRNA concentration in the dentate gyrus (CON n=12, EX n=12). **(B)** 7 days of forced exercise (a) resulted in a significant decrease in NGF protein concentration in the perirhinal cortex (* represent $P<0.05$, $t_{(17)}=2.591$), (CON n=11, EX n=8). (b) 7 days forced exercise had no affect on β-NGF mRNA in the perirhinal cortex (CON n=12, EX n=11). **(C)** 7 days of forced exercise had no affect on (a) NGF protein concentration (CON n=11, EX n=12) or (b) β-NGF mRNA concentration in the hippocampus (CON n=12, EX n=12). Data presented as means ± SEM. Statistical analyses: unpaired Student's *t* tests.

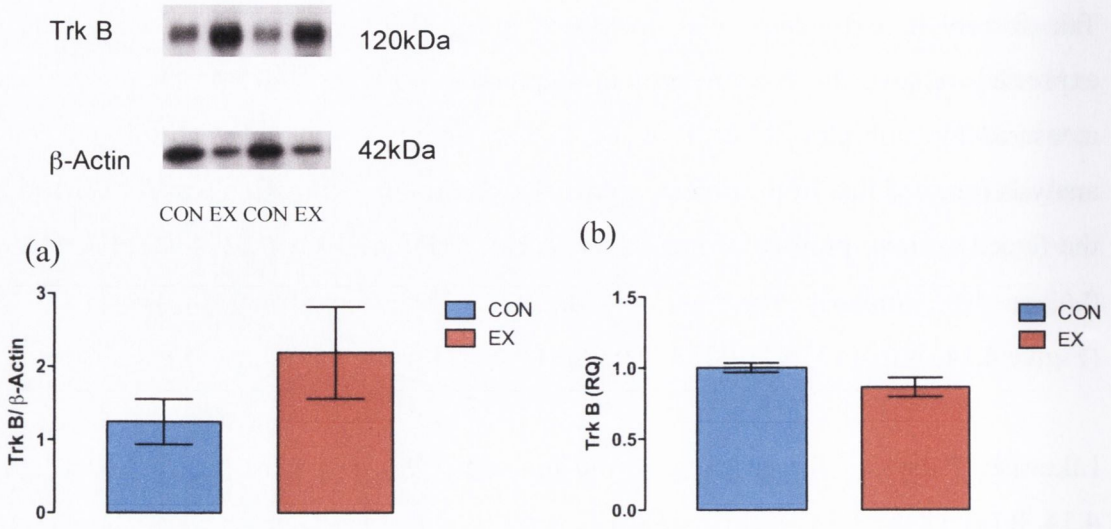
4.3.5. Analysis of Trk B receptor expression

7 days of forced exercise did not affect Trk B expression in any brain region analysed. Trk B protein expression was measured using Western immunoblotting and is expressed relative to ß-actin protein expression. Trk B mRNA expression was measured by multiplex RT-PCR using ß-actin as an endogenous control. Statistical analysis revealed that in the dentate gyrus, Trk B protein expression was not altered by the forced exercise protocol (Figure 4.14, A (a): CON: 1.248 ± 0.31, n=11; EX: 2.89 ± 0.63, n=10). Similarly, there was no difference between groups in Trk B mRNA (Figure 4.14, A (b): CON: 1.002 ± 0.03, n=11; EX: 0.8646 ± 0.07, n=11).

Likewise, 7 days of forced exercise did not affect Trk B protein expression (Figure 4.14, B (a): CON: 1.142 ± 0.10, n=8; EX: 1.308 ± 0.21, n=12) or Trk B mRNA (Figure 4.14, B (b): CON: 1.051 ± 0.03, n=12; EX: 1.051 ± 0.04, n=10) in the perirhinal cortex. Also, 7 days forced exercise did not affect Trk B protein expression (Figure 4.14, C (a): CON: 1.011 ± 0.15, n=10; EX: 1.249 ± 0.27, n=11) or Trk B mRNA (Figure 4.14, C (b): CON: 0.6681 ± 0.06, n=12; EX: 0.6461 ± 0.06, n=11) in the hippocampus. Data are presented as RQ values, mean ± SEM. The statistical analyses used were unpaired Student's *t* tests.

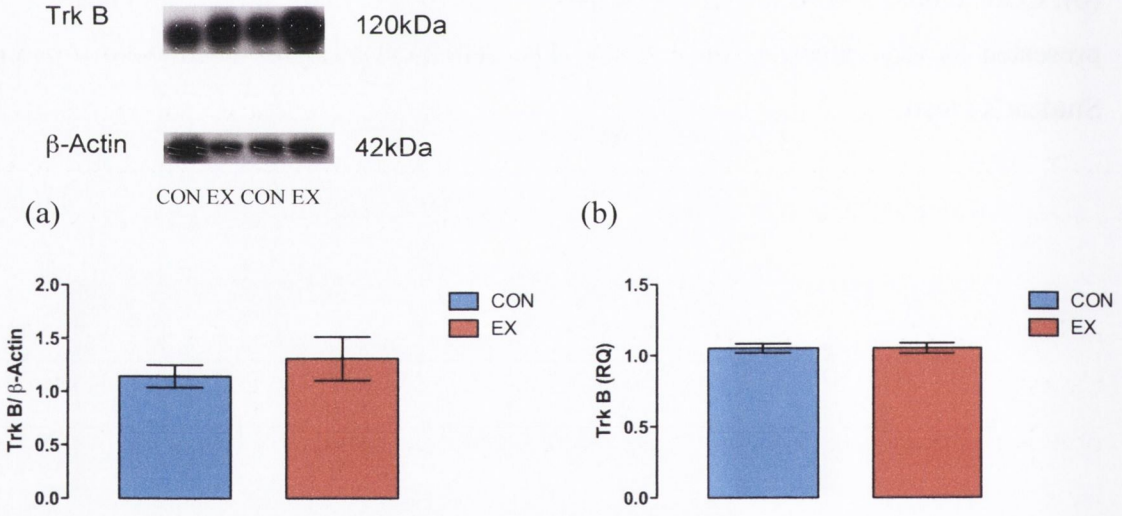
Dentate Gyrus

A



Perirhinal Cortex

B



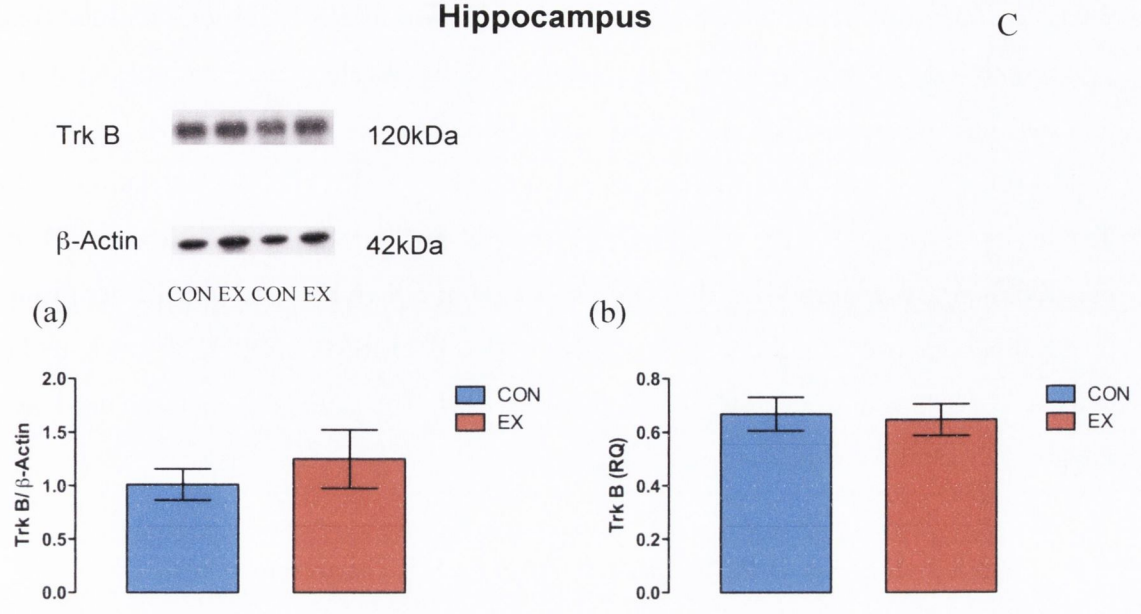


Figure 4.14: Effect of 7 days of forced exercise on Trk B expression in the dentate gyrus, perirhinal cortex and hippocampus

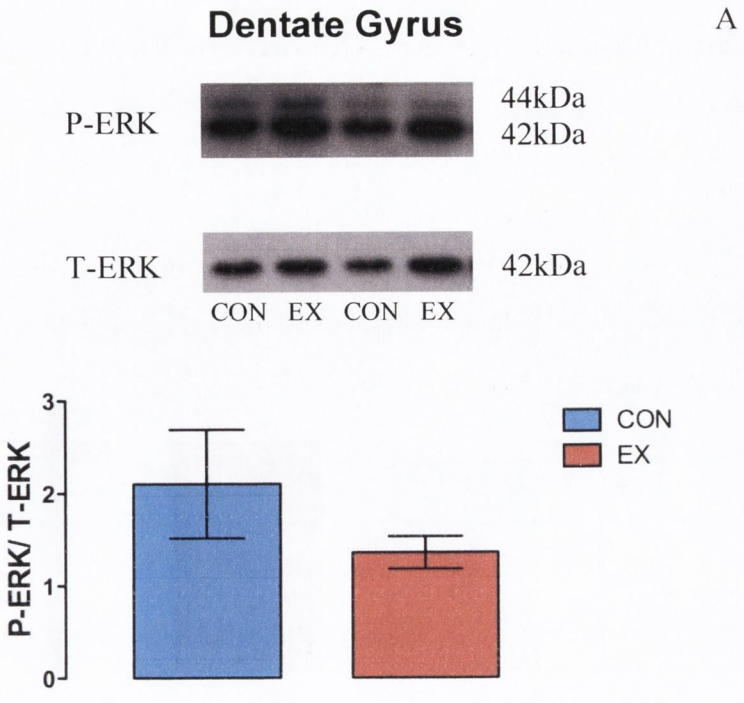
(A) 7 days forced exercise did not affect (a) Trk B protein expression (CON n=11, EX n=10) or (b) Trk B mRNA (CON n=11, EX n=11) in the dentate gyrus. **(B)** 7 days forced exercise did not affect (a) Trk B protein expression (CON n=8, EX n=12) or (b) Trk B mRNA (CON n=12, EX=10) in the perirhinal cortex. **(C)** 7 days forced exercise did not affect (a) Trk B protein expression (CON n=10, EX n=11) or (b) Trk B mRNA (CON n=12, EX=11) in the hippocampus. Sample blot pictures and histograms are given for Trk B protein expression. Data are presented as Trk B per β-actin for protein and mRNA expression, mean ± SEM. Statistical analysis: Unpaired Student's *t* tests.

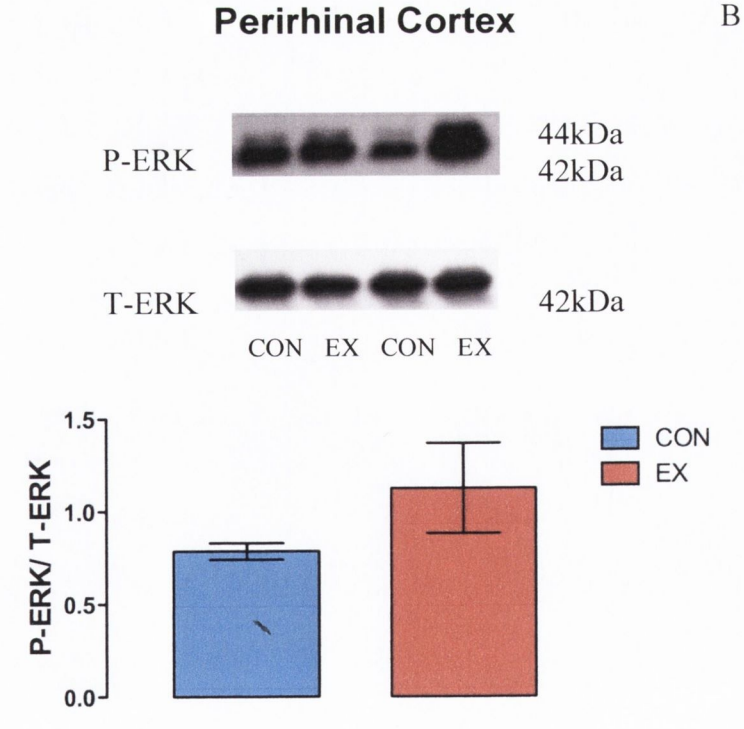
4.3.6. Analysis of ERK activity

7 days of forced exercise did not affect ERK activation in any brain region at the time-point analysed. ERK activation was measured using Western immunoblotting and is reported as Phospho-ERK (P-ERK) expression relative to total-ERK (T-ERK) expression. 7 days of forced exercise had no effect on ERK phosphorylation in the dentate gyrus (Figure 4.15, A: CON: 2.1 ± 0.59, n=11; EX: 1.39 ± 0.18, n=12), the perirhinal cortex (Figure 4.15, B: CON: 0.79 ± 0.04; EX: 1.13 ± 0.24, n=12 per group), or the hippocampus (Figure 4.15, C: CON: 1.13 ± 0.25, n=12; EX: 1.24 ± 0.1, n=11). P-ERK is expressed as a % of T-ERK, mean ± SEM. The statistical analyses used were unpaired Student's *t* tests.

4.3.7. Analysis of P-Synapsin 1 and P-CaMKii expression

7 days of forced exercise had no effect on P-Synapsin 1 expression in the dentate gyrus (Figure 4.16, A: CON: 0.437 ± 0.071, n=13; EX: 0.416 ± 0.065, n=15). 7 days of mild forced exercise also had no effect on P-CaMKii expression in the dentate gyrus (Figure 4.16, B: CON: 0.743 ± 0.037, n=13; EX: 0.705 ± 0.036, n=14). P-Synapsin 1 and P-CamKii expression are reported per β-Actin expression, mean ± SEM. The statistical analyses used were unpaired Student's *t* tests.


Dentate Gyrus
A
P-ERK
44kDa
42kDa
T-ERK
42kDa
CON EX CON EX
P-ERK/ T-ERK
3
2
1
0
CON
EX


Perirhinal Cortex
B
P-ERK
44kDa
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T-ERK
42kDa
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1.5
1.0
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CON
EX

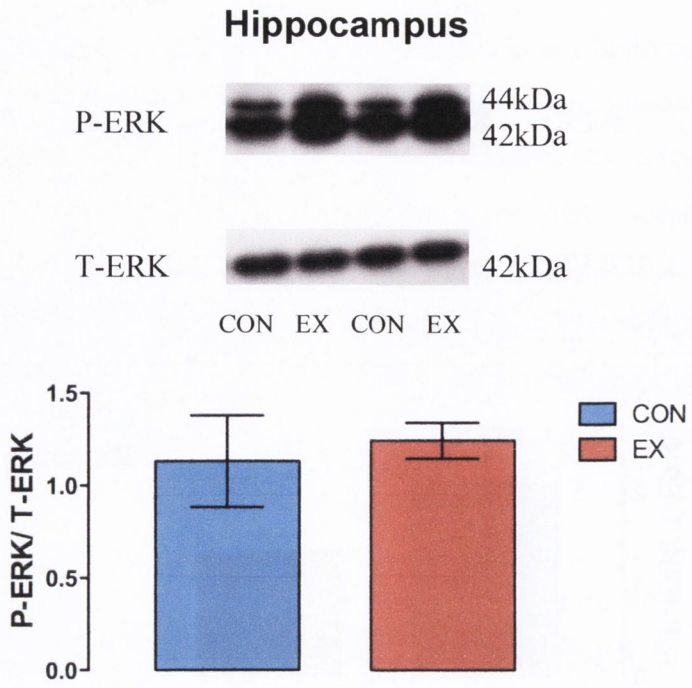

Figure 4.15: Effect of 7 days of forced exercise on ERK activation in the dentate gyrus, perirhinal cortex and hippocampus

(A) 7 days of forced exercise had no effect on ERK activity in the dentate gyrus (CON n=11, EX n=12). **(B)** 7 days of forced exercise had no effect on ERK activity in the perirhinal cortex (CON n=12, EX n=12). **(C)** 7 days of forced exercise had no effect on ERK activity in the hippocampus (CON n=12, EX n=11). Sample blot pictures and histograms are given. ERK activity is reported as P-ERK expression per T-ERK expression, mean ± SEM. Statistical analysis: Student's *t* tests.

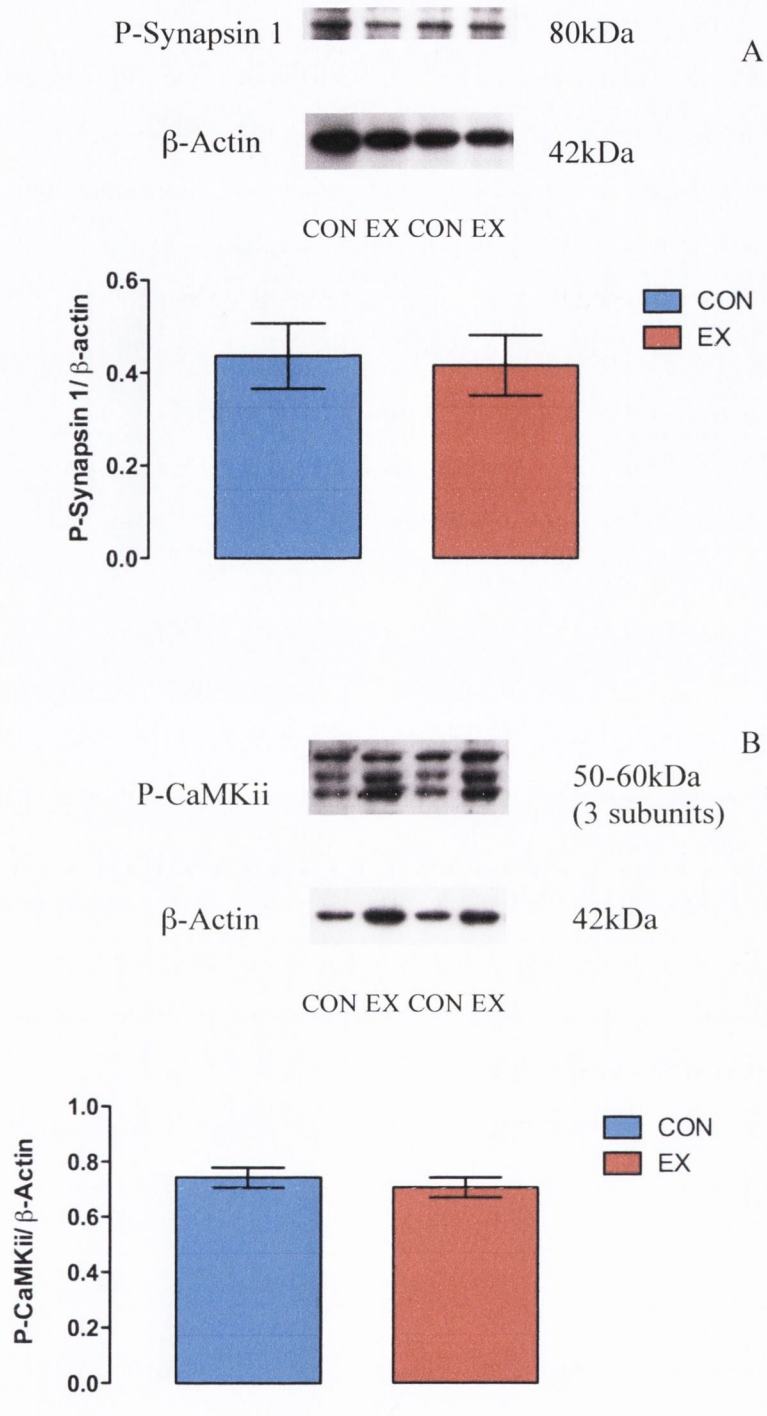

Figure 4.16: Effect of 7 days of forced exercise on P-Synapsin 1 and P-CaMKii expression in the dentate gyrus

(A) 7 days of mild forced exercise had no effect on P-Synapsin 1 expression in the dentate gyrus (CON n=13, EX n=15). ***(B)*** 7 days of mild forced exercise had no effect on P-CaMKii expression in the dentate gyrus (CON n=13, EX n=14). Sample blot pictures and histograms are given. P-Synapsin 1 and P-CamKii expression are reported per β-Actin expression, mean ± SEM. Statistical analysis: Student's *t* tests.

4.3.8. Analysis of BrdU positive cells

7 days of moderate forced exercise had no significant effect on the expression of BrdU positive cells in the dentate gyrus (Figure 4.17: CON: 2.375 ± 0.756%, n=7; EX: 5.125 ± 2.511%, n=5). BrdU positive cells were stained by immunohistochemistry and are expressed as a percentage of the total number of cells counted per slide, mean ± SEM. The statistical analysis used was an unpaired Student's *t* test.

4.3.9. Analysis of Ki67 mRNA and VEGF mRNA

Ki67 mRNA and VEGF mRNA were analysed by RT-PCR. 7 days of forced exercise had no effect on Ki67 mRNA or VEGF mRNA in the brain. Ki67 mRNA was not altered in the dentate gyrus (Figure 4.18 A (a): CON: 1.048 ± 0.092, EX: 1.278 ± 0.175, n=12 per group), the perirhinal cortex (Figure 4.18 B (a): CON: 1.248 ± 0.291; EX: 0.928 ± 0.078, n=11 per group) or the hippocampus (Figure 4.18 C (a): CON: 1.052 ± 0.104, n=11; EX: 0.928 ± 0.078, n=12). Similarly, the forced exercise protocol had no affect on VEGF mRNA in the dentate gyrus (Figure 4.18 A (b): CON: 1.018 ± 0.061, EX: 1.106 ± 0.103, n=12 per group), the perirhinal cortex (Figure 4.18 B (b): CON: 1.005 ± 0.029, n=12; EX: 1.068 ± 0.051, n=11) or the hippocampus (Figure 4.18 C (b): CON: 1.026 ± 0.073, EX: 0.979 ± 0.055, n=12 per group). Data presented as RQ values with β-actin as an endogenous control, mean ± SEM. The statistical analyses used were unpaired Student's *t* tests.

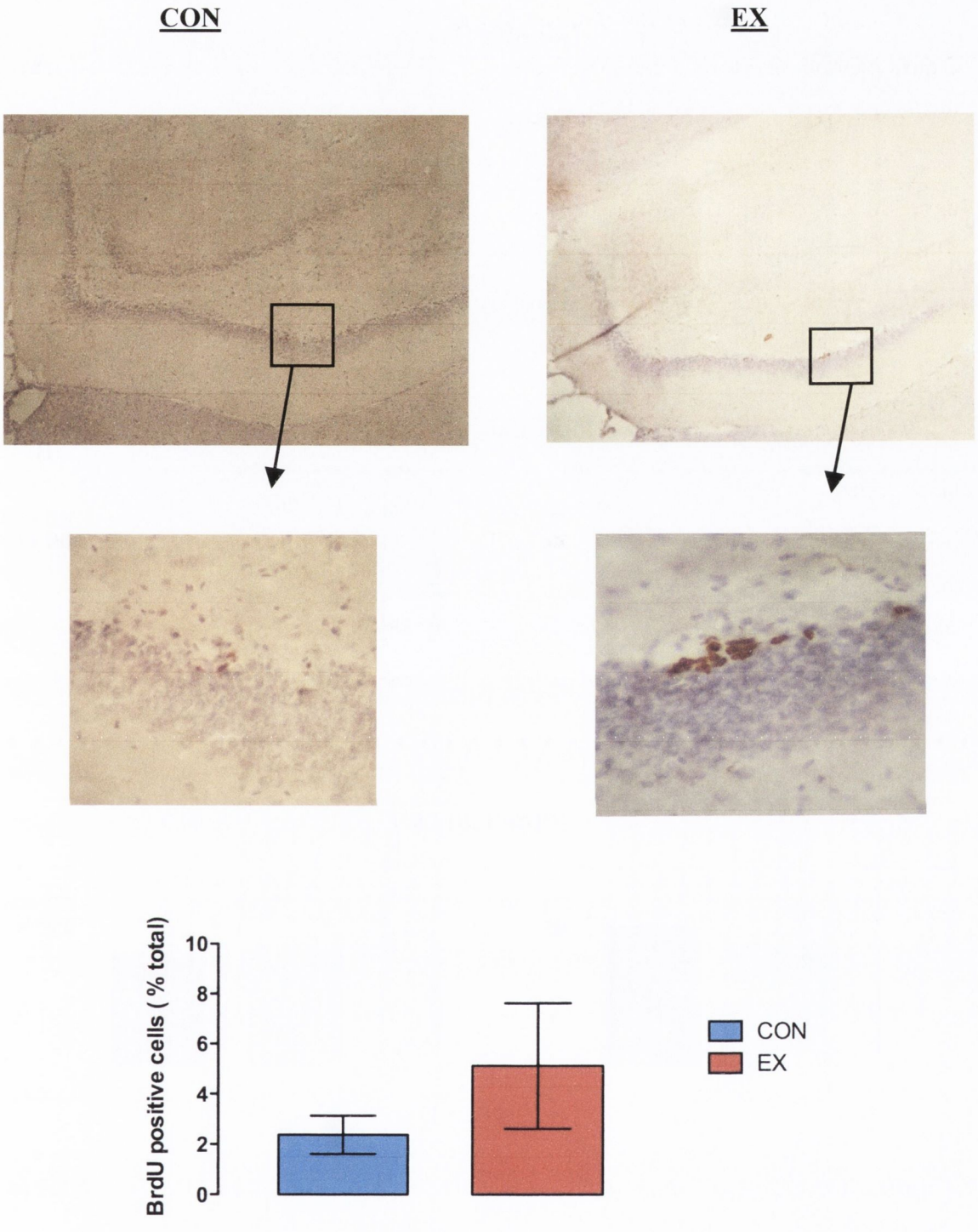

Figure 4.17: Effect of 7 days of forced exercise on BrdU positive staining

7 days of forced exercise had no significant effect on the expression of BrdU positive cells in the dentate gyrus (P=0.2533, $t_{(10)}$=1.212). Sample slide photographs for each group and a histogram are given. BrdU positive cells are expressed as a percentage of the total number of cells counted per slide, mean ± SEM. Statistical analysis: unpaired Student's *t* tests, CON n=7, EX n=5.

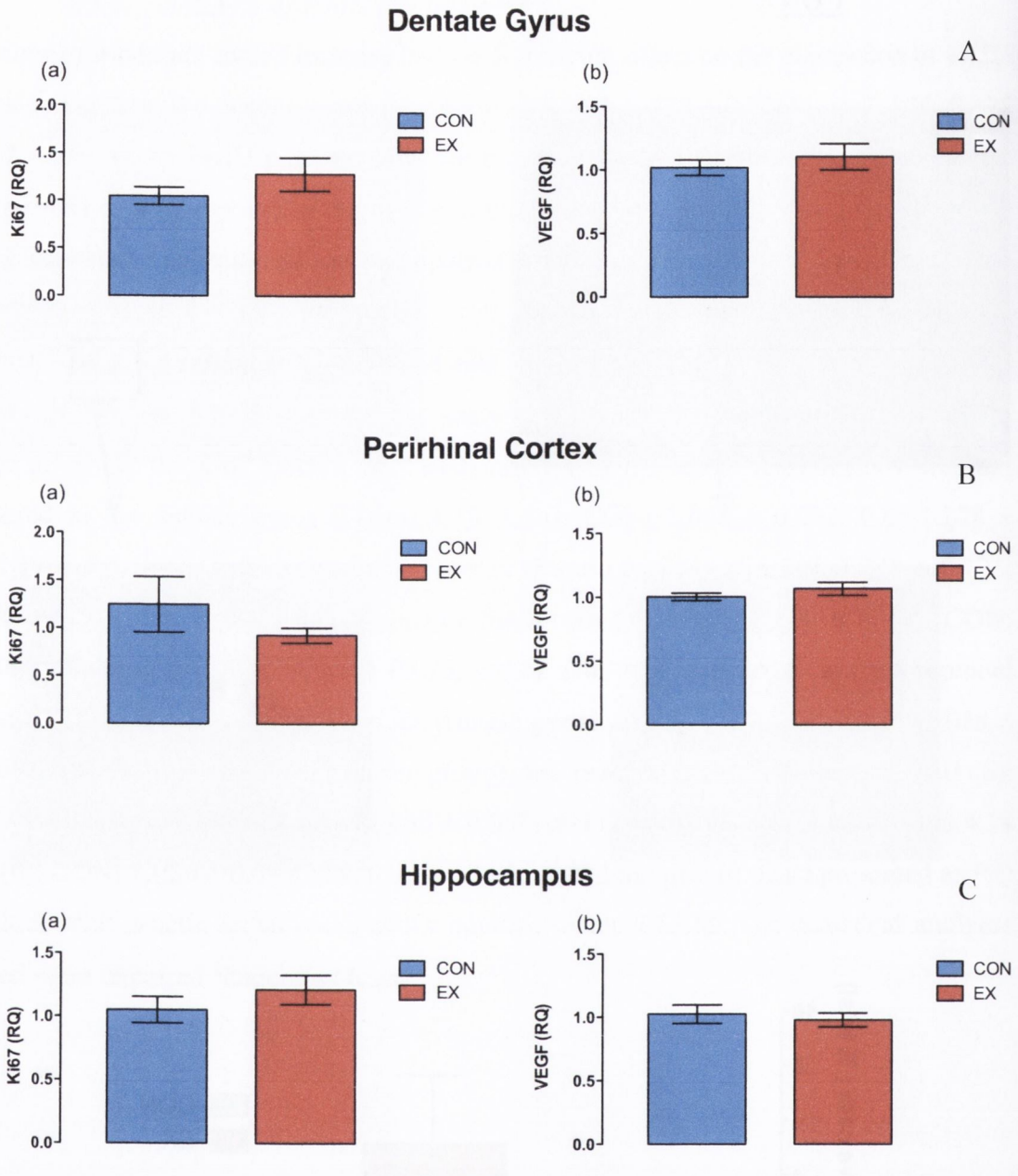

Figure 4.18: Effect of 7 days of forced exercise on Ki67 mRNA and VEGF mRNA in the dentate gyrus, perirhinal cortex and hippocampus

(A) 7 days of forced exercise had no effect on (a) Ki67 mRNA and (b) VEGF mRNA in the dentate gyrus (CON n=12, EX n=12). **(B)** 7 days of forced exercise had no effect on (a) Ki67 mRNA (CON n=11, EX n=12) and (b) VEGF mRNA (n=12 per group) in the perirhinal cortex. **(C)** 7 days of forced exercise had no effect on (a) Ki67 mRNA (CON n=11, EX n=12) and (b) VEGF mRNA (n=12 per group) in the hippocampus. Data presented as RQ values with β-actin as an endogenous control, mean ± SEM. Statistical analyses: unpaired Student's *t* tests.

4.3.10. Analysis of IGF-1 concentrations

7 days of forced exercise did not affect the concentration of IGF-1 in the serum (Figure 4.19, CON: 1064 ± 22.60pg.ml^{-1}, EX: 997.7 ± 33.83pg.ml^{-1}). Data are presented as pg IGF-1 per ml serum, mean ± SEM, CON n=12, EX n=10. IGF-1 concentrations were also analysed in an exercising muscle of the hind limb and in the liver. The forced exercise protocol had no affect on IGF-1 concentrations in the muscle (Figure 4.20 A; CON: 6.375 ± 1.17pg.mg protein^{-1}, EX: 6.734 ± 1.6pg.mg protein^{-1}) or the liver (Figure 4.20 B; CON: 216.1 ± 8.35pg.mg protein^{-1}, CON: 206.8 ± 9.75pg.mg protein^{-1}). Data are presented as pg IGF-1 per mg protein, mean ± SEM. CON n=10, EX n=10.

Similarly, 7 days of forced exercise had no affect on IGF-1 protein concentrations in the dentate gyrus (Figure 4.21 A (a): CON: 263.1 ± 37.04pg.mg protein^{-1}, n=10; EX: 217.1 ± 48.85pg.mg protein^{-1}, n=10), the perirhinal cortex (Figure 4.21, B (a): CON: 286.7 ± 46.42pg.mg protein^{-1}, n=10; EX: 232.2 ± 44.12pg.mg protein^{-1}, n=11) or the hippocampus (Figure 4.21, C (a): CON: 149.6 ± 15.70pg.mg protein^{-1}, n=11; EX: 150.8 ± 12.18pg.mg protein^{-1}, n=12). Data are presented as pg IGF-1 per mg protein, mean ± SEM. Likewise, 7 days of forced exercise had no affect on IGF-1 mRNA in any of the brain regions analysed. IGF-1 mRNA expression was measured by multiplex RT-PCR using ß-actin as an endogenous control. IGF-1 mRNA in the dentate gyrus (Figure 4.21, A (b): CON: 1.076 ± 0.159, n=12; EX: 1.228 ± 0.169, n=12), the perirhinal cortex (Figure 4.21, B (b): CON: 1.030 ± 0.076, n=12; EX: 0.959 ± 0.064, n=11) and the hippocampus (Figure 4.21, C (b): CON: 1.169 ± 0.253, n=12; EX: 0.908 ± 0.145, n=12) was not altered by forced exercise. Data presented as RQ values, mean ± SEM. The statistical analyses used were unpaired Student's *t* tests.

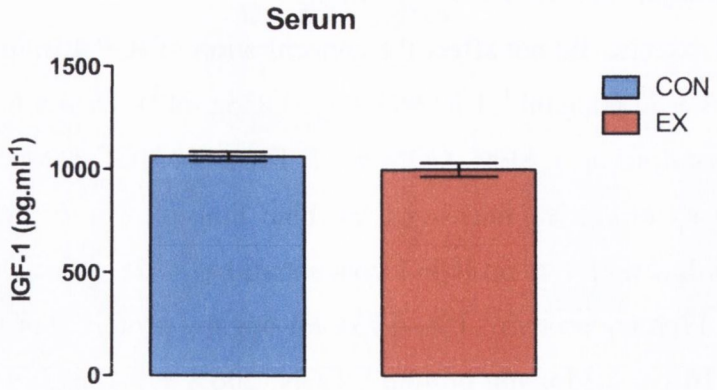

Figure 4.19: Effect of 7 days of forced exercise on serum IGF-1 concentration

7 days of forced exercise had no effect on serum IGF-1 concentration. Data presented as pg IGF-1 per ml serum, mean ± SEM. Statistical analyses: unpaired Student's *t* tests, CON n=12, EX n=10.

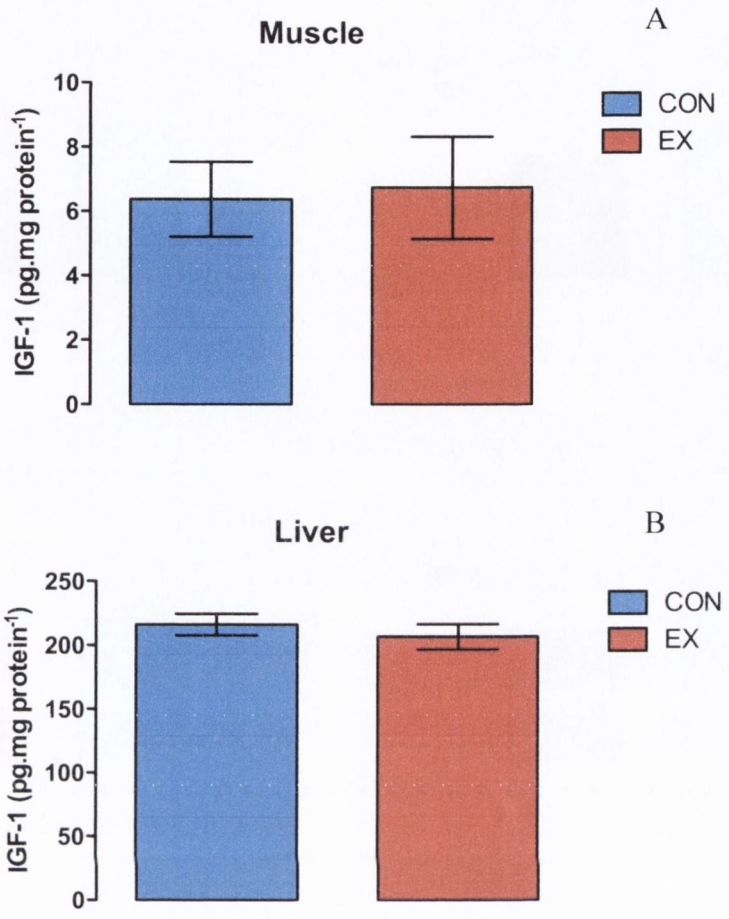

Figure 4.20: Effect of 7 days of forced exercise on IGF-1 concentrations in muscle and liver

(A) 7 days of forced exercise had no effect on IGF-1 concentrations in the muscle. **(B)** There was no difference in the concentration of IGF-1 between EX and CON groups in the liver. Data presented as pg IGF-1 per mg protein, mean ± SEM. Statistical analyses: unpaired Student's *t* tests, CON n=10, EX n=10.

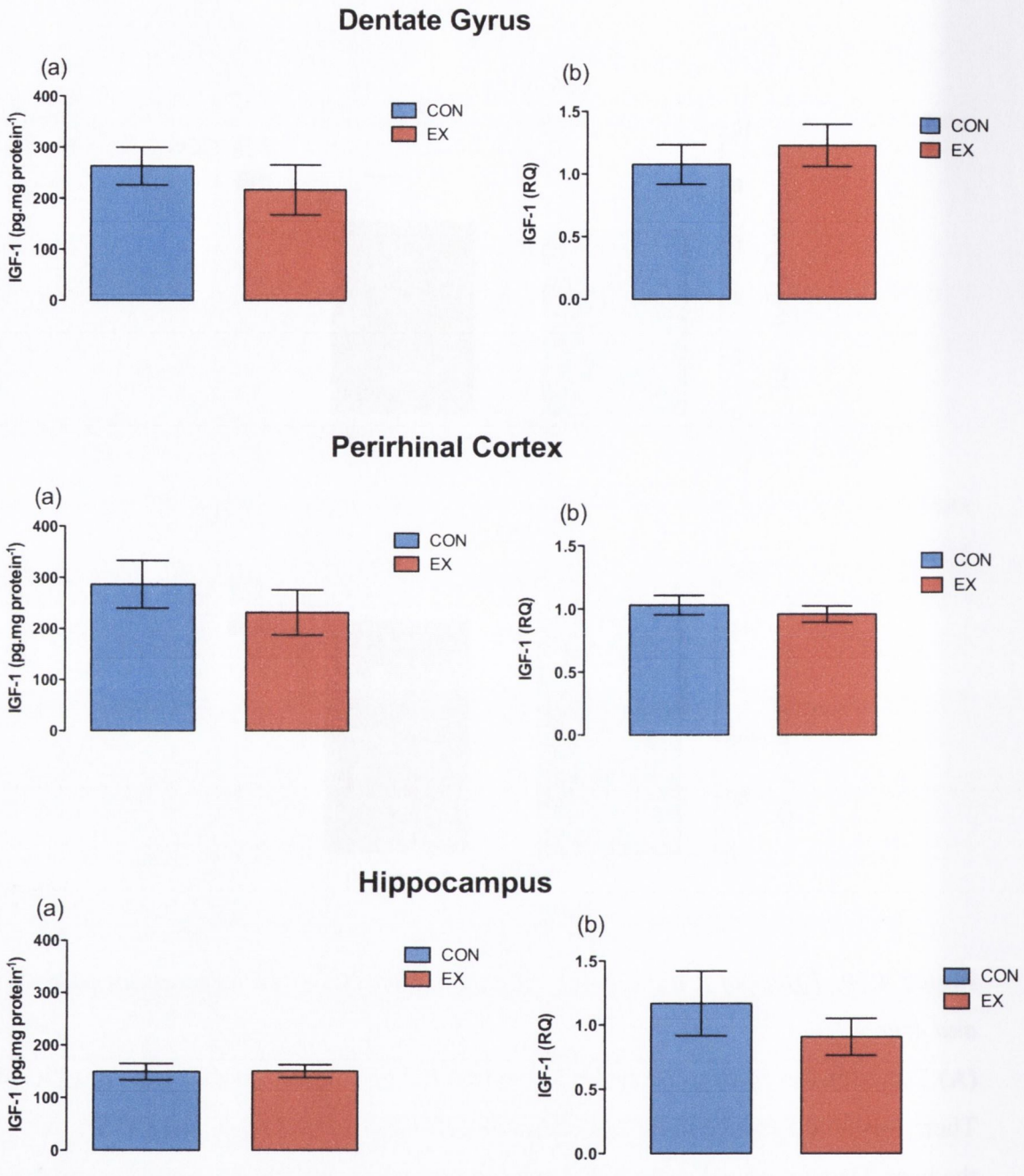

Figure 4.21: Effect of 7 days of forced exercise on IGF-1 concentrations in the dentate gyrus, perirhinal cortex and hippocampus

(A) 7 days of forced exercise had no effect on (a) IGF-1 protein concentration (CON n=10, EX n=10) or (b) IGF-1 mRNA concentration (CON n=12, EX n=12) in the dentate gyrus. **(B)** 7 days of forced exercise had no effect on (a) IGF-1 protein concentration (CON n=10, EX n=11) or (b) IGF-1 mRNA concentration (n=12, EX n=11) in the perirhinal cortex. **(C)** 7 days of forced exercise had no effect on (a) IGF-1 protein concentration (CON n=11, EX n=12) or (b) IGF-1 mRNA concentration (CON n=12, EX n=12) in the hippocampus. Protein data presented as pg IGF-1 per mg protein, mean ± SEM, mRNA data presented as RQ values with β-actin as an endogenous control, mean ± SEM. Statistical analyses: unpaired Student's *t* tests.

4.3.11. Recognition memory and exogenous BDNF infusion

One bolus infusion of exogenous BDNF icv enhanced novel object recognition memory as assessed by the 3-object variant of the object substitution task. Both groups spent equal amounts of time exploring objects A, B and C during the training phase (Figure 4.22, A). However, during the testing trials (Figure 4.22, B) there was an overall effect of object ($P<0.0001$, $F_{(2,45)}=26.70$) and a significant interaction ($P=0.0095$, $F_{(2,45)}=5.171$), indicating that a preference occurred for one of the objects but that the two groups responded differently. *Post hoc* analysis revealed a significant preference for the novel object D in the exogenous BDNF group (object A: 23.271 ± 4.01%; object B: 15.957 ± 4.898%; object D: 58.845 ± 6.746%, $P<0.001$). There was also a slight preference for object D in the CON group (object A: 34.57 ± 3.291%; object B: 24.373 ± 2.568%; object D: 41.057 ± 2.989%, $P<0.05$), although this was only significant relative to object B. Data are presented as a percentage of the total exploration time, mean ± SEM. The statistical analyses used were two-way ANOVA and *post hoc* Bonferroni, CON n=9, EX n=8.

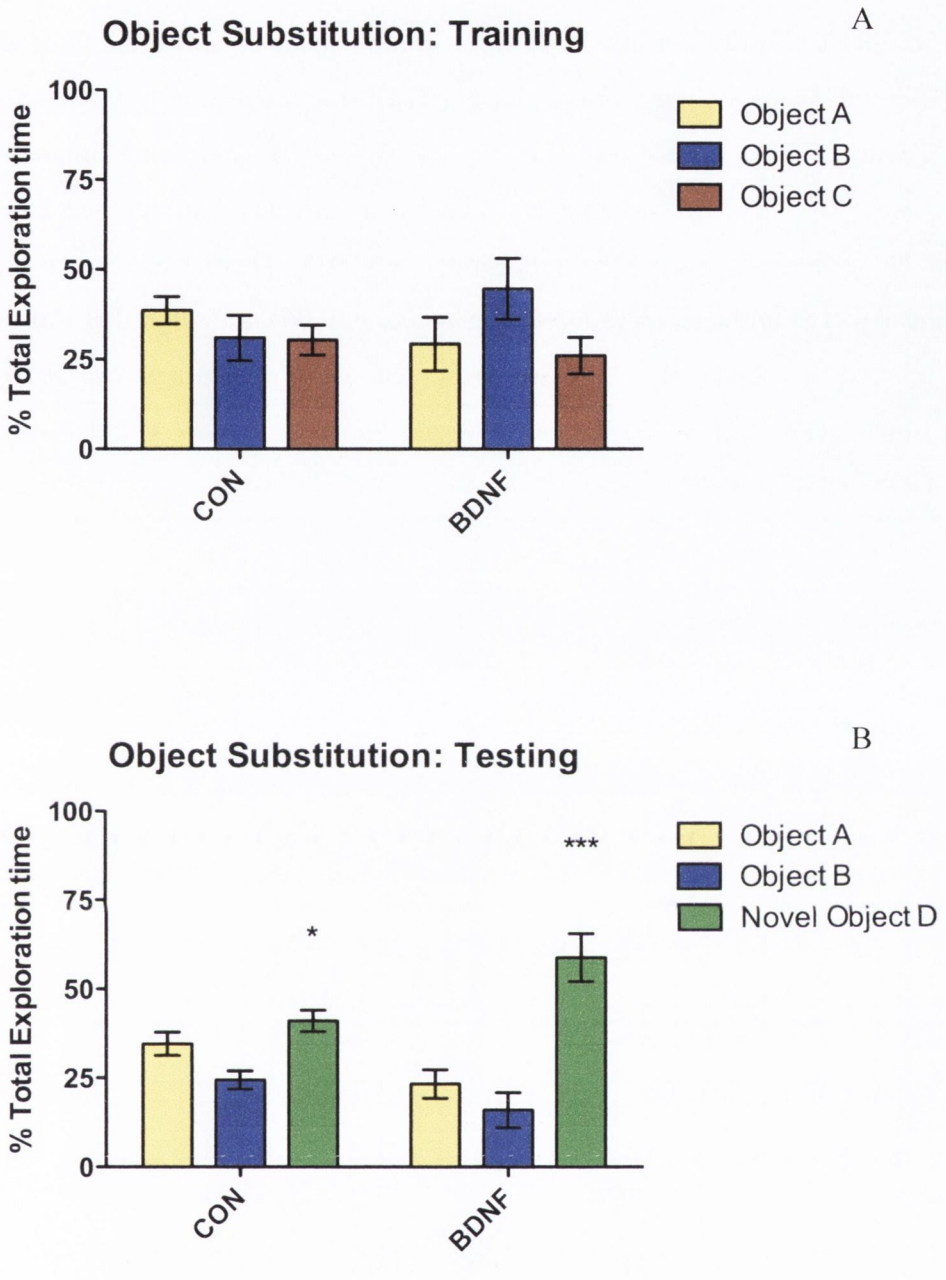

Figure 4.22: Effect of exogenous BDNF application on the 3-object variant of the object substitution task

One bolus infusion of exogenous BDNF enhanced performance in the 3-object variant of the object substitution task. **(A)** Both groups spent equal amounts of time exploring the 3 objects during the training phase. **(B)** During the testing trials there was an overall effect of object ($P<0.0001$, $F_{(2,45)}=26.70$) and a significant interaction ($P=0.0095$, $F_{(2,45)}=5.171$). *Post hoc* analysis revealed a significant preference for the novel object D in the EX group (*** represents $P<0.001$). There was also a slight preference for object D in the CON group (* represents $P<0.05$), although this was only significant relative to object B. Data are presented as a percentage of the total exploration time, mean ± SEM. Statistical analysis: two-way ANOVA and *post hoc* Bonferroni, CON n=9, EX n=8.

4.3.12. Recognition memory, forced exercise and anti-BDNF infusion

7 days of forced exercise and anti-BDNF icv infusions resulted in an alteration in performance in the object substitution task. Both groups spent equal amounts of time exploring the 3 objects during the training phase (Figure 4.23, A). During the testing trials (Figure 4.23, B) there was a significant effect of object ($P=0.0012$, $F_{(2,39)}=8.024$) and a significant interaction ($P=0.0018$, $F_{(2,39)}=7.498$). *Post hoc* analysis revealed a significant preference for the novel object D in the EX group (object D: 44.597 ± 4.110%, $P<0.01$, relative to object C: 19.762 ± 3.912%). However, there was unequal exploration of the familiar objects (object A: 35.641 ± 2.860%, $P<0.05$ relative to object C). Similarly, in the anti-BDNF group there was unequal exploration of the objects (object A: 24.428 ± 4.808%, $P<0.05$ relative to object D: 39.327 ± 1.697%; object C: 36.244 ± 4.175%). Data are presented as a percentage of the total exploration time, mean ± SEM. Statistical analysis: two-way ANOVA and *post hoc* Bonferroni, EX n=7, Anti-BDNF n=8.

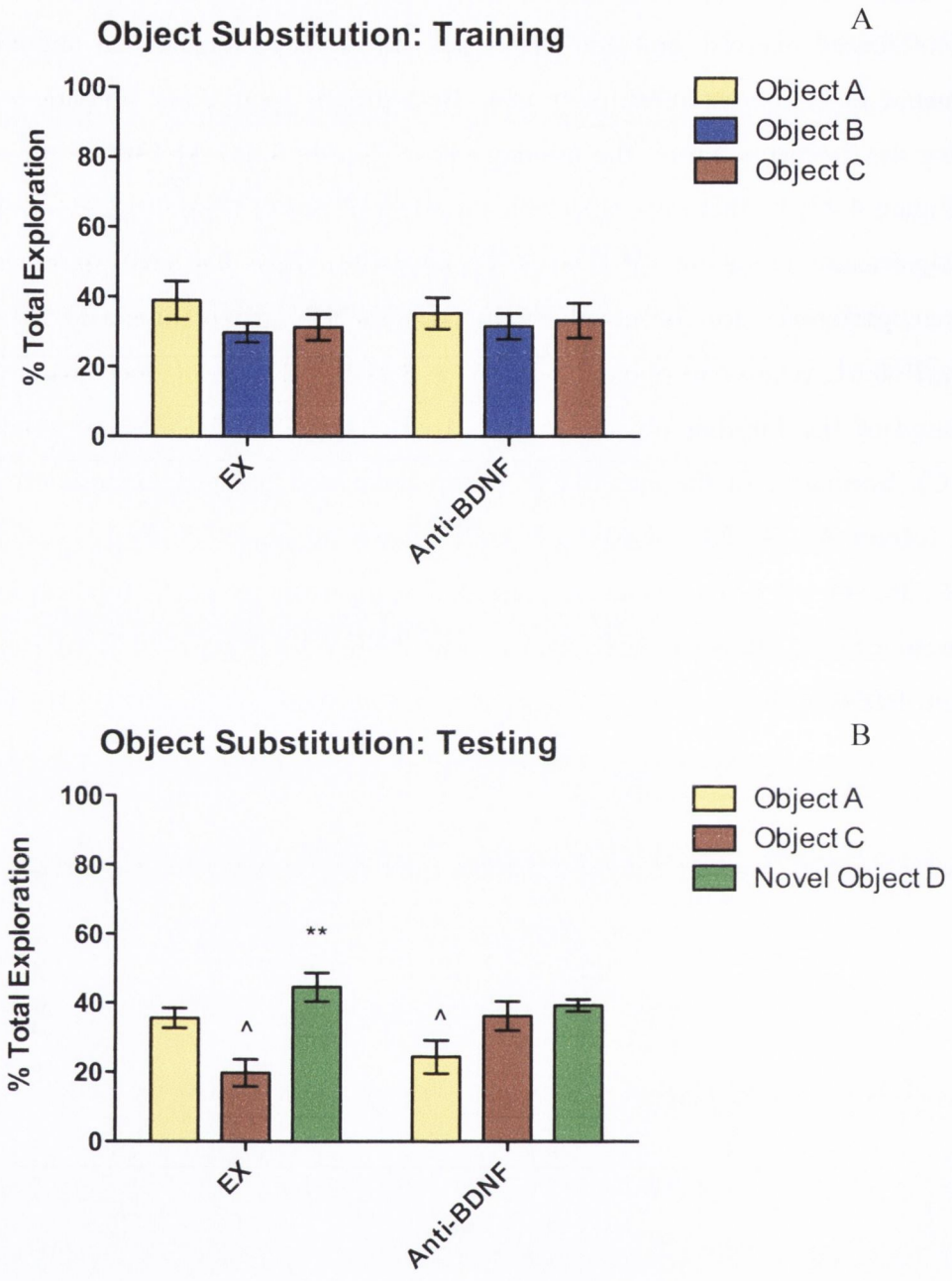

Figure 4.23: Effect of forced exercise and anti-BDNF infusion on the 3-object variant of the object substitution task

7 days of forced exercise and anti-BDNF infusions resulted in an alteration in performance in the object substitution task. **(A)** Both groups spent equal amounts of time exploring the 3 objects during the training phase. **(B)** During the testing trials there was a significant effect of object ($P=0.0012$, $F_{(2,39)}=8.024$) and a significant interaction ($P=0.0018$, $F_{(2,39)}=7.498$). *Post hoc* analysis revealed a significant preference for the novel object D in the EX group (** represents $P<0.01$, relative to object C). However, there was unequal exploration of the familiar objects in both groups (^ represents $P<0.05$). Data are presented as a percentage of the total exploration time, mean ± SEM. Statistical analysis: two-way ANOVA and *post hoc* Bonferroni, EX n=7, Anti-BDNF n=8.

4.4. Discussion

The overall objective of this study was to further elucidate the roles of neurotrophins in the link between exercise and improved cognition, using a rodent model. The aim was firstly to establish the effect of treadmill running on both spatial and non-spatial recognition memory in the male Wistar rat. Secondly, the effect of the forced exercise protocol on neurotrophin concentrations, both in the circulation and in specific regions of the brain, was determined. Analysis was completed in order to investigate the underlying mechanisms at the molecular level and an assessment of the contribution of neurogenesis to the exercise-induced cognitive enhancements was made. Finally, infusions of exogenous BDNF and anti-BDNF into the CNS were carried out, in an effort to support the emerging evidence for a functional role of BDNF in mediating exercise-induced enhancements in cognitive function.

4.4.1. Recognition memory and exercise

Forced exercise had no effect on spatial memory, as demonstrated by the results from the object displacement task. Both groups displayed increased exploration of the displaced object, indicating that both sedentary and exercised rats learned the task equally well. This confirmed previous results from our laboratory in which one week of treadmill running had no effect on spatial memory, as assessed by the Morris water maze in young rats (O'Callaghan *et al.*, 2007). The object displacement task used in the present study involves intrinsically-motivated spatial learning (Poucet, 1989). A similar task has been used previously to illustrate chronic mild stress induced impairments in spatial learning in mice (Li *et al.*, 2008). Arguably, this task is a less stressful paradigm for testing spatial memory. It is reliant on the spontaneous exploratory behaviour of the rat and is independent of external motivators, such as the desire to escape the Morris water maze. To the best of my knowledge, this is the first time that the object displacement task has been used to assess the effect of exercise on cognitive function.

There is some debate in the literature on the merits of using forced versus voluntary exercise paradigms in rodents. Voluntary exercise programmes are said to simulate aspects of human behaviour as they allow rodents to choose the how much to run (Vaynman *et al.*, 2004). However, it is proposed that a forced exercise paradigm better simulates the supervised-training regime used in experimental studies on human

subjects, in which the experimenter sets the exercise durations. Voluntary exercise reportedly eliminates problems associated with the stress of forced treadmill running and investigator handling (Cotman & Berchtold, 2002), but investigator handling is required in order to perform the behavioural tests; therefore more frequent handling could be considered beneficial, as the animals become better accustomed to the experimenter. Furthermore, parameters such as exercise intensity, duration and frequency are highly variable and dependent on the spontaneous motor activity of the animal with voluntary exercise protocols (Soya *et al.*, 2007). A forced exercise paradigm allows for the intensity, duration and timing of the exercise to be controlled for and planned to accommodate behavioural testing.

There is conflicting information in the literature on the subject of exercise-induced enhancements in spatial memory. In agreement with the present study, Barnes *et al* (1991) reported no improvement in spatial learning following 10-weeks of treadmill running in older rats (Barnes *et al.*, 1991). However, other studies saw improvements in the Morris water maze following both three weeks and one week of voluntary wheel running in young rats (Adlard *et al.*, 2004; Vaynman *et al.*, 2004). Differences in the modality, intensity and duration of the exercise paradigms may account for the different results reported. It had been shown that neuronal activation is exercise-intensity dependent, as evidenced by differences in c-fos induction (Soya *et al.*, 2007). Cell proliferation in the dentate gyrus of rats is also modulated by both the intensity and duration of exercise (Kim *et al.*, 2003). It may be the case that the stress associated with treadmill running, or the intensity of the exercise in the present study, prevented any exercise-induced enhancement in spatial memory from occurring.

Alternatively, exercise may be ameliorating the effects of stress on spatial memory. Commonly, voluntary wheel running is performed with individually-housed animals, as is the case with both of the studies mentioned showing exercise-induced improvements in the water maze (Adlard *et al.*, 2004; Vaynman *et al.*, 2004). Isolation is not a normal condition for social animals such as rats, and may have adverse effects on their health and behaviour (Boggiano *et al.*, 2008). Chronic mild stress has been shown to impair performance in both spatial and non-spatial recognition memory tasks (Li *et al.*, 2008). Furthermore, exercise has been consistently shown to rescue impaired spatial memory, such as occurs with age-related cognitive decline (Albeck *et al.*, 2006), kainic acid or

LPS infusion (Gobbo & O'Mara, 2005; Wu *et al.*, 2007) and pre-natal ethanol exposure (Christie *et al.*, 2005), It is possible that exercise may only noticeably improve spatial learning when there is an existing cognitive deficit, such as isolation stress-induced impairment or age-related cognitive decline, neither of which was a factor in the present study.

Exercise induced an enhancement in non-spatial recognition memory, as evidenced by the results from the object substitution task. Exercised rats spent significantly more time exploring the novel object, relative to their exploration of the familiar object, while there was a trend towards increased exploration of the novel object in the control rats. In agreement with the present finding, an exercise-induced enhancement in the discrimination between one familiar and one novel object was previously demonstrated in our laboratory (O'Callaghan *et al.*, 2007). Furthermore, the results from the 3-object variant of the object substitution task demonstrated that exercise enhanced the ability of rats to discriminate between one novel object and two familiar objects. This confirmed previous observations of exercise-induced enhancements in non-spatial recognition memory, using a more challenging object substitution paradigm. This is the first time the 3-object version of this task has been used to assess the effect of exercise on cognitive function.

Previously it has been shown that sedentary rats can discriminate between one novel and one familiar object in the open field (Hennigan *et al.*, 2007; O'Callaghan *et al.*, 2007), while control rats in the present study showed a similar trend. The results from the 3-object variant of the object substitution task demonstrated that sedentary rats were unable to discriminate between one novel object and two familiar objects, following the standard three 5min training trials. Overtraining, involving nine 5min training trials, resulted in successful learning of the task by sedentary rats, as evidenced by increased exploration of the novel object during the testing phase. These results support the assertion that the 3-object version of the object substitution task is a more difficult test of non-spatial recognition memory. The difference between the exercise-induced results in spatial and non-spatial memory tasks may be due to an inherently superior spatial learning ability in rats, which left no room for improvement in the object displacement task. It is possible that a more difficult version of the object displacement task may reveal any exercise-induced alterations to this form of learning in young rats.

On the other hand, exercise may be selectively enhancing a particular type of learning. The object displacement and object substitution tasks are thought to enlist two distinct forms of learning. Object displacement recruits spatial memory, which is widely accepted to be a hippocampal-dependent form of learning. The hippocampus processes information related to the familiarity of arrangements of items. Evidence suggests that the novel arrangement of familiar objects activates the CA1 region of the hippocampus, with a significant decrease in activation of the dentate gyrus and subiculum, when compared to activation patterns created by a familiar arrangement of familiar objects (Wan *et al.*, 1999). Central to the object substitution task is the discrimination between the novelty and familiarity of objects. While this task also recruits the hippocampus, with an evident role for the dentate gyrus subfield (Kelly *et al.*, 2003), the perirhinal cortex is likely to be the most crucial cortical area that supports object recognition memory (Buckley, 2005). The perirhinal cortex is associated with the recognition of the familiarity of individual items, novel items activate the perirhinal cortex and area TE of the temporal lobe more than familiar items (Wan *et al.*, 1999). Considering the evidence of differential activation of the the hippocampus and associated cortical areas by spatial and non-spatial recognition memory, the disparity in the results from the behavioural tasks in the present study may be a result of differential effects of exercise on specific regions of the brain.

4.4.2. BDNF concentrations and exercise

There was an increase in the concentration of serum BDNF during the forced exercise protocol in both the exercised and the control groups. The lack of a significant difference between exercised and sedentary rats indicated that the increase was not a result of exercise, but was induced by some other factor. Serum samples were collected through the saphenous vein on day 1 and day 5 of the forced exercise protocol, but the final serum sample was collected as trunk blood, following the behavioural testing on day 8. Activity-dependent release and learning induced increases in BDNF have previously been demonstrated (Bramham & Messaoudi, 2005; Silhol *et al.*, 2007). Hence, it was hypothesized that the learning involved in the behavioural test induced a robust increase in serum BDNF, which may have masked any exercise-related effects.

To test this theory, another group of animals completed the forced exercise protocol and were sub-divided into learning and non-learning groups. Exercise induced an increase in serum BDNF concentrations, with the principal increase occurring in the exercise with learning group. This data supports the hypothesis that both exercise and learning had an effect on serum BDNF concentrations. However, this hypothesis is based on the assumption that BDNF is crossing the blood brain barrier. There is evidence to suggest that BDNF crosses the blood-brain barrier in both directions, with BDNF efflux from brain to blood occurs via bulk flow in association with the reabsorption of cerebrospinal fluid (Pan *et al.*, 1998). Moreover, circulating BDNF concentrations are known to reflect BDNF concentrations in the brain during development and aging (Karege *et al.*, 2002). It is proposed that the robust serum BDNF increase in the exercise with learning group occurred as a result of both a learning-induced increase in BDNF in the CNS, resulting in increased efflux of BDNF from brain to blood, in addition to an exercise-induced increase in BDNF in the periphery.

In contrast to the results for serum BDNF, 5 days of treadmill running had no effect on plasma BDNF concentration. Furthermore, the quantity of BDNF in the plasma samples was significantly lower than that of the serum samples at the end of the exercise protocol. Evidence suggests that the majority of circulating BDNF is stored in platelets (Radka *et al.*, 1996). It has been reported that the amount of BDNF stored in the platelets accounts for the difference in serum BDNF concentration, relative to plasma (Rosenfeld *et al.*, 1995; Lommatzsch *et al.*, 2005). However, platelets do not synthesize BDNF; they sequester and store it, through as yet unidentified mechanisms (Fujimura *et al.*, 2002). Stored BDNF is released upon agonist stimulation, via platelet degranulation (Rosenfeld *et al.*, 1995; Radka *et al.*, 1996). Therefore, exercise may induce increases in serum BDNF by triggering the release of stored BDNF from the platelets. This hypothesis merits further investigation as little is currently known about the source of exercise-induced increases in circulating BDNF.

Evidence suggests that BDNF is synthesised by certain peripheral tissues, including vascular smooth muscle and visceral epithelia (Donovan *et al.*, 1995; Lommatzsch *et al.*, 1999). While these tissues are not targets for BDNF action, it has been suggested that visceral-epithelia derived BDNF is necessary for normal function of the adult

peripheral nervous system (Lommatzsch *et al.*, 1999). In the present study, forced exercise induced an increase in BDNF protein in the soleus muscle, while BDNF protein concentration was not altered in the liver. In agreement with the present finding, voluntary exercise has previously been shown to elevate both BDNF protein and mRNA expression in the soleus muscle after 3 and 5 days of wheel running, an effect which was blocked by botulinum toxin paralysis (Gomez-Pinilla *et al.*, 2002). Muscle-derived BDNF has been implicated in the regulation of motoneuron excitability (Gonzalez & Collins, 1997). Hence, the present increase in muscle BDNF has implications for muscle function via modulation of the motoneurons. Furthermore, skeletal muscle is proposed as a potential peripheral source for the exercise-induced increase in circulating BDNF.

The forced exercise protocol induced significant increases in BDNF protein in the dentate gyrus, perirhinal cortex and the hippocampus. The largest expression and induction of BDNF was observed in the dentate gyrus, with smaller but statistically significant increases in the hippocampus and the perirhinal cortex. This confirmed previous results from our laboratory showing an exercise-induced increase in expression of BDNF in the dentate gyrus (O'Callaghan *et al.*, 2007) and agreed with evidence from the literature demonstrating exercise-induced increases in hippocampal BDNF protein (Vaynman *et al.*, 2004; Ding *et al.*, 2006). To the best of my knowledge, this is the first evidence of an exercise-induced enhancement in BDNF protein in the perirhinal cortex. This result is of particular interest given that the exercise-induced enhancements in cognitive function were specific to the object substitution task, and the perirhinal cortex is reportedly specialized for processing object identity (Buckley, 2005).

Interestingly, forced exercise did not alter BDNF mRNA levels in the dentate gyrus, perirhinal cortex or hippocampus. However, evidence suggests that alterations in BDNF mRNA expression do not always translate to changes at the protein level (Tropea *et al.*, 2001). In contrast to the present data, 5 days of voluntary exercise has been shown to significantly increased BDNF mRNA levels in the hippocampus, an effect that was attenuated by blocking the IGF-1 receptor (Ding *et al.*, 2006). It has been suggested that there is a threshold level of activity beyond which BDNF mRNA expression is potentiated (Oliff *et al.*, 1998). However, an inverse relationship between

forced exercise intensity and BDNF mRNA has also been reported. Evidence suggests that BDNF mRNA is elevated in response to low-intensity but not moderate-intensity treadmill running, an effect that the authors attribute to a stress-induced reduction in BDNF expression (Soya *et al.*, 2007; Lou *et al.*, 2008). However, the protocol used in the present study was similar to the mild intensity condition, although the duration of exercise was twice as long. Hence, stress may be a factor in the lack of effect of exercise on BDNF mRNA.

Given that BDNF readily crosses the blood-brain-barrier in both directions (Pan *et al.*, 1998), exercise-induced increases in circulating BDNF may contribute to the concentration of BDNF in the CNS, or vice versa. Interactions between blood-borne growth factors and exercise-induced cognitive improvements have been reported previously, with evidence of a role for serum IGF-1 in mediating the effects of exercise on the brain. Peripheral infusions of labelled IGF-1 mimicked the effects of exercise on neuronal activity, as demonstrated by neuronal accumulation of IGF-1 and increased neuronal c-Fos labelling (Carro *et al.*, 2000). Considering the present increase in BDNF protein in specific regions of the brain and the lack of a corresponding alteration in BDNF mRNA, the exercise-induced increase in serum BDNF may have contributed to the concentration of BDNF in the brain, which could have functional implications for exercise-induced cognitive enhancement.

4.4.3. NT-4/5 and NGF concentrations

The forced exercise protocol had no effect on NT-4/5 protein concentration in the dentate gyrus, perirhinal cortex and hippocampus. Similarly exercise had no effect on NT-4/5 mRNA expression although there was insufficient cDNA remaining to complete analysis of hippocampal NT-4/5 mRNA expression. As NT-4/5 is one of the more recently discovered neurotrophins (Hallbook *et al.*, 1991), there is relatively little research reported in the literature on the effect of exercise on expression levels, and even less evidence for a role in the exercise-induced enhancements in cognitive function. Exercise was shown to increase NT-4/5 immunoreactivity in white matter of the lumbar spinal cord (Skup *et al.*, 2002) and to increase skeletal muscle derived NT-4/5 in an activity dependent manner, which supports the growth and remodelling of adult motor neurons (Funakoshi *et al.*, 1995). Furthermore, NT-4/5 is known to be a high affinity ligand for the Trk B receptor and application of NT-4/5 to hippocampal

neurons in culture enhanced excitatory synaptic transmission (Binder, 2007). However, NT-4/5$^{-/-}$ knockout mice are normal and long-lived with no obvious neurological deficits (Ibanez, 1996). This suggests that NT-4/5 plays, at most, a minor role in memory processes and is not a candidate mediator of the exercise-induced enhancements in cognitive function.

NGF protein and mRNA concentrations were not altered following treadmill running in the dentate gyrus or hippocampus. However, although expression levels were low in the perirhinal cortex, a small but statistically significant decrease in NGF protein was measured in the exercised group, while mRNA expression was unchanged. There is limited evidence in the literature on the effect of exercise on NGF concentrations. NGF mRNA has been shown to increase in the brain following 12 weeks of high intensity treadmill running in rats, a result that was linked to neuroprotection and a significant decrease in infarct volume caused by middle cerbral artery occlusion. However, following 4 weeks and 8 weeks of this forced exercise protocol, no alterations in NGF were recorded (Ang *et al.*, 2003). Another study reported an increase in hippocampal mRNA following 2 nights of voluntary wheel running which had returned to control levels by day 7 of the exercise protocol (Neeper *et al.*, 1996). In contrast, acute exercise had no effect on serum NGF concentrations in healthy human controls (Gold *et al.*, 2003).

NGF is implicated in the stress response of the hypothalamic-pituitary-adrenal axis (Aloe *et al.*, 2002). Increases in NGF concentration in plasma and hypothalamus have been linked to stress (Alleva & Santucci, 2001), which may be functional in promoting remodelling of damaged tissues following stress (Aloe *et al.*, 2002). Conversly, a decrease in NGF concentration in the hippocampus was previously reported and attributed to stress caused by forced motor activity (over a 2 hour and 10 hour period), although acute physical threat did not elicit the same response (von Richthofen *et al.*, 2003). The effect of exercise on NGF concentrations in the perirhinal cortex has not previously been reported. It is possible that the stress of treadmill running caused a decrease in NGF concentration, as von Richthofen and colleagues suggest for the hippocampus. However, considering the limited amount of literature available in this area, there may be other as yet unknown mechanisms involved in the physical exercise stimulated decrease in NGF protein concentration reported.

4.4.4. Signalling

We saw no alterations in the expression of Trk B protein in the dentate gyrus, perirhinal cortex or the hippocampus, as a result of the forced exercise protocol. Furthermore, the forced exercise protocol had no affect on Trk B mRNA expression in those areas. In contrast, 7 days of voluntary wheel running elevated Trk B mRNA in the lumbar spinal cord of rats (Gomez-Pinilla *et al.*, 2002), providing evidence that exercise can augment expression of the receptor. Furthermore, 5 weeks of treadmill running has been shown to restore the diminished expression of Trk B, following both LPS-infusion and age-related decline (Wu *et al.*, 2007; Wu *et al.*, 2008). However, it may be more functionally relevant to analyse Trk B phosphorylation, as this is an indication of activation of the receptor. Unfortunately, there were no antibodies commercially available to complete this analysis during this study.

Evidence from the literature suggests that the molecular mechanisms involved in the beneficial effects of physical exercise on brain function include activation of the MAPK and ERK pathway, partly through promoting expression of BDNF. 7 days of voluntary exercise increased both phosphorylated p44/ERK and p42/ERK significantly in the hippocampus (Shen *et al.*, 2001). Furthermore, 5 days of voluntary exercise significantly elevated proteins downstream of BDNF activation, important for synaptic function, such as synapsin 1, and signal transduction cascades associated with memory processes, such as CaMKii (Ding *et al.*, 2006). In contrast, in the present study ERK activation was not affected in the dentate gyrus, perirhinal cortex or hippocampus as a result of the forced exercise protocol. Similarly, 7 days of forced exercise had no effect on the expression of phospho-synapsin 1 and phospho-CaMKii in the dentate gyrus, perirhinal cortex and hippocampus. However, this does not necessarily imply that the cognitive enhancements reported do not involve the MAPK/ERK pathway.

Rapid and transient activation of the MAPK/ERK cascade was previously shown following the exploration of objects, and its involvement in long-term recognition memory was demonstrated through the use of the MEK inhibitor U0126 (Kelly *et al.*, 2003). Furthermore, induction of LTP (the cellular analogue of learning and memory) in the dentate gyrus has previously been shown to increase MAPK/ERK activity (Davis *et al.*, 2000). The increases in MAPK/ERK activity reported by Kelly *et al* was seen 5-

10min after exposure to the objects in the training phase of the object recognition task. The samples in the present study were collected 6hrs post-training and immediately post-testing in the object recognition task. Furthermore, Davis *et al* (2000) reported that MAPK/ERK activation is transient and quickly returned to baseline as a result of negative feedback involving up-regulation of MKP-1, the MAPK phosphatase. It is possible that the timing of sample collection in the present study may be responsible for the lack of ERK activation recorded.

4.4.5. Neurogenesis

The 7-day forced exercise protocol had no significant effect on the expression of $BrdU^+$ cells in the dentate gyrus, indicating that the rate of neurogenesis was not affected by exercise. While BrdU labelling is the "gold standard" in measuring neurogenesis, it is a marker of both neurogenesis and cellular repair. However, there was also no exercise-induced alteration in Ki67 mRNA expression in the dentate gyrus, perirhinal cortex or hippocampus. Ki67 is solely a marker of cell proliferation (Markakis & Gage, 1999; Wojtowicz & Kee, 2006). In agreement with the present result, one week of moderate treadmill exercise did not increase BrdU labelling in the dentate gyrus (Lou *et al.*, 2008). In contrast, 5 weeks of treadmill running has been shown to restore impaired neurogenesis, following both LPS-infusion and age-related decline (Wu *et al.*, 2007; Wu *et al.*, 2008). Moreover, 2-3 weeks of voluntary exercise increased the number of Ki67 labelled cells in the dentate gyrus (Eadie *et al.*, 2005). However, considering the length of time it takes neurons to mature and become integrated into a network, any potential functional consequences of adult neurogenesis are likely to occur as long-term adaptations, rather than acute benefits (Kempermann *et al.*, 2004). In support of this hypothesis, voluntary wheel running for 2 to 4 months has been shown to increase BrdU labelling, improve water maze performance and selectively enhance dentate gyrus LTP (van Praag *et al.*, 1999).

VEGF is a secreted protein generally associated with angiogenesis, although recent evidence indicates that VEGF also has neurotrophic and neuroprotective effects and can actually stimulate neurogenesis *in vivo* (Jin *et al.*, 2002). The concentration of VEGF mRNA was not altered by the forced exercise protocol in the dentate gyrus, perirhinal cortex or hippocampus. However, a lack of exercise, via hindlimb suspension, has been shown to decrease VEGF plasma concentration (Yasuhara et al.,

2007), indicating that physical activity can affect VEGF expression. Furthermore, peripheral blockade of VEGF abolished running-induced neurogenesis but had no detectable effect on baseline neurogenesis in non-running animals (Fabel *et al.*, 2003). Taken together, the evidence from the literature suggests that exercise can indeed modulate the rate of adult neurogenesis, possibly via mechanisms involving VEGF, which may have functional consequences for learning and memory. However, considering the timing of the present experiments, neurogenesis does not appear to play a role in the exercise-induced cognitive enhancements reported here.

4.4.6. IGF-1 concentrations

7 days of forced exercise did not affect the concentration of IGF-1 protein in the serum, muscle or liver. Furthermore, the forced exercise protocol had no affect on IGF-1 protein concentration or mRNA expression in the dentate gyrus, perirhinal cortex and hippocampus. Previously, Carro and colleagues demonstrated that treadmill running did not alter serum IGF-1 levels. Similarly, brain IGF-1 mRNA levels were not altered by exercise, in agreement with the present study. However, treadmill running was found to increase levels of IGF-1 protein in the brain, via uptake of serum IGF-1 through the blood-CSF pathway (Carro *et al.*, 2000). The exercise protocol used in the present study was milder in intensity than that used by Carro and colleagues and may have been insufficient to enhance uptake of serum IGF-1 into the brain. Hence, the disparity in the results between these two studies is likely to be a result of differences in exercise protocols.

Exercise-stimulated uptake of circulating IGF-1 into the brain has been linked to neurogenesis. Blocking the entrance of IGF-1 into the brain prevented exercise-induced increases in neurogenesis, while subcutaneous administration of IGF-1 mimicked the effect of exercise on neurogenesis (Trejo *et al.*, 2001). Furthermore, mutant mice with low levels of serum IGF-1 displayed reduced adult hippocampal neurogenesis and impaired spatial learning, effects which were reversed by peripheral IGF-1 infusion but not exercise. Exercise could not recover hippocampal function because of the lack of availability of circulating IGF-1, confirming the hypothesis that exercise exerts an effect on hippocampal neurogenesis via enhanced entrance of serum IGF-1 into the brain (Trejo *et al.*, 2008). However, environmental enrichment (including access to running wheels) was shown to improve spatial memory by a neurogenesis-independent

mechanism (Meshi *et al.*, 2006). Furthermore, use of an anti-mitotic agent demonstrated that not all types of hippocampal-dependent memory are associated with neurogenesis (Shors *et al.*, 2002). Overall, this indicates that while exercise can enhance cognitive function via increased neurogenesis, possibly via mechanisms involving IGF-1, exercise can also enhance learning and memory through neurogenesis-independent mechanisms, in agreement with the present study.

4.4.7. Recognition memory and exogenous BDNF infusion

Acute intracerebroventricular infusion of exogenous BDNF enhanced object substitution learning. These data provide further evidence of a role for BDNF in this form of learning, in agreement with previous studies demonstrating BDNF-induced facilitation in long term memory retention (Alonso *et al.*, 2005) and reports of exogenous BDNF-induced enhancements in synaptic plasticity (Messaoudi *et al.*, 1998), in particular transcription-dependent, late phase LTP (Messaoudi *et al.*, 2002). In the present study, exogenous BDNF was administered at a dosage based on the magnitude of the exercise-induced increase in BDNF protein in the hippocampus. By replicating the effect of exercise, our observation of a BDNF-induced improvement in this form of learning strengthens the proposed link between BDNF expression and exercise-induced cognitive enhancement. This is the first evidence of an exogenous BDNF-induced enhancement in object recognition memory, particularly at a dose equivalent to the exercise-induced increase in hippocampal-BDNF.

Alonso and colleagues infused a dose 25 times greater than that of the present study, into each side of the brain in order to elicit a cognitive enhancement. Furthermore, Messaoudi and colleagues (1998) reported that a minimum dose of 2μg BDNF was necessary for maximum BDNF-induced potentiation, whereas 0.2μg (a 20-fold greater dosage than that used in the present study) reportedly induced only submaximal potentiation. Reduced food intake, weight loss, sprouting responses from non-targeted neuronal populations (Tuszynski *et al.*, 1996), hyper-excitability and seizure activity (Xu *et al.*, 2004) and poor diffusion of BDNF, attributed to the dense expression of truncated TrkB receptors on the ependymal choroids epithelium (Burke *et al.*, 1994) are some of the disadvantages associated with icv administration of neurotrophins. However, Tuszynski and colleagues reported side-effects, and Xu and colleagues

described an epileptogenic effect following BDNF infusions that were 120,000-fold and 500-fold greater than the present study respectively. Furthermore, a BDNF infusion 6000-fold less than that used by Burke and colleagues enhanced recognition memory in the present study, indicating that exogenous BDNF can penetrate into the brain parenchyma and have functional implications at considerably lower concentrations than those previously reported.

4.4.8. Recognition memory, forced exercise and anti-BDNF

Previously, exercise was shown to enhance learning in the 3-object variant of the object substitution task, along with a concomitant increase in BDNF protein in the dentate gyrus, hippocampus and perirhinal cortex. Similarly, an infusion of exogenous BDNF into the brain mimicked the effect of exercise on recognition memory. In an effort to inhibit this enhancement in recognition memory, rats completing a 7-day forced exercise protocol received anti-BDNF or a control infusion of sheep serum, on days 2, 4 and 6 of the exercise protocol. The function-blocking BDNF antibody, generated via the immunization of sheep with recombinant human BDNF, neutralizes BDNF but not the other related neurotrophins, NT-3, NT-4/5 or NGF (Mu *et al.*, 1999). Unfortunately, both groups of rats demonstrated unequal exploration of the familiar object during the testing trial, which complicates interpretation of the results. However, while the exercise group demonstrated some preference for the novel object, the exercise and anti-BDNF group did not appear to recognise the novel object, further supporting the role of BDNF in the exercise-induced enhancement in recognition memory.

In agreement with the present study, 7 days of continuous anti-BDNF infusion (120μg/day) into the right lateral ventricle was shown to inhibit spatial memory in the Morris water maze (Mu *et al.*, 1999). Similarly, mutant mice with a deletion in one copy of the BDNF gene displayed decreased BDNF mRNA and impaired performance in the Morris water maze task (Linnarsson *et al.*, 1997). In contrast, a 25μg injection of anti-BDNF into the left cerebral ventricle following training in the Morris water maze had no effect on memory retention (Cirulli *et al.*, 2000). It has been suggested that BDNF exerts its role in long-term memory formation in a time-dependent manner. Bilateral infusions of anti-BDNF antibody (0.5μl/side) into the dorsal CA1 region of the hippocampus 15min prior or 1, 3 or 4hrs after training completely blocked long-

term memory in a one-trial inhibitory avoidance task, but when administered immediately or 6 hours after training anti-BFNF had no effect (Alonso *et al.*, 2002). Indicating that BDNF has differential effects on acquisition and consolidation of memory.

4.4.9. Summary

Exercise had no effect on spatial recognition memory, as evidenced by the results from the object displacement task. However, one week of treadmill running enhanced non-spatial recognition memory, as assessed by the object substitution task. These results confirm the efficacy of forced exercise in selectively enhancing cognition in the rat, in agreement with previous results from our laboratory (O'Callaghan *et al.*, 2007), using a novel spatial-memory task. Furthermore, exercise was shown to induced enhancements in a more challenging measure of non-spatial recognition memory, the 3-object substitution task. This exercise-induced cognitive enhancement was associated with increased expression of BDNF in the serum and muscle of exercised rats but not in the plasma or liver. A parallel increase was observed in BDNF protein concentrations in the dentate gyrus, hippocampus and perirhinal cortex, while no alteration to BDNF mRNA expression was observed. The exercise-induced increase in BDNF protein in the perirhinal cortex has not been reported previously, and is particularly interesting considering the role of the perirhinal cortex in object recognition memory.

The one-week forced exercise protocol was found not to affect neurogenesis. However, given the timescale of the experiment, any alterations to the rate of neurogenesis would be unlikely to have had functional implications. Furthermore, IGF-1 concentrations and VEGF mRNA expression were not altered by the treadmill exercise. Overall, evidence from the literature suggests that exercise can enhance cognitive function by up-regulation of neurogenesis, through mechanisms involving increased serum IGF-1 uptake into the brain. However, in agreement with data from the present study, exercise can also enhance cognitive function through neurogenesis-independent mechanisms.

Expression of the BDNF receptor, Trk B, was not altered by the forced exercise protocol, although receptor activation may have proved a more functionally relevant analysis. Furthermore, we did not see any changes in the expression or activation of downstream signalling molecules of Trk B activation; ERK activation, phosphorylated

synapsin 1 expression and phosphorylated CaMKii expression. It is likely that the time of sampling was responsible for this, as changes in the expression and activation of these proteins are known to be transient. While evidence for Trk B activation is not presented here, strong evidence supporting the role of BDNF in mediating exercise-induced cognitive enhancement is presented, including both the correlative data of concomitant increases in BDNF protein in relevant regions of the brain and the results from the exogenous BDNF infusion study. This finding was supported by the infusion of the function blocking BDNF antibody in conjunction with exercise.

Chapter 5
General Discussion

Chapter 5: Discussion

5.1. General Discussion

The main objectives of this study were to investigate the effect of exercise on cognitive function and assess the role of neurotrophins in any exercise-related changes observed. There is evidence that physical exercise plays a role in the maintenance of cognitive function, including reduced risk of AD and an amelioration of age-related cognitive decline (McAuley *et al.*, 2004; Kramer *et al.*, 2006; Larson *et al.*, 2006). Therefore, exercise may be viewed as a simple means of maintaining brain function and promoting brain plasticity (Cotman & Berchtold, 2002). While this idea of 'healthy body, healthy mind' is not novel, the fact that recent research has also shown acute exercise-induced cognitive enhancements in young healthy men (Tomporowski, 2003; Ferris *et al.*, 2007; Winter *et al.*, 2007), prompts the need to establish the mechanism behind this link between acute exercise and cognitive function and explore any potential targets for therapeutic application.

Results from the present study, which assessed the effect of an acute bout of exercise on cognitive function in healthy young men, have confirmed the ability of acute exercise to enhance cognitive function. Furthermore, this cognitive enhancement was found to be selective for a MTL-dependent task. Further research using a rodent model revealed that one-week of treadmill running enhanced novel object recognition in young rats, but had no effect on a spatial recognition memory task. Physical exercise is associated with increases in cardiac output and blood flow to skeletal and cardiac muscles, to support increasing metabolic demands. There is some evidence to suggest that regionally specific increases in cerebral blood volume also occur as a result of exercise; Pereira and colleagues found that two weeks of exercise increased the cerebral blood volume in the dentate gyrus of the hippocampus in mice (Pereira *et al.*, 2007). Moreover, cerebral blood flow and metabolism have also been shown to increase with cardiac output (Ide & Secher, 2000). Increased cerebral blood flow may result in an increased level of alertness, which could potentially explain the reported cognitive enhancements. However, it is important to note that exercise-induced changes in metabolism manifest during, not after exercise (Ide & Secher, 2000). Furthermore, the pattern of neural activity associated with physical exercise rapidly returns to baseline following cessation of the exercise, therefore any alteration in the performance of a

cognitive task following, rather than during an exercise bout is unrelated to exercise-induced neural activity. Exercise actually impairs prefrontal dependent cognition, when cognitive tasks are performed during the exercise bout (Dietrich & Sparling, 2004). Therefore, whilst increased regional cerebral blood flow does not fully explain the current finding of selective cognitive enhancements after an acute exercise bout in men, or a short-term training programme in rats, it does provide a mechanism for increased influx of blood-borne trophic factors to specific regions of the brain.

The role of BDNF in neuronal survival and differentiation during development is well defined. In addition this neurotrophin mediates neuronal plasticity in adults. Hence, BDNF is proposed as a mediator of the cognitive enhancements described in the present study. BDNF is expressed throughout the CNS, and reportedly co-localizes with its receptor, Trk B, at glutamate synapses (Bramham & Messaoudi, 2005). Activity-dependent secretion of BDNF potentiates synaptic transmission in glutamatergic synapses, by enhancing neurotransmitter release and membrane excitability (Schinder & Poo, 2000). The binding of BDNF to pre-synaptic Trk B facilitates small synaptic vesicle docking, which potentiates neurotransmitter release, leading to the facilitation of LTP in the post-synaptic neuron and a reduction in synaptic fatigue (Hilfiker *et al.*, 1999; Jovanovic *et al.*, 2000; Xu *et al.*, 2000; Bramham & Messaoudi, 2005). BDNF is also a potent neuroexcitant at the post-synaptic membrane, inducing extremely rapid depolarisations, leading to modulation of calcium influx through voltage-dependant calcium channels and NMDA receptors (Kafitz *et al.*, 1999; Kovalchuk *et al.*, 2004; Bramham & Messaoudi, 2005). The reported ability of BDNF to increase neuronal excitability occurs within milliseconds of ligand binding, while enhanced vesicle docking takes at least 3 hours to complete (Bramham & Messaoudi, 2005). Considering the timescales involved in the enhancement of cognitive function in the present study, the aforementioned actions of BDNF are of particular relevance to the acute exercise-induced enhancement of cognition in men, and short-term training induced enhancements in rats, respectively.

Acute-exercise has been repeatedly shown to increase serum BDNF concentrations in young men (Gold *et al.*, 2003; Rojas Vega *et al.*, 2006; Ferris *et al.*, 2007; Winter *et al.*, 2007; Tang *et al.*, 2008), a finding that was confirmed in the present study. Further research revealed an increased expression of BDNF protein in the serum and muscle of

exercised rats, following the 7-day training protocol. These peripheral increases in BDNF protein concentration may be functionally relevant for exercise-induced enhancements in learning and memory, based on the assumption that BDNF crosses the blood brain barrier. There is evidence to support the influx of BDNF into the brain via a specific saturable transport system (Pan *et al.*, 1998). There is also evidence of elevated blood volume in the dentate gyrus following exercise (Pereira *et al.*, 2007), which may increase delivery of circulating BDNF to the MTL. In addition to the elevated expression of circulating BDNF in exercised rats, a parallel increase was observed in BDNF protein concentrations in the dentate gyrus, hippocampus and perirhinal cortex. In contrast, no alteration to BDNF mRNA expression was observed. This evidence supports the proposed influx of circulating BDNF into the brain, a mechanism for exercise-induced enhancement in cognitive function that merits further investigation to assess potential therapeutic applications of peripheral BDNF infusions.

On the other hand, one week of treadmill running was shown to increase serum BDNF concentrations in rats, but BDNF concentrations also increased in the serum of corresponding sedentary controls. Further research indicated that this increase occurred as a result of both a learning-induced increase in BDNF in the CNS, and an exercise-induced increase in BDNF in the periphery. BDNF is known to cross the blood-brain-barrier bidirectionally, with BDNF efflux occurring in association with CSF reabsorption (Pan *et al.*, 1998). Moreover, learning-induced increases in BDNF protein expression have previously been reported in the hippocampus, but not the hypothalamus or frontal cortex (Silhol *et al.*, 2007). Therefore, it may be the case that in sedentary animals the increased BDNF concentration observed in the serum is as a result of transport of BDNF from brain to blood following learning. Data from the present study demonstrated an exercise-induced enhancement in the performance of the face-name task, which is MTL-dependent, and a lack of effect of acute exercise on performance of the pre-frontal dependent Stroop task in young men. This evidence suggests that elevated BDNF concentrations in the brain, either as a result of increased influx from the periphery or increased local secretion in the CNS, mediated the enhanced cognitive function reported in men. In contrast, spatial and non-spatial object recognition tasks both recruit the hippocampus and associated cortical areas, but exercise failed to alter performance on a spatial memory task in the rat. Spatial memory formation occurs in association with increases in hippocampal BDNF mRNA (Mizuno

et al., 2000) and Trk B receptor expression in the hippocampus of aged animals (Silhol *et al.*, 2007). This indicates that the selectivity of the exercise-induced cognitive enhancement from the rat studies is mediated not only by BDNF protein concentration, but also by some other mechanism, possibly one that involves BDNF mRNA expression and Trk B receptor expression.

There is strong evidence to support the role of the MAPK/ERK pathway in BDNF-Trk B signalling (Davis *et al.*, 2000; Kelly *et al.*, 2003). However, given the putatively transient nature of changes in the expression and activation of the proteins downstream of Trk activation, the window of analysis chosen for the present study could not facilitate the analysis of the signalling cascades involved in Trk activation. Nonetheless, evidence of the outcome of BDNF-Trk B signalling in terms of cognitive enhancement is presented. An investigation of Trk B activation in different regions of the brain would ultimately give a better understanding of the location of BDNF activity. However, the unavailability of an antibody specific for phosphorylated-Trk B prevented this analysis.

The results from the study assessing the impact of exogenous BDNF infusion on learning provide further evidence supporting the role of BDNF in mediating exercise-induced cognitive enhancement. BDNF was infused at a physiologically relevant dose, based on the exercise-induced increases in BDNF protein in the hippocampus in the present study. Kafitz *et al* (1999) administered BDNF to CA1 pyramidal neurons at a similar dose (6.43nM), in a hippocampal slice preparation, and found it evoked rapid depolarization of the neuron, through activation of voltage-gated sodium channels, coupled directly to Trk B independently of second messenger signalling, resulting in a train of action potentials (Kafitz *et al.*, 1999). This finding implies that icv infusion of BDNF in the present study enhanced non-spatial recognition memory through an increased excitability of neurons in the hippocampus and associated cortical areas, and that BDNF-Trk B binding can also enhance synaptic plasticity in the absence of activation of second messenger systems. The role of BDNF in enhancing recognition memory is further supported by the infusion of the function blocking BDNF antibody in conjunction with exercise. These results merit further investigation to assess the potential therapeutic application of BDNF infusion in the treatment and prevention of cognitive decline.

BDNF not only enhances synaptic transmission and neuronal excitability but is also important for the proliferation of neural stem cells (Lee *et al.*, 2002) and reportedly mediates exercise-induced increases in adult neurogenesis (Wu *et al.*, 2008). Data from the present study demonstrated that one-week of treadmill running in rats does not affect neurogenesis, nonetheless it is worth noting that given the timescale of the experiment, any alterations to the rate of neurogenesis would be unlikely to have had functional implications (Kempermann *et al.*, 2004). However, while a 3-week training programme was insufficient to improve aerobic fitness or augment memory test performance in men, 5 weeks of aerobic exercise resulted in enhanced fitness scores and improvements in MTL-dependent cognition in young men. Hence, an exercise-induced increase in hippocampal neurogenesis is proposed as a possible mechanism behind this enhancement. Further research is required to confirm this hypothesis.

Shors *et al* reported neurogenesis related enhancements in cognitive function and Trejo *et al* showed that the uptake of serum IGF-1 into the brain is necessary for exercise-induced increases in neurogenesis and that exercise-induced enhancements in cognitive function are associated with increased hippocampal neurogenesis (Trejo *et al.*, 2001; Shors *et al.*, 2002; Trejo *et al.*, 2008). Exercise increases uptake of serum IGF-1, through the blood-CSF pathway (Carro *et al.*, 2000). The present study demonstrates decreased serum IGF-1 concentrations immediately post-acute exercise, following both 3 and 5 weeks of aerobic training. One possible explanation for this is that aerobic training enhances the efficacy of IGF-1 uptake into target tissues, resulting in transient decreases in circulating IGF-1 as the rate of uptake temporarily exceeds the rate of synthesis, leading to an increased rate of neurogenesis.

In summary, the present study supports the association between physical activity and cognitive function, with evidence that suggests a causal link between increased cardiovascular fitness and improvements in memory performance. Specific cognitive enhancements were demonstrated in previously sedentary young men after just 5 weeks of 3 sessions per week aerobic training. This moderate and manageable training regime has the potential to attenuate age-related cognitive decline. Furthermore, the finding that acute exercise enhances cognitive function immediately post-exercise, could possibly have implications for improving information processing and learning ability in

school children if, for example, daily exercise regimes were included in the curriculum. Although the mechanism behind these effects has yet to be fully elucidated, this study presents strong evidence for the involvement of BDNF in mediating exercise-induced cognitive enhancements. In addition, this study shows that BDNF infusion at a relatively low dose enhances cognitive function, a result that could potentially have therapeutic applications for the treatment of cognitive decline. In general, BDNF appears to mediate both short-term and long-term enhancements in learning and memory, through its actions on neuronal excitability, synaptic plasticity and neurogenesis. Therefore, it may be possible to use exercise programmes that increase endogenous BDNF, or alternatively to infuse exogenous BDNF, or a BDNF mimetic, to attenuate or ameliorate cognitive decline associated with brain trauma, disease and aging.

5.2. Future Directions

This study strongly supports a role for BDNF in mediating exercise-induced cognitive enhancement. However, it also provides a starting point for further research to answer the many remaining questions.

Was the lack of an effect of exercise on performance of a spatial learning task in rats the result of an innately superior spatial learning ability, relative other forms of memory?

In order to clarify whether exercise selectively enhanced non-spatial recognition memory, a more challenging version of the object displacement task could be used to assess whether exercise can enhance performance in a task that is at the limits of spatial memory ability in sedentary rats.

Are "standard" housing conditions actually deprived housing conditions?

In order to eliminate potential confounds and explain the disparity in results between different studies in the effect of exercise on spatial memory, an assessment of the effect of housing conditions on learning and memory could be made. A comparison of isolation versus group housing, and standard caged versus enriched housing (nest boxes and toys, with and without running wheels) could elucidate the impact of housing

conditions on cognitive function. Data that could be used to eliminate potential confounds from future exercise studies, such as the influence of isolation-induced stress on sedentary animals, or the role of exercise in cognitive enhancements attributed to environmental enrichment.

Does circulating BDNF cross the blood-brain barrier and impact on central BDNF concentrations?

To assess the effect of serum BDNF influx on BDNF protein concentrations in the brain, a peripheral injection of exogenous BDNF could be administered. Intra muscular injections could be given, in an attempt to mimic the effect of exercise on BDNF protein concentration in the muscle. Alternatively, intravenous injections may require smaller quantities of BDNF to achieve similar effects, which could limit costs somewhat. In either case, brain BDNF concentrations could be measured and an assessment of the effect peripheral BDNF infusion on cognitive function could be made.

Does central BDNF cross the blood-brain barrier and impact on circulating BDNF concentrations?

It is possible that efflux of centrally derived BDNF, in association with CSF reabsorption, is an important factor in determining the concentration of circulating BDNF. A repeat of the exogenous BDNF intracerebroventricular infusion study could clarify this, if analysis of serum and muscle BDNF concentrations were included. Furthermore an assessment of the effect of central exogenous BDNF infusion on a more challenging object substitution task could further elucidate the potential for BDNF-mediated enhancement of spatial memory.

What is the role of exercise intensity and duration on BDNF induction and cognitive function in rats?

In order to clarify the minimum amount of exercise necessary to induce increases in BDNF concentrations, the effect of an acute bout of treadmill running on BDNF concentrations in rats could be completed. Furthermore, an assessment of the effect of different intensities of exercise on BDNF protein induction and mRNA expression could also be completed, including analysis of corticosterone concentrations as a measure of stress. An extended forced exercise protocol could also be used to assess the

ability of forced exercise to increase the rate of neurogenesis and establish the role of serum IGF-1 uptake.

What is the effect of repeated exogenous icv infusions of BDNF?

An assessment of the ability of repeated exogenous BDNF infusions to increase the rate of neurogenesis could be completed. Furthermore, an assessment of any negative implications of exogenous BDNF infusion could have implications for the potential use of BDNF as a therapeutic agent.

What is the time course of induction of downstream mediators of BDNF pathway?

A time course analysis of BDNF-Trk B signalling could reveal the timing of activation and expression of the downstream signalling molecules of Trk B ligand binding. Western immunoblotting could be used to assess the involvement of the MAPK/ERK pathway and CREB phosphorylation at different time points following both exercise and learning. Furthermore, an assessment of Trk B phosphorylation, using Western immunoblotting, would be useful as a marker of receptor activation in specific brain regions, if a phospho-specific antibody becomes commercially available.

Was the training-induced enhancement in cognitive function in young men a result of increased neurogenesis?

Pereira and colleagues (2007) provided evidence that increased cerebral blood volume may be used as an *in vivo* correlate of neurogenesis. Hence magnetic resonance imaging (MRI) could be used to assess the impact of an aerobic training protocol on cerebral blood volume in humans, which could indicate any exercise-related changes in neurogenesis. Furthermore, the face-name task used to assess medial temporal lobe dependent learning had recently been adapted for use with MRI (Nestor *et al.*, 2008). Therefore, the pre and post-training cognitive testing sessions could be performed in the scanner to give a clearer understanding of the differences in activation of particular brain regions between sedentary and trained participants.

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VIII Appendix

Solutions used

- **Electrode running buffer:**

Glycine	200mM
Tris base	25mM
SDS	17mM
Distilled water	

- **Krebs Solution:**

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

- **Phosphate-buffered saline (PBS, pH 7.4):**

NaCl	100mM
Na2HPO4	80mM
NaH2PO4	20mM
Distilled water	
Containing Tween (PBS-T)	0.1% Tween 20 (v/v)

- **Sample Buffer:**

Tris-HCl	0.5mM, pH6.8
Glycerol	10% (v/v)
SDS	0.05% (w/v)
β-mercaptoethanol	5% (v/v)
Bromophenolblue	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)
TEMED	0.1% (v/v)
Distilled water	

- **Stacking gel:**

Bisacrylamide	6.5% (w/v)
Tris-HCL	0.5M, pH 6.8
SDS	1% (w/v)
Ammonium persulphate	0.5% (w/v)
TEMED	0.1% (v/v)
Distilled water	

- **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	20mM
NaCl	150mM
Tween-20	0.05% (v/v)
Distilled water	

DEPARTMENT OF PHYSIOLOGY, TRINITY COLLEGE, DUBLIN.

Medical Questionnaire

The purpose of this survey is to keep a record of all subject/participant personal, medical and general health details for later comparison and data analysis. It is also essential to ensure any unnecessary risk or injury is avoided to all involved in the experimental series. Please complete all of the personal information at the top of this page and answer all of the questions accurately. All information will be kept as confidential as possible.

Subject Name:_____ Date:_____

Height:_____ Weight:_____

Sex:_____ Age & D.O.B.:_____

Contact Telephone Numbers:_____

Please circle the appropriate answer and provide details in all cases.

1. Are you a smoker? YES NO _____
2. Do you suffer from asthma? YES NO _____
3. Do you drink alcohol? YES NO _____
4. Do you drink tea/coffee? YES NO _____
5. Do you drink Coke/Pepsi etc? YES NO _____
6. Are you a diabetic? YES NO _____
7. Are you lactose intolerant? YES NO _____
8. Have you ever had any soft tissue injuries (ie: broken bones, ligament damage...)?
   YES NO _____
   _____
9. Does your family have a history of stroke and/or heart disease?
   YES NO _____
   _____
10. Do you have any allergies? YES NO _____
    _____
11. Do you have any other medical/health related complaints that should be made aware to the investigators? YES NO _____
    _____

12. Do you perform any regular physical activity? YES NO

If YES, please indicate type, duration and frequency._____

_____

_____

13. Are you currently taking any prescribed medication? YES NO

If YES, please indicate which drugs, and reasons for prescription._____

_____

_____

14. Have you ever knowingly or unknowingly taken any performance enhancing agents (eg: anabolics, steroids, β-blockers...)? YES NO

If YES, please indicate which agents, and why._____

_____

_____

15. Are you currently taking any other dietary supplements (eg: vitamins, iron, proteins...)? YES NO

If YES, please indicate which supplements, and why._____

_____

_____

Please sign and date this survey below if the answers you have given are, to the best of your knowledge, true and correct. If you are unsure of any questions or have any information you think may be important, but not specifically addressed by these questions, please make it known to the principal investigator of the study.

Signature of Subject:_____Date:_____

INFORMED CONSENT DOCUMENT

Project Title: An Investigation of the effects of exercise on circulating neurotrophins and whether this has implications for cognitive function.

Principal Investigator: _____

Physician: _____

Subject Name: _____ Date: _____

Subject Contact info: _____

In signing this form you are agreeing that:

a) You are between 18 and 30 years of age, and legally independent.
b) You are a volunteer in a research project being conducted by the principal investigator named above, the purpose of this research has been outlined.
c) All experimental procedures in which you will be involved have been explained to you fully, including the expected duration of these procedures.
d) All reasonably foreseeable risks or discomforts of any experimental procedures being performed have been described for you.
e) You have completed a brief questionnaire regarding your medical history and informed the principal investigator of any medical condition you feel they should be aware.
f) All data from any experimental procedures, surveys or the like, completed by you will be kept confidential and all data and experimental samples will not be used for any purpose outside the goals of this study without your express permission.
g) You will not disclose any information regarding the specific goals of the project to those not directly involved.
h) As a volunteer you can withdraw at any stage from the project or any other experimental procedures.
i) The principal investigator also has the ability to exclude or withdraw you from any experimental procedures or the project in general at any time.
j) The principal investigator has offered to answer any pertinent questions about the experimental procedures, the research project, and the research-related risk of injuries.
k) You have a contact (generally the principal investigator), which can be used in case of any concerns or complications regarding the experimental procedures, following your participation in the research project.

STATEMENT

I acknowledge that this study and this consent form have been explained to me. The investigator(s) has/have answered all my questions to my satisfaction and I understand what will happen if I agree to be part of this study

I have read, or had read to me, this consent form.

Eadaoin Griffin (Principal Investigator) has fully explained to me the risks involved and the need for the research; has informed me that I may withdraw from participation at any time without prejudice; and has offered to answer any inquiries which I may make concerning the procedures to be followed. I freely consent to my participation in the experimental procedures of the above research project though without prejudice to me legal and ethical rights. I have received a copy of this agreement and I understand that if there is a sponsoring company, a signed copy will be sent to that sponsor.

I have read and understand all of the above points.

Signature of Subject: _____ Date: _____

Signature of Principal Investigator: _____ Date: _____

Signature of Physician: _____ Date: _____

Declaration of Helsinki

The World Medical Association has developed the Declaration of Helsinki as a statement of ethical principles to provide guidance to physicians and other participants in medical research involving human subjects. Medical research involving human research includes research on identifiable human material or identifiable data

Recommendations Guiding Physicians in Biomedical Research involving Human Participants.
Adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964 and amended by the 29th World Medical Assembly, Tokyo, Japan, October 1975, the 35th World Medical Assembly, Venice, Italy, October 1983 and the 41st World Medical Assembly, Hong Kong, September 1989.

Introduction
It is the mission of the physician to safeguard the health of the people. His or her knowledge and conscience are dedicated to the fulfilment of this mission.

The declaration of Geneva of the World Medical Association binds the physician with the words, "The health of my patient will be my first consideration" and the International Code of Medical Ethics declares that "A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient".

The purpose of biomedical research involving human participants must be to improve diagnostic, therapeutic and prophylactic procedures and the understanding of the aetiology and pathogenesis of disease.

In current medical practice most diagnostic, therapeutic or prophylactic procedures involve hazards. This applies especially to biomedical research.

Medical progress is based on research which ultimately must rest in part on experimentation involving human participants.

In the field of biomedical research a fundamental distinction must be recognised between medical research in which the aim is essentially diagnostic or therapeutic for a patient, and medical research, the essential object of which is purely scientific and without direct diagnostic or therapeutic value to the person subjected to the research.

Special caution must be exercised in the conduct of research which may effect the environment, and the welfare of animals used for research must be respected.

Because it is essential that the results of laboratory experiments be applied to human beings to further knowledge and to help suffering humanity, the World Medical Association has prepared the following recommendations as a guide to every physician in biomedical research involving human participants. They should be kept under review in the future. It must be stressed that the standards as drafted are only a guide to physicians all over the world. Physicians are not relieved from criminal, civil and ethical responsibilities under the law of their own countries.

Basic Principles

1. Biomedical research involving human participants must conform to generally accepted scientific principles and should be based on adequately performed laboratory and animal experimentation and on a thorough knowledge of the scientific literature.

2. The design and performance of each experimental procedure involving human participants should be clearly formulated in an experimental protocol which should be transmitted for consideration, comment and guidance to a specially appointed committee independent of the investigator and the sponsor provided that this independent committee is in conformity with the laws and regulations of the country in which the research experiment is performed.

3. Biomedical research involving human participants should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human participant must always rest with a medically qualified person and never rest on the subject of the research, even though the participant has given his or her consent.

4. Biomedical research involving human participants cannot legitimately be carried out unless the importance of the objective is in proportion to the inherent risk to the participant.

5. Every biomedical research project involving human participants should be preceded by careful assessment of the predictable risks in comparison with foreseeable benefits to the participant or to others. Concern for the interests of the participant must always prevail over the interests of science and society.

6. The right of the research participant to safeguard his or her integrity must always be respected. Every precaution should be taken to respect the privacy of the participant and to minimize the impact of the study on the participant's physical and mental integrity and on the personality of the participant.

7. Physicians should abstain from engaging in research projects involving human participants unless they are satisfied that the hazards involved are believed to be predictable. Physicians should cease any investigation if the hazards are found to outweigh the potential benefits.

8. In publication of the results of his or her research, the physician is obliged to preserve the accuracy of the results. Reports of experimentation not in accordance with the principles laid down in this Declaration should not be accepted for publication.

9. In any research on human beings, each potential participant must be adequately informed of the aims, methods, anticipated benefits and potential hazards of the study and the discomfort it may entail. He or she should be informed that he or she is at liberty to abstain from participation in the study and that he or she is free to withdraw his or her consent to participation at any time. The physician should then obtain the participant's freely-given informed consent, preferably in writing.

10. When obtaining informed consent for the research project the physician should be particularly cautions if the participant is in a dependent relationship to him or her or may consent under duress. In that case the informed consent should be obtained by a physician who is not engaged in the investigation and who is completely independent of this official relationship.

11. In the case of legal incompetence, informed consent should be obtained from the legal guardian in accordance with national legislation. Where physical or mental incapacity makes it impossible to obtain informed consent, or when the participant is a minor, permission from the responsible relative replaces that of the participant in accordance with national legislation.

Whenever the minor child is in fact able to give a consent, the minor's consent must be obtained in addition to the consent of the minor's legal guardian.

12. The research protocol should always contain a statement of the ethical considerations involved and should indicate that the principles enunciated in the present Declaration are complied with.

Medical Research Combined with Professional Care (Clinical Research)

1. In the treatment of the sick person, the physician must be free to use a new diagnostic and therapeutic measure, if in his or her judgement it offers hope of saving life, re-establishing health or alleviating suffering.

2. The potential benefits, hazards and discomfort of a new method should be weighted against the advantages of the best current diagnostic and therapeutic methods.

3. In any medical study, every patient – including those of a control group, if any – should be assured of the best-proven diagnostic and therapeutic method.

4. The refusal of the patient to participate in a study must never interfere with the physician-patient relationship.

5. If the physician considers it essential not to obtain informed consent, the specific reasons for the proposal should be stated in the experimental protocol for transmission to the independent committee (1,2).

6. The physician can combine medical research with professional care, the objective being the acquisition of new medical knowledge, only to the extent that medical research is justified by its potential diagnostic or therapeutic value for the patient.

Non-Therapeutic Biomedical Research involving Human Research Participants (Non-Clinical Biomedical Research)

1. In the purely scientific application of medical research carried out on a human being, it is the duty of the physician to remain the protector of the life and health of that person on whom biomedical research is being carried out.

2. The participants should be volunteers – either healthy persons or patients for whom the experimental design is not related to the patient's illness.

3. The investigator or the investigating team should discontinue the research if in his/her or their judgement it may, if continued, be harmful to the individual.

4. In research on man, the interest of science and society should never take precedence over considerations related to the well-being of the participant.

IX Publications

Griffin, É. W., Bechara, R., Birch, A., Kelly, Á. M. (2009). Exercise enhances recognition memory in the rat: evidence for a BDNF related mechanism. *Hippocampus* (In press).

O'Callaghan, R. M., Griffin, É. W., Kelly, Á. M. (2009). Long-Term Treadmill Exposure Protects Against Age-Related Neurodegenerative Change in the Rat Hippocampus. *Hippocampus* (In press).

Published Abstracts:

Griffin, É., Foley, C., Mullally, S., O'Mara, S. & Kelly, Á. (2007). The effect of acute exercise on hippocampal based learning and serum growth factor concentration in sedentary young men. *European Neuropsychopharmacology*, **17**(S1), S63-S64

Griffin, É.W., Foley, C., Mullally, S., O'Mara, S.M., & Kelly, Á.M. (2006). The effect of acute and chronic exercise on serum BDNF concentration; implications for cognitive function. *Irish Journal of Medical Science*, **175** (3), es1: 67

Griffin, É.W., Mullally, S., O'Mara, S.M., Warmington, S.A. and Kelly, Á.M. (2005) Exercise-induced changes in cognitive function: a role for IGF? Acta Neurobiologiae Expermentalis, *37th Annual General Meeting of the European Brain and Behaviour Society, August*, **65**, pp56S